EFFECTS OF WATERCRESS FARMING ON FISH POPULATIONS

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Dedicated to the memory of Janet Susan White 1948-2017

Abstract

Watercress (*Nasturtium officianale*) is a salad crop grown commercially in watercress beds irrigated with water from chalk aquifers. The effluent from irrigation, and in some instances, salad washing processes, are discharged into adjacent chalk streams. There is concern that macroinvertebrate assemblages downstream of discharges reflect organic pollution, which has been attributed to siltation and more recently the release of phenethyl isothiocyanate (PEITC). An antiherbivore metabolite produced by watercress in response to physical damage, PEITC has the potential to be released into chalk streams *via* two pathways: (i) *via* irrigation water emanating from watercress beds following disturbances such as harvesting and other crop-damaging activities; (ii) *via* the discharge of salad wash effluent from the rinsing of watercress, a process carried out on small number farms. While PEITC toxicity to macroinvertebrates is well-studied, the impact that PEITC may be having on fish populations has received little attention. Watercress farms discharge into chalk stream headwaters where fish embryos are incubated, so there is a potential for this sensitive early life stage to be directly exposed to PEITC.

To determine the impact watercress farm discharges are having on habitat, macroinvertebrate and fish, three watercress farms each were surveyed biannually. Each farm varied in its utilisation of salad washing, with the aim of the study to investigate whether any changes in physicochemistry, habitat, macroinvertebrate prey availability from watercress bed irrigation and salad wash effluent rendered sites suboptimal to support fish populations. Organic pollution stress was assessed through macroinvertebrate assemblages using the Walley Hawkes Paisley Trigg (WHPT) biotic index, which provides a score based on the relative abundances of pollution sensitive and pollution tolerant taxa. Sites receiving salad wash effluent had significantly lower WHPT scores than expected and higher total macroinvertebrate abundance, while sites receiving watercress bed irrigation water only scored higher than predicted. Fish species other than brown trout (*Salmo trutta*) were found at higher densities below discharges. However, *S. trutta* found at lower densities were in better condition, possibly due to decreased intraspecific competition and greater macroinvertebrate prey abundances. In contrast to sites that received just watercress bed irrigation discharge, sites below salad wash effluent had densities of young-of-year *S. trutta* that were lower than expected, suggesting that PEITC release from salad washing may reduce embryo survival.

To assess embryotoxicity of PEITC, a series of laboratory trials exposed *S. trutta*, common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) embryos to 0.01, 0.1 and 1µg/L PEITC during embryonic development. In all three species, exposure to 1µg/L resulted in complete mortality between 1-3 dosing days, while embryos exposed to 0.1 µg/L PEITC suffered higher mortality rates, significantly delayed hatching, higher incidence of spinal deformations and significantly altered behaviour

compared to controls. These levels of exposure were orders of magnitude below estimates of PEITC discharges from salad washing, suggesting that salad wash effluent may have been a factor in the low densities of young-of-year *S. trutta* downstream of salad wash effluent discharge.

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Abbreviations

BMWP Biological Monitoring Working Party

DMR Daily Mortality Rate

DMSO Dimethyl Sulfoxide

DPF Days Post Fertilisation

DPH Days Post Hatch

EA Environment Agency

ELS Early Life Stage

EPT Ephemeroptera, Plecoptera and Trichoptera

NTAXA number of taxa considered in the analysis

PEITC Phenethyl isothiocyanate

PPB parts per billion

PSI Proportion of Sediment-sensitive Invertebrates

RICT River Invertebrate Classification Tool

RIVPACS River Invertebrate Prediction and Classification System

SS suspended solids

WHPT Walley, Hawkes, Paisley Trigg

YOY Young of Year

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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed

Dated

26th September 2020

INTRODUCTION

1.1 The current state of rivers and streams

Of all habitats on the planet, freshwater ecosystems are currently considered the most threatened by anthropogenic activities (Reid et al. 2018). Freshwaters are disproportionately high in biodiversity, which Martens (2009) describes as the 'freshwater paradox'. Rivers and streams cover just 0.58% of the world's non-glacial surface, yet they account for almost 6% of all described species including 33% of all vertebrates (Dudgeon et al. 2006). Declines in freshwater organisms, estimated to be 83% between 1970 and 2014, exceed those in both terrestrial and marine ecosystems (Reid et al. 2018). Rivers and streams provide a multitude of services for humankind, including a vital source of water for domestic, industrial and agricultural applications, waste disposal, food, navigation, hydropower and leisure activities (Adeloye 2009). As a result, running waters have long attracted human settlement, which has bought upon them problems associated with over-exploitation (Dudgeon et al. 2006). It has been estimated that 10,00 -20,000 riverine species are at risk of extinction due to anthropogenic activities (Vörösmarty et al. 2010). It is therefore unsurprising that rivers have been described as the most impacted ecosystems on the planet (Malmqvist & Rundle 2002).

1.2 The chalk stream habitat

1.2.1 Distribution

Globally, the geomorphological conditions required to form chalk streams are extremely scarce, being mostly confined to southern and eastern England and a small area of northern France. The key component in the formation of chalk streams is the occurrence of cretaceous chalk bedrock, which in the UK is found in a band stretching from the north east as far as the Eastern Wolds in Yorkshire, to East Anglia, and into the South West (Figure 1a). The Environment Agency (2004) considers there to be 161 chalk rivers and streams in England, the pattern of distribution closely follows the chalk bedrock (Figure 1b). The global scarcity of chalk streams, and their unique biodiversity have seen them assigned a priority habitat under the EU Habitats Directive (92/43/EEC).

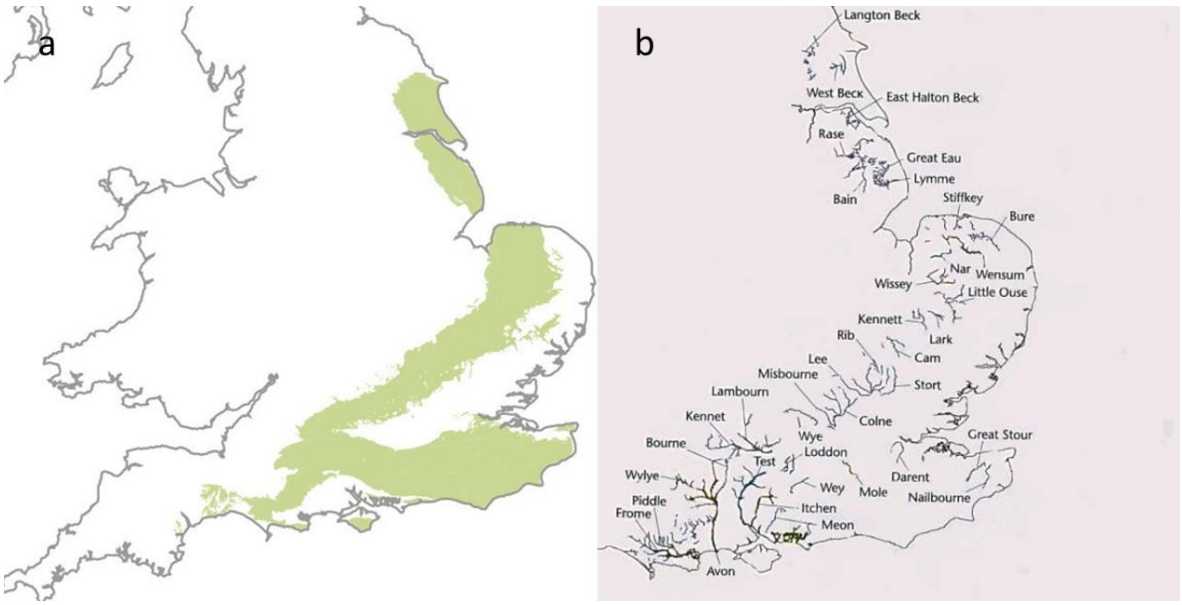

Figure 1 Cretaceous bedrock in the United Kingdom (a) (Image: British Geological Survey P785839) and the distribution of the major chalk rivers in England (b) (Natural England, 2009)

1.2.2 Hydrology

Chalk is highly porous, allowing chalk bedrock to become saturated by precipitation. Subsurface saturated bedrocks are called aquifers, and retain precipitated water underground (Berrie 1992). Where chalk bedrock encounters an impermeable stratum of rock, water emerges at the surface in a spring. Springs may 'break' – the term for commencement of flow – as the water table in the aquifer rises (Mainstone 1999). Similarly, springs at higher elevations become dry as the water levels in an aquifer fall. Typically, the water table in an aquifer is highest in winter following autumn rains when precipitation is at a maximum, and lowest in the drier summer months (Berrie 1992). Chalk streams are typically fed by springs along their length, and the longitudinal position of the springs are hydrologically important. The springs in the upper reaches cease to flow during spring and summer as the rate of discharge in the aquifer is greater than recharge from precipitation. They will break in the winter as aquifer levels rise, giving rise to the name 'winterbournes' for the ephemeral upper reaches of a chalk stream (Mainstone 1999). Further down the gradient, some springs will rarely, if ever, dry out, and this point of the stream is known as the perennial head.

Water percolates slowly through chalk aquifers, the residence time is often in excess of 20 years (Foster et al. 1986). The steady release of water from aquifers attenuates sporadic precipitation, resulting in the relatively stable hydrological cycles of chalk streams (Mainstone 1999). Chalk streams on pure chalk geologies which are largely spring-fed are stable biological habitats, with ratios of maximum and minimum mean daily discharges as low as 3:1. This contrasts strongly with typical surface water rivers, which in clay catchments can have ratios greater than 100:1 (Ladle and

Westlake 1995). However, some chalk streams are of mixed geology, or may start out on chalk bedrock, before entering areas of impermeable soils where run-off can distort the hydrograph. Mainstone (1999) defines mixed geology chalk streams as those that maintain strong summer flows but are influenced by the presence of other solid geology or quaternary deposits, to differentiate from classic chalk streams, which have <80% chalk in the underlying geology.

1.2.3 Physicochemistry

In southern England, spring water emerges from underground chalk aquifers at around 11 °C regardless of season (Crisp et al. 1982). This results in chalk streams that remain cool in summer and warm in winter relative to ambient air temperatures, providing a relatively stable biological habitat (Crisp et al. 1982; Berrie, 1992). Compared to typical surface water rivers, the annual temperature regime has a relatively narrow amplitude (Mackey and Berrie 1991; Berrie 1992). Actual water temperatures at a temporal and spatial scale will naturally depend on season (air temperature), the distance from spring aquifers, the size of the river and the amount of riparian shading (Broadmeadow et al. 2011). A typical annual temperature range of a large southern chalk river is around 5-17 °C (Mainstone 1999). Alongside temperature, the chemical composition and high nutrient status of un-impacted chalk streams remains stable with seasonality (Casey 1969; Bowes et al. 2005) particularly in the upper reaches (Cox 2009). Extended percolation though chalk imbues chalk stream waters with high alkalinity and conductivity, with pH values in the range of 7.4 to 8 (Table 1). Of the two major plant nutrients in chalk streams, nitrate tends to be in excess while phosphorus is often very low at less than 0.02mg/L (Casey et al. 1993; Cox 2009). As relatively little surface water enters chalk streams, suspended solid levels can be extremely low with very low turbidly (Berrie 1992). This is particularly true for headwaters, while lower reaches tend to encounter non-chalk geologies and so may carry higher suspended solids through run-off, resulting in substrates with higher levels of sedimentation (Mainstone 1999).

Table 1 Indicative annual means of key water quality parameters in near-pristine chalk streams (after Mainstone 2009)

Parameter	*Upper reaches*	*Middle reaches*	*Lower reaches*
Suspended solids (mg/L)	<2	4	6
SRP (mg P mg/L)	<0.01	0.02	0.03
Total Phosphorus (mg/L)	0.02	0.04	0.06
Nitrate (mg/L)	0.2	0.5	1
Total Ammonia (mg/L)	0.01	0.03	0.05
pH	7.8-8.0	7.8	7.4

1.2.4 Channel structure

The original and natural state of most UK chalk streams was likely to be an ill-defined, braided channel, however, over centauries, the course of rivers have undergone restructuring to suit the needs of agriculture, provide flood defence, and to provide water power for industries (Ladle and Westlake 1995). Comparisons of First Edition Ordnance Survey maps dating from 1890 to the present day reveal extensive modifications of chalk stream paths, with the removal of meanders and channel straightening, principally to increase channel capacity, the maintenance of water meadows, for navigation and for water mills (Brunner et al. 2010). The presence of mills and head-retaining structures on many chalk streams presents obstacles to migration for anadromous fish such as salmon *Salmo salar* (L.) and sea trout *Salmo trutta* (*L.*) and the catadromous eel *Anguilla anguilla* (L.) (Mainstone 1999). Chalk stream channels have been artificially deepened to increase drainage capacity, which can lead to loss of gravel substrates, which are of great importance to chalk stream ecosystems. These gravel substrates would naturally be revealed from deposits in the alluvial plane as the path of the river migrates across the landscape (Mainstone 1999). This is a slow process, and with the course of many chalk streams artificially restricted and channelled, it is a process that is often no longer occurring. Once lost, these substrates are irreplaceable by natural processes (Mainstone 1999).

1.3 Flora and Fauna of chalk streams

Chalk streams are among the most productive and species-rich temperate freshwater ecosystems (Wright 1992; Woodward et al. 2008). The biological communities characteristic of chalk streams vary longitudinally; from winterbourne sections which cease to flow when springs dry out each summer, to perennial headwaters to classic chalk streams that never dry out and finally to large chalk streams generally wider than 10 metres (Mainstone 1999). The following overview describes some of the characteristic in-stream biota found in perennial chalk stream reaches. It largely focusses on headwater streams, as these are the typical location of watercress farms.

1.3.1 Riparian vegetation and macrophytes

Prior to land management, most UK chalk streams were banked by woodland dominated by alder (*Alnus glutinosa*) and willow (*Salix* spp. L.) (Ladle and Westlake 1995). Presently, little of this vegetation remains following extensive clearance for agriculture, and the majority of chalk streams now flow through catchments dominated by arable and pasture. Shading of a river by riparian vegetation modulates the thermal regime of a watercourse, with the loss of riparian shade over chalk rivers considered a factor that may exacerbate impacts of rising temperatures under climate warming (Broadmeadow et al. 2011). Channel shading, particularly by tree canopy, limits the

growth of the aquatic macrophytes which provide refugia for macroinvertebrates and fish (Dawson and Kern-Hansen 1979; Flynn et al. 2002; Davis et al. 2018). Moreover, while shading may reduce autochthonous production, increased leaf litter input from tree canopy can increase allochthonous productivity (Halliday et al. 2016). In the absence of shade, macrophyte growth is typically so vigorous that it requires annual cutting back by managers to retain effective water conveyance and to maintain angling amenity (Dawson et al. 1991; Old et al. 2014). The ideal chalk river habitat therefore is generally considered to be one which combines both attributes, being a heterogenous mosaic of openings between a riparian canopy (Mainstone 1999; Broadmeadow et al. 2011). As both riparian vegetation cover and macrophyte cover enhance salmonid densities, they are both key variables in predictive models such as HABSCORE used in the present study (Milner et al. 1998).

The low gradients, and the clear, shallow and nutrient rich waters of chalk streams promote the abundant growth of a diverse range of submerged macrophytes (Kronvang et al. 2006). The spatial distribution of macrophyte species within chalk stream channels are driven by substrate composition, water velocities (Mainstone 1999) and light intensity (Sand-Jinsen et al. 1989). There are approximately 30 macrophyte species common to chalk streams (Mainstone 1999), two of which are important to the present study, water crowfoots *Ranunculus spp*. and watercress (*Nasturtium officianale* (R.Br)) (Figure 2).

The dominant instream submerged macrophyte species of chalk streams are the water crowfoots *Ranunculus* spp. (Cotton et al. 2006). Chalk stream management for fisheries has tended to favour these for the refuge they provide for popular sport fish such as brown trout (*Salmo trutta* L.) and their macroinvertebrate prey. These macrophytes are conspicuous in chalk stream channels from wetted winterbournes to large lowland rivers, and can provide over 80% cover of a given reach (Cotton et al. 2006). *Ranunculus* is of high ecological importance and is scheduled as a priority habitat under the EC Habitats Directive (92/43/EEC). Coarse gravel beds with a low silt content, turbulent currents and high light intensities are key requirements for *Ranunculus* growth and it is often absent in shade, its presence being dependent on high levels of solar radiation (Flynn et al. 2002)

Watercress is a common annual emergent that is farmed as a salad crop, and commonly occurs the entire length of chalk streams; from ephemeral winterbourne sections to the lower reaches of mixed geology chalk rivers (Mainstone 1999). Watercress is a semi-aquatic and fast growing Brassica with recognised human health benefits when consumed (Pinela et al. 2018). Watercress develops rapidly and in some perennial sections of chalk stream it may out-compete the usually dominant *Ranunculus* by autumn. By winter, most watercress dies back to root, or is washed away, while *Ranunculus* remains and flourishes in the absence of watercress (Mainstone 1999).

Figure 2 Common macrophytes of chalk streams; a, water crowfoot (*Ranunculus spp*.); b, watercress (*Nasturtium officianale* (R.Br)). Images by the author

1.3.2 Macroinvertebrates

Chalk streams support abundant and very diverse instream macroinvertebrate communities (Mainstone 1999; Wright and Symes 1999; Visser et al. 2019). Macroinvertebrates are consumers at intermediate trophic levels, and often play an important role in nutrient cycling in streams, consuming primary productivity and constituting an important source of food for numerous fish (Wallace and Webster 1996). In chalk streams, macroinvertebrate communities vary spatially, longitudinally from winterbourne to perennial, and within these zones utilise a range of mesohabitats.

1.3.2.1 Distribution of macroinvertebrates in chalk streams

Perennial chalk streams can be broadly divided into headwater, middle and lower reaches, with longitudinal differences in hydrology, sediments, water chemistry and mesohabitats favouring different macroinvertebrate taxa. In the more swiftly flowing headwaters in the upper reaches, taxa favouring clean gravel beds and high current velocities will flourish, such as the orders Plecoptera, Ephemeroptera and Coleoptera. The middle reaches do not tend to have the broadest range of species of any particular order, but may have greatest diversity of arthropods (Dunn et al. 2006). Lower reaches are typically slower flowing with greater sedimentation, and these tend to support the greatest diversity of Gastropoda, Hirudinea and Trichoptera (Mainstone 1999).

Within the longitudinal continuum, mesohabitats provide additional niches for invertebrates to utilise. A year-long study of invertebrates in five distinct mesohabitats, including three vegetative

habits; *Ranunculus*, *Berula*, *Callitriche* and two sediment habitats; gravel and silt, on the River Lambourne, UK, found that species richness, abundance and biomass was significantly higher on macrophytes than in gravels and silt (Wright 1992). Vegetative habitats can be broken down into instream submerged (*Ranunculus*, *Potamogeton, Zannichellia*, *Callitriche)*, instream emergent (*Rorippa, Berula*) and bankside emergent (*Phragmites, Phalaris, Glyceria, Carex, Rorippa, Apium/Berula, Sparganium*) (Mainstone 1999). Instream submerged macrophytes are often dominated by suspension feeders such as Simuliidae and Trichoptera (Harrod 1964), while marginal emergent macrophytes may contain a broader range of taxa including insects that use emergent shoots for emergence into winged adults, such as Ephemeroptera and Odonata (Painter 1999; Harrison and Harris 2002; Stewart and Samways 2008).

Sediment mesohabitats can be broken down into eroding and depositional sediments. Fine depositional sediments are mainly characterised by deposit and suspension feeders such as Tubificidae, pea mussels (Sphaeriidae) and the burrowing mayfly *Ephemera danica* Müller (Ladle and Westlake 1995). Eroding sediments, such as gravels and cobbles, can be more taxon-rich than depositional sediments and support a wide range of functional feeding groups, typically detritivores, grazers and scrapers, including Chironomidae, Ephemeroptera and Gammaridae (Wright and Symes 1999).

1.3.2.2 Gammaridae

Colloquially, gammarids are variously known as ‘freshwater shrimp’, ‘side swimmers’ and ‘scuds’, though are not proper shrimps, belonging to the order Amphipoda. There are two native Gammaridae species which may be encountered in chalk streams, the widespread *Gammarus pulex* (L.) (Figure 3), and *G. lacustris* (Sars) having a much more limited UK range. In addition, there are several invasive species which have recently appeared in British waters including those belonging to the genera *Dikerogammarus* and *Echinogammarus* (Dobson et al. 2013; Jermacz and Kobak 2018). Although gammarids encountered in chalk streams will almost certainly be *Gammarus pulex*, to account for the possibility that other species may be present, this study will use the term gammarid rather than *Gammarus pulex*, unless directly referring to specific studies involving the species.

Figure 3 *Gammarus pulex*. Image © Jan Hamrsky (www.lifeinfreshwater.net), reproduced with permission

In chalk streams, gammarids are the most abundant macroinvertebrate in terms of biomass, and being the principal detritivore, the play an essential role in the benthic community by shredding autumn leaves (Wright and Symes 1999; Kunz et al. 2010). In experimental manipulation experiments, the absence of *Gammarus pulex* has been shown to dramatically reduce detrital processing rates, and as such they are considered a keystone species (Woodward et al. 2008). In addition, with their high propensity to drift (Rader 1997) they are an important dietary component for chalk stream fish, particularly during the winter months when insect larvae abundances are typically low (Mann and Orr 1969; Macneil et al. 1999; French et al. 2016). Due to their key position in aquatic food webs and their importance as winter forage for fish, gammarids are a key taxon of interest in the present study.

Gammarids are often used as indicators for water quality in biomonitoring (Ciliberti et al. 2017) and are frequently used in ecotoxicology trials (Kunz et al. 2010). Despite their frequent use as an indicator species, they are only rated as moderately pollution-sensitive in macroinvertebrate biotic indices such as BMWP and WHPT (Clarke and Davey-Bowker 2014). However, so ubiquitous and abundant are they in healthy chalk streams one can expect several hundred or even over one thousand individuals to be netted in a three-minute kick sample (Medgett and Court 2008). Being easy to capture and identify, they are particularly useful in citizen science. One such nationwide project is the Riverfly Project, where the Environment Agency in conjunction with Salmon and Trout Conservation charity agreed a target of >500 gammarids per kick sample to indicate a healthy chalk stream population (S&TC 2019).

1.3.2.3 Ephemeroptera, Plecoptera and Trichoptera (EPT)

The insect orders Ephemeroptera (E), Plecoptera (P) and Trichoptera (T) are collectively referred to as EPT in biotic assessments of running waters. Due to the sensitivity of the aquatic larval stages of EPT to a range of stressors, their presence or absence in macroinvertebrate surveys have been widely used in bioassessment (Wright and Ryan 2016). Many riverine EPT require the high water velocities, high dissolved oxygen and coarse substrates that typify chalk stream habitats (Rasmussen et al. 2012). EPT taxa play important roles in nutrient cycling, with their emergence as winged adults providing a key link in in aquatic/terrestrial energy subsidies (Wallace and Webster 1996; Marcarelli et al. 2011). Moreover, the aquatic larval stages are important prey items for many fish species. For example, an 84% reduction in EPT densities due to increasing agricultural intensification in a New Zealand stream from the 1960s to 2016 was considered the key driver in the decline of wild trout fishery (Stewart et al. 2019).

Ephemeroptera, or mayflies (Figure 4a) are particularly abundant in chalk streams, where the larval stages can be found in great densities in macrophytes, feeding chiefly on periphyton, and on the stream bed feeding on detritus and epilithon (Sartori and Brittain 2015). In Britain, forty-nine species are known to occur, with over 50% of those species found in chalk streams (Wright et al. 1998). Larval Ephemeropterans can contribute up to 25% of the total zoobenthos production in cool and unpolluted streams and are an important component in the diets of fish (Elliott and Humpesch 2010). Due to their widespread occurrence, their importance in food webs, particularly in fish production, they are considered important components of bioassessment protocols (Sartori and Brittain 2015).

Plecoptera, or stoneflies (Figure 4b) are typically far less numerous in chalk streams than Ephemeroptera and Trichoptera (Wood and Petts 1999; Wright et al. 2002). There are 34 recorded species in the UK (Elliott 2009), most of which require highly oxygenated waters and are found in fast-flowing riffle habitat where coarse substrates dominate (DeWalt et al. 2015). Stoneflies are among the most sensitive freshwater fauna to environmental degradation and so are considered excellent bioindicators (Fochetti and De Figueroa 2006).

Trichoptera, or caddisflies (Figure 4c), play an important role in nutrient processing and energy flows in chalk streams, providing an important source of food for trout and other fish (Holzenthal et al. 2015). A key feature of Trichoptera larvae is their use of silk to construct cases, in which they reside, or to spin nets to trap food particles from water column (Holzenthal et al. 2015). In the UK, there are 152 cased caddis and four true caseless species, which may feed by filter feeding using silk nets, scraping, gathering detritus or predation, depending on the species (Wallace et al. 2003).

Most Trichopterans are intolerant of pollution, making them a key order in bioassessments (Holzenthal et al. 2015).

Figure 4 Examples of EPT macroinvertebrates; a, Ephemeroptera (Baetidae); b, Plecoptera (Perlodidae); c, Trichoptera (Limnephilidae). Images © Jan Hamrsky (www.lifeinfreshwater.net), reproduced with permission

1.3.3 Fish

Many chalk streams are managed for sport fisheries, with the management practices prioritising salmonid species, in particular the iconic native brown trout *Salmo trutta* (L.) (Figure 5a), an economically-valuable species which has become synonymous with chalk streams (Elliott 1989; Mann et al. 1989). While chalk streams are well-renowned for their wild trout fisheries, some lower reaches may support coarse fisheries (Mann et al. 1989). *S. trutta* abundances are frequently nurtured and/or bolstered by fisheries managers, with grayling, dace and coarse fish often removed from chalk streams by angling clubs in an effort to encourage trout production through decreased interspecific competition of resources (Ladle and Westlake 1995; Mainstone 1999). Of the fish species native to chalk streams, three are listed on Annex II of the EU Habitats Directive as considered threatened throughout Europe; the Atlantic salmon *Salmo salar* (L.), bullhead *Cottus gobio* (L.) (Figure 5b) and the brook lamprey *Lampetra planeri* (Bloch) (JNCC 2017). The Atlantic salmon's current UK range is centred on the west of the British Isle and their UK population is in decline through climate change and direct and indirect human influence. Populations of *S. salar* were healthy in most chalk streams until stocks declined rapidly in the last 30 years (JNCC 2017). Despite being on Annex II of the Habitats Directive, *C. gobio* are widespread throughout the UK and typically found in high densities in chalk streams (Mills and Mann 1983; Cowx and Harvey 2003). However, their population trend is presently unknown (JNCC 2017). The European eel *Anguilla anguilla* (L.) , is commonly encountered in chalk streams, but globally is placed on the IUCN red list as critically endangered (Jacoby et al. 2015).

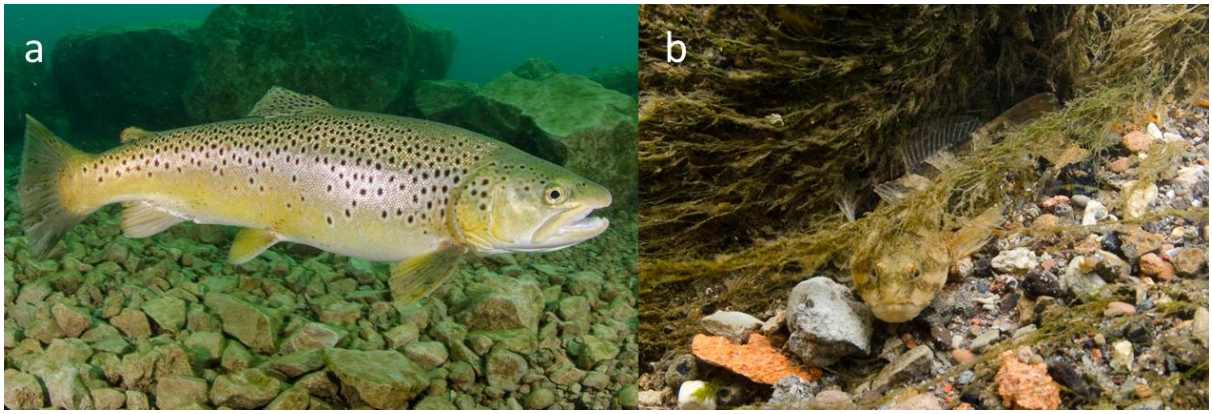

Figure 5 Two commonly encountered chalk stream fish; a, brown trout (*Salmo trutta*); b, bullhead (*Cottus gobio*). Images © Rob Cuss, reproduced with permission

Table 2 describes the species that utilise four of the major habitat types of chalk streams, from ephemeral winterbournes to the larger lowland rivers. The distribution of fish species along chalk streams is congruous with typical rivers as described by Vannote et al. (1980) who proposed the River Continuum Concept. Lower reaches tend to have increased abundances of cyprinids, while upper and middle reaches have the highest densities of salmonids. Chalk stream headwaters in their natural state are characterised by clean gravel beds, which make them important spawning grounds for salmonids (Crisp 1993; Milner et al. 1998; Armstrong et al. 2003; Collins and Walling 2007). *S. trutta* and *S. salar* lay eggs interstitially in a series of gravel excavations called redds. The eggs are deposited by the female as she flexes her body to create a depression, laying the eggs into the streambed before burying them in the substrate. The subsequent hatching and survival of alevins (salmonid fry or hatchlings) is dependent on clean gravel substrates that perennial headwaters provide, by allowing a flow through of water to provide oxygen and remove waste products (Turnpenny and Williams 1980; Heywood and Walling 2007; Soulsby et al. 2001).

Table 2 Characteristic fish assemblages of the different longitudinal sections of chalk streams; R1, winterbournes; R2, perennial headwaters; R3, classic chalk streams; R4 large chalk rivers (after Mainstone 1999)

Species	Scientific name	Spawning/juvenile habitat				Growing/adult residence habitat			
		R1	R2	R3	R4	R1	R2	R3	R4
Atlantic salmon	*Salmo salar*	■	■	■	■				
Brown trout	*Salmo trutta*	■	■	■	■		■	■	■
Brook lamprey	*Lampetra planeri*		■	■			■	■	
Sea lamprey	*Petromyzon marinus*				■				
River lamprey	*Lampetra fluviatilis*			■	■				
Grayling	*Thymallus thymallus*			■	■				■
Minnow	*Phoxinus phoxinus*	■	■	■			■	■	■
Bullhead	*Cottus gobio*		■	■			■	■	
Dace	*Leuciscus leuciscus*			■	■			■	■
3-sp. stickleback	*Gasterosteus aculeatus*	■	■				■	■	
Stone loach	*Noemacheilus barbatulus*		■	■			■	■	
Pike	*Esox lucius*			■	■			■	■
Eel	*Anguilla anguilla*						■	■	■
Chub	*Leuciscus cephalus*			■	■				■
Gudgeon	*Gobio gobio*			■	■				■
Roach	*Rutilus rutilus*				■				■
Perch	*Perca fluviatilis*				■				■
Barbel	*Barbus barbus*				■				■

1.4 Sources of pollution in rivers and streams

The linear and unidirectional flow of rivers makes them uniquely sensitive to activities within river catchments; any pollutant entering a river is likely to exert effects for a considerable distance downstream (Malmqvist and Rundle 2002). Rivers and stream ecosystems are commonly impacted by multiple sources of contaminants from their catchments (Rasmussen et al. 2013; Leps et al. 2015), which are broadly divided into two categories; 'point source' and 'non-point source'.

Non-point pollution (also known as 'diffuse pollution'), emanate from diffuse sources such as run-off and leaching from agricultural or urban land (Stevenson et al. 2008; Withers et al. 2014; Ramião 2015). Non-point pollution is principally flow dependent, occurring intermittently around periods of high precipitation (Bowes et al. 2008). The application of fertilisers to arable land and animal wastes from intensive livestock rearing are known pathways for diffuse nutrients loads to enter rivers (Burkholder et al. 2007) which may be transient following periods of heavy precipitation (Mainstone et al. 1997). In addition, pesticides applied to fields in river catchments may also enter river systems through runoff and leaching (Reichenberger et al. 2007) which can have adverse impacts on riverine flora and fauna (Daam and Van Den Brink 2010; Mottes et al. 2014). Run-off

presents a route for mobilized fine sediments to enter a rivers, which are considered more detrimental to many riverine ecosystems than nutrient loadings (Wagenhoff et al. 2012; Turunen et al. 2016). Fine sediments enter river channels primarily through poor land management, bank erosion by livestock and run-off from arable land, and represent a significant problem in many rivers (Myers and Swanson 1992; Stevens and Cummins 1999; Sovell et al. 2000). The impacts that increased fine sediment loads may have on chalk streams are discussed in section 1.5.1.2.

Point source pollution enters a river channel *via* a specific location such as an outflow pipe or drainage ditch. These may be consented discharges, or illegal unlicensed discharges which typically occur as a result of accidental failure in the transport or storage of polluting substances (Hendry et al. 2003). In contrast to non-point source pollution, locating the source of point source pollution is generally easier. Sewerage treatment works (STWs) outflows are perhaps the most extensively studied consented point source discharges. In industrialised countries such as the UK, much progress has been made in the treatment of waste water to remove nutrients which formally caused eutrophication downstream of discharges (Carey and Migliaccio 2009; Oliveira and Machado 2013). However, wastewater discharges may still contain micropollutants in the form of personal care products, surfactants and pharmaceuticals. As the concentrations of these compounds in effluent are extremely low, the risk of acute environmental toxicity is considered negligible (Franzellitti et al. 2013), though chronic long-term exposure and additive effects have only recently started receiving attention (Luo et al. 2014; Margot et al. 2015).

Industrial discharges from mining and industry can be significant point sources of heavy metal pollution. In the developed world considerable progress in reducing water pollution from industrial point sources has been made (Dudgeon et al. 2006), however the legacy of such activities may remain for considerable time. For example, copper mining ceased in 1890s in the River Hayle in Cornwall, UK, yet a section of river still has copper levels that exceed Environment Quality Standards, and it is subsequently devoid of much of the expected macroinvertebrate and fish life (Durrant et al. 2011).

Trout farms and watercress farms are typically situated on headwater chalk streams to take advantage of high quality water supply, with the spent water discharged back into the river channel (Cox 2009; Tello et al. 2010). Discharges from trout farms may elevate levels of suspended solids and nutrients resulting from uneaten feed, fish faeces and excretion (Tello et al. 2010; Guilpart et al. 2012). Guilpart et al. (2012) report that such inputs into eight headwater rivers in France typically increased the biomass of pollution-tolerant oligochaetes and chironomids, with a concomitant decrease in abundance of pollution-sensitive taxa. The degree of shifting from pollution-sensitive to pollution-tolerant macroinvertebrate assemblages tracked the size and intensity of the trout farm, with the largest farms seeing the greatest shift. Discharges from watercress farms present a

potential point source input of pollution in many chalk streams (Casey and Smith 1994; Mainstone 1999). As watercress farms are the focus of the present study, the remainder of the chapter will focus specifically on watercress farming practices and the impacts they may have on the hydrology and ecology of chalk streams.

1.5 Watercress farming

Historically, watercress has been manually harvested from streams for food and records show medicinal usage from the first to 19th centuries (Manton 1935). Watercress does not remain fresh for long after harvest, so it was not until the advent of the railway in the 19th century that commercial production became viable. The first commercial watercress farm in Great Britain was created in Gravesend in 1808 to supply the London Markets. There followed a growth of watercress farming in the south east of England, with modern production focussed around the southern counties of Wiltshire, Hampshire and Dorset (Cox 2009). As of 2009 there were 32 watercress farms in the UK situated on chalk streams that are Sites of Special Scientific Interest (SSSI), totalling over 52.6 hectares of production, and 6 farms on non-SSSI chalk streams totalling over 7 hectares (Cox 2009). The watercress industry has been continually growing in recent years, with UK annual sales growing from £18m in 2006 to £55m in 2010 (Cotter 2012). In 2016, it was reported that year-on year sales of bagged watercress had increased by 52% (Produce Buisness UK 2016). The rise in popularity is partly down to its designation as a 'superfood', being high in antioxidants, vitamin C, calcium, iron and folate (Zeb 2015; Fallah and Ebrahimi 2016; Ek et al. 2018). Its popularity has also been boosted by numerous studies extolling its anti-carcinogenic properties, partly due to the high levels of phenethyl isothiocyanate (PEITC) it contains (Traka and Mithen 2009; Pinela et al. 2020)

Watercress is grown in shallow gravel beds (Figure 6) typically constructed near the perennial head of chalk streams, where boreholes sunk into the underlying chalk aquifer provide water to irrigate beds of the growing macrophyte. Watercress production has favoured chalk stream headwaters for the stable temperature of spring water which protects the plants from frost in winter, and the high nutrient content enabling vigorous growth (Berrie 1992). Borehole water is at first introduced slowly into watercress beds, and then at ever-increasing volumes as the bed matures. The water leaving the beds is channelled into the nearby chalk stream, usually *via* a settlement pond and/or screens to reduce sediment loading in the receiving river.

Figure 6 Watercress beds on a large conventional watercress farm in southern England in November 2018 (photograph by the author)

Historically, watercress production was seasonal, with watercress considered a winter crop. In traditional watercress production, the watercress beds are planted in early summer and harvested between September to April, the relatively warm spring water allowing for winter growth (Cox 2009). Watercress beds are then typically harvested on rotation, with cut stubbles allowed to regrow for further harvest. This traditional method requires that harvesting ceases in summer when the crop runs to seed. To avoid watercress bed substrates becoming clogged, the watercress bed gravels are routinely cleaned of debris, with the sediment largely discharged into the chalk stream (Cox 2009). Bed cleaning on traditional farms typically occurred annually, with the newly cleaned gravel bed reseeded with watercress seed or cuttings.

In the 1980s most watercress farms began to adopt what is now known as conventional watercress production, which principally diverges from traditional watercress farming in that it allows for year-round crop production (Cox 2009). Conventional production practices vary from farm to farm (Casey and Smith 1994; Cox 2009) but some clear distinctions between traditional techniques and modern conventional production can still be drawn. Unlike traditional methods, peak production in conventional production occurs in the summer months where growth rates can be more rapid (Cox 2009). Conventionally produced watercress is either grown from seed or through vegetative propagation depending on the time of year, using crops grown from seed in greenhouses and poly-tunnels. At the peak of the growing season (May – September) the crop may go from seed to harvest in just 25 days (Steve Rothwell, personal comms. 2017).

Although far less-common, a small number of smaller watercress farms continue to operate traditional production methods, and are subject to different regulations to conventional farms (Cox 2009). Unlike conventional farms, there is no stipulation for settlement lagoons to trap sediments. Instead, they are licenced to clean and replant watercress beds once a year only to limit silt and debris entering the receiving watercourse. However, as watercress beds are cleaned on rotation (Cox 2009), this may still lead to a number of annual sediment discharge events for each traditional watercress farm.

In response to surveys showing negative impacts on macroinvertebrate taxa below watercress farm discharges (Smith 1992; Mainstone 1999; Cox 2009), in the early 1990s the National Rivers Authority (NRA) began to licence and monitor a number of key water parameters in the discharges of watercress farms to limit impacts on chalk stream ecosystems. These regulations have since transferred to the jurisdiction of The Environment Agency and will be discussed in relation to specific discharge components.

1.5.1 Impacts of watercress farming on chalk streams

Chalk streams are susceptible to a range of human activities, such as water abstraction, channel modification, fish farming, watercress farming, intensive agricultural activity and urban development in their catchments (Mainstone 1999). The extent of impacts to chalk streams from watercress production will vary from farm to farm, as the cultivation methods employed and the scale of operation are not uniform (Casey and Smith 1994; Cox 2009). Historically, the use of zinc to control crook root disease and Malathion to control flea and mustard beetles was common, however, the use of pesticides has long been discontinued (Cox 2009; AFS 2016). Presently, the primary pressures arising from watercress farming on chalk streams are thought to be altered hydrological regimes, the release of particulates leading to sedimentation of gravel substrates, nutrient enrichment and the release of PEITC (Cox 2009; Dixon 2010; Cotter 2012; Zhang et al. 2017).

1.5.1.1 Hydrology

The water used in the production of watercress is largely for the irrigation of watercress beds, but also may include salad washing. Abstraction is licenced by the Environment Agency, which sets limits on the volume removed, and stipulates that the abstraction is non-consumptive, requiring at least 95% of the water to be returned locally to chalk streams (Cox 2009). Water resources on a catchment scale are unlikely to be impacted to a large degree by watercress production, but local hydrological regimes have been shown to be altered (Cox 2009). There is evidence that drawdown on aquifers from boreholes reduces the flow in rivers upstream of the abstraction (Owen 1991).

Decreased flows typically result in decreased water velocity, water depth and wetted channel width, and increased water temperatures (Dewson et al. 2007) which may increase concentrations of pollutants and fine sediments within gravel beds (Wood and Armitage 1997; Milan et al. 2000). Low flow conditions can result in alterations of the typical macroinvertebrate community (Dewson et al. 2007; Graeber et al. 2017) and may result in decreased salmonid spawning success (Hendry et al. 2003; Elliott et al. 2006; Jonsson and Jonsson 2009). However, downstream of watercress beds, the returned water can make up a significant component of river flow and so may mitigate impacts of low discharge in dry years. Casey and Smith (1994) calculated that nationally, watercress farms could contribute up to 1 million m^3 of water to chalk streams daily. On a local scale, two studies, one on the Bourne Rivulet in 2004, and one at Abbotts Ann watercress farm in 2007 found that in summer months, discharge from watercress beds can contribute over 90% of the total flow in the receiving rivers (Cox 2009).

1.5.1.2 Suspended solids and siltation

The deposition of fine sediments in reaches downstream of watercress farm discharges may be due to the release of suspended solids in discharge water, the extent of which is largely down to management practices (Casey and Smith 1994). This can take the form of fragments of plant matter and silts with a wide variation in organic content (Casey and Smith 1994). The release of suspended solids is generally pulsed, with the major peaks occurring during the routine cleaning of watercress beds, the frequency of which depends largely on the size of the farm, but will occur several times a year throughout the growing season (Mainstone 1999). To meet their licence conditions, conventional farms must limit suspended solids in their discharge water to 20mg/L and have been obliged to install silt settlement systems. Compliance is monitored by the Environment Agency, and regular sampling has shown good compliance with the consent targets only infrequently missed (Cox 2009). However, as typical chalk stream waters have suspended solids of less than 5mg/L (Casey and Smith 1994), even with full compliance, watercress farms may still be a net contributor to suspended solids in chalk streams.

Silts in chalk stream substrates are dominated by mobilisations from cultivated soils and erosion from pastures (Collins and Walling 2007). Bank erosion by livestock, or poaching, can also contribute large loads of sediment to a river (Myers and Swanson 1992; Stevens and Cummins 1999; Sovell et al. 2000; Neal and Anders 2015). Relative to diffuse inputs from agriculture, watercress farm discharges are thought to contribute relatively small quantities (Hendry et al. 2003). The stable hydrology of chalk streams make them particularly susceptible to the retention of silt in their gravel beds, with limited freshets and scouring events to dislodge accumulations (Acornley and Sear 1999).

With a lack of natural flushing events, It is therefore important that sediment inputs are effectively minimised (Collins and Walling 2007).

Excessive siltation in the main river channel can have adverse impacts on many of the characteristic biota evolved to inhabit chalk streams (Gammon 1970; Nutall and Bielby 1973; Rabeni and Smale 1995; Strand and Merritt 1999; Jones et al. 2012). Increased sediment loads have been cited as a contributing factor in what has been termed 'chalk stream malaise' – a term describing the degradation of classic chalk stream habitat. Symptomatically, this covers degraded water quality, diminished macrophyte growth and increased algal growths, a reduction of salmonid breeding success and reduced abundance of some Ephemeroptera species (Heywood and Walling 2007).

The classic habitat-forming macrophyte of chalk streams, *Ranunculus*, has a strong preference for silt-free gravels (Mainstone 1999). Excessive siltation can reduce *Ranunculus* root growth lessening anchorage, ultimately leading displacement by river flow (Jones et al. 2012). Increased sedimentation can reduce macroinvertebrate diversity, density and species richness (Gammon 1970; Nutall and Bielby 1973; Jones et al. 2012). The addition of fine sediment can change the composition of riverbeds as the average size of particles becomes smaller and the interstices between larger particles become filled (Kaufmann et al. 2009). Most macroinvertebrates have specific substrate requirements and will avoid patches that fail to meet these requirements. The addition of fine sediments will favour deposit feeders and can increase drift in motile taxa that require the interstices in clean gravel beds as refugia (Jones et al. 2012). In addition, silt accumulation in epilithic periphyton can diminish its nutritional value to macroinvertebrate consumers (Graham 1990).

Arguably the most studied impact of siltation relates to the reproductive success of salmonids, which require clear gravel substrate for reproduction (Soulsby et al. 2001; Heywood and Walling 2007; Hauer et al. 2020). The accumulation of fine sediments in river bed substrates reduces its permeability resulting in reduced dissolved oxygen supply to the nest (Greig et al. 2007) and increase embryo mortality (Sear et al. 2016). In addition, it may physically abrade embryos (Lisle and Lewis 1992; McHenry et al. 1994) and inhibit the emergence of alevins by the smothering gravel surfaces (Rubin and Glimsäter 1996; Rubin 1998).

1.5.1.3 Nutrient enrichment

The two major aquatic plant nutrients are nitrate (N) and phosphorus (P). While N is generally in excess in the chalk aquifers used to irrigate watercress beds, P is often very low, making it a limiting nutrient in most chalk streams (Casey et al. 1993; Cox 2009). Consequently, watercress growers may add P fertilisers to watercress beds during the growing season, which may increase P loading in chalk rivers. In contrast, N is typically lower in discharges than abstracted borehole water as it is

assimilated in watercress growth (Casey and Smith 1994; Cox 2009). Phosphorus levels in chalk streams are measured as the concentration of soluble reactive phosphate (SRP), which is the biologically available P in the water column. English Nature in collaboration with the Environment Agency developed common standards for conservation objectives for target concentrations of SRP in chalk rivers, which are set at of 0.04 mg/L for perennial headwaters, 0.06 mg/L for classic chalk streams and 0.1 mg/L for large chalk rivers (Mainstone et al. 2008). Abstracted water typically has SRP levels of 0.01 mg/L, while watercress bed outflow concentrations were on average raised to 0.06-0.08 mg/L during the growing season (Cox 2009). However, due to concerns over eutrophication, many watercress growers have markedly reduced or ceased their application of P since around 2010.

The input of excess nutrients into a water body causes eutrophication (Smith et al. 2006). Chalk streams have short residence times, so the input of excess nutrients does not express itself as it does in lentic water bodies, where phytoplankton blooms occur (Hilton et al. 2006). Typically, in chalk streams it can result in increased growth of epiphytes on instream macrophytes such as *Ranunculus*, which may limit their growth (Wilby et al. 1998; Yates and Johnes 2013), the increased production of filamentous algae on substrates (Carr and Goulder 1990; Neif et al. 2017), which can have a significant effect on macrophyte community structure (Dawson et al. 1999; Davis et al. 2018).

1.5.1.4 Phenethyl Isothiocyanate (PEITC)

Phenethyl Isothiocyanate (PEITC) also known as 2-Phenethyl Isothiocyanate (formula C_9H_9NS, molecular weight 163.24 g/mol) is a secondary metabolite produced by brassicas (e.g broccoli, Brussels sprouts, cabbage, cauliflower, kale and watercress) in response to, and as a defence against herbivory (Di Gioia et al. 2020). This secondary metabolite is produced *via* the hydrolysis of glucosinolates by the enzyme myrosinase (β-thioglucoside glucohydrolase) (Dinkova-Kostova and Kostov 2012) (Figure 7). Glucosinolates occur in within the cell vacuoles of tissues of cruciferous plants and are physically separated from myrosinase. Glucosinolates are hydrolysed by myrosinase into PEITC when they are bought together by physical damage to plant tissues, for example following grazing (for reviews; Halkier and Gershenzon, 2006 and Dinkova-Kostova and Kostov, 2012). There is a large and growing body of evidence indicating that PEITC may reduce the risk of carcinogenesis and heart disease when consumed by humans (Traka and Mithen 2009; Dinkova-Kostova and Kostov 2012; Pan et al. 2018; Abbaoui et al. 2018) which has led to vegetables rich in PEITC, such as watercress, being described as superfoods (Rodrigues et al. 2016). While consumption of PEITC is potentially beneficial to humans, it has well-documented allelopathic and genotoxic properties (Shelton 2005). To a range of aquatic and terrestrial invertebrates it is a

chemical deterrent to consumption (Newman et al. 1990, 1996; Kerfoot et al. 1998). Moreover, the physical abrasion of watercress tissues such as may occur during harvesting and crop washing have been demonstrated to release PEITC into solution, which can exert a toxic effect on macroinvertebrates (Dixon 2010; Dixon and Shaw 2011; Ntalli et al. 2017).

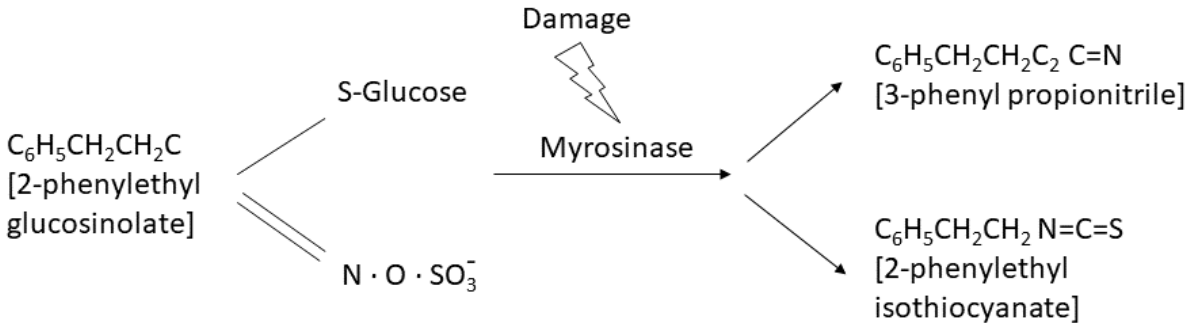

Figure 7 The glucosinolate-myrosinase system. The water soluble glucosinolate (predominantly 2-phenylethyl form in watercress) is compartmentalised separately from the myrosinase enzyme which is released upon tissue damage. The myrosinase catalyzes the hydrolysis, resulting in noxious volatiles, especially isothiocyanate, which is the putative defensive agent (after Newman et al. 1992)

Watercress is the richest natural source of PEITC, which imbues it with its distinctive hot peppery taste (Gill et al. 2007). Because physically damaged watercress tissue leeches PEITC into solution (Worgan and Tyrell 2005; Dixon and Shaw 2011), watercress farming activities such as harvesting and salad washing can result in PEITC being discharged into chalk streams. Watercress is harvested with cropping machines that cut and sweep the crop into bins (Cox 2009). Typically, this is performed above the water surface in drained watercress beds which would limit PEITC release during harvest. However, once the watercress bed is irrigated following harvest, the cut stubbles may cause a pulse of PEITC to be released in the discharge water (Dixon 2010). The annual harvest timing, quantity of crop harvested, and number of cropping events differ from producer to producer and with product demand, so release of PEITC from harvesting is likely to vary widely in magnitude and frequency from farm to farm. Some large-scale modern watercress farms house washing and packing facilities for watercress and imported salad leaves. Salad washing, which involves tumbling crops in borehole water which is subsequently discharged into chalk streams is likely to contribute a more significant input of PEITC than harvesting (Dixon 2010). On one watercress farm in Hampshire, Dixon (2010), noted a strong smell of PEITC in salad wash effluent water prior to discharge into a chalk stream. In contrast to intermittent harvesting, salad washing may occur seven days a week during working hours, so may present a more chronic load than release from harvesting.

Due to its function as an anti-herbivore defence against grazing, it is unsurprising that when in solution, PEITC exerts a toxic effect on macroinvertebrates. Most research has centred on gammarids, which have a 48hr LC_{50}s between 0.96 and 3.62 mg/L (Newman et al. 1990) and have been shown to exhibit avoidance behaviour to PEITC liberated by crushed watercress tissues (Worgan and Tyrell 2005). Just 1µg/L PEITC has been shown to alter natural behaviour in pre-copular gammarid pairs by causing them to release (Dixon and Shaw 2011). The decline in gammarid abundances downstream of discharges and a more general decline in pollution-sensitive macroinvertebrate taxa below discharges from watercress farms have been attributed to PEITC release (Medgett and Court 2008; Cox 2009; Dixon 2010; Dixon and Shaw 2011; Cotter 2012). The role of PEITC in altered macroinvertebrate assemblages is discussed further in section 3.1.3**.** The sensitivity of fish embryos to PEITC is explored using ecotoxicological trials in chapter five.

1.5.1.5 Extent of PEITC release from watercress farming and salad washing

As far as the author is aware, environmental concentrations of PEITC released from the harvesting and washing of watercress have not been accurately measured. The volatile nature of the compound (Chen and Ho 1998; Doheny-Adams et al. 2018) allied to the inherent problems of measuring the concentration of a compound in a fluvial environment make this a challenge. However, two separate studies have estimated PEITC concentrations in discharge water from a single watercress farm on the Bourne Rivulet, Hampshire. Worgan and Tyrell (2005) calculated an estimate of the PEITC released from the harvesting of watercress using as a base the level of 1.92-3.60 mg PEITC per wet g of watercress calculated by Newman et al. (1990). They assumed that 25% of the tissue is damaged through harvesting, and used the estimated mass of watercress harvested, the summer maximum flows of water through beds and the duration of harvesting activity to estimate that 320-590 µg/L is released over a 24-hour period. However, Newman et al. (1990) derived their PEITC content levels by freeze and thawing watercress tissue which is likely to cause considerably more tissue damage than stem cutting. It is expected that more PEITC is released from salad washing activities, where the cut watercress crop is tumbled in spring water, which is later discharged into the chalk stream. Dixon (2010) calculated an estimate of the PEITC concentration found in salad wash water from the watercress farm on the Bourne Rivulet. By using a measured 397-696 µg of PEITC liberated per g of damaged watercress tissue, and the estimate of 15% of the crop being damaged in the salad wash process, it was estimated that 60-104 µg PEITC per g of leaf would be washed from fresh plant. Using the ratio of salad crop to wash water used on the Bourne Rivulet farm (10g leaf per litre of water), provided an estimate of 600-1040 µg/L PEITC released in factory salad wash water. In an aqueous matrix, the degradation of PEITC is temperature dependent. A study by Ji et al. (2005) found higher temperatures to cause more rapid degradation of PEITC. In their controlled laboratory study, at pH 7.4, PEITC had a half-life of 56 hrs at 25°C and

108 hrs at 4°C. Assuming a linear relationship between temperature and PEITC half-life, at the average chalk stream aquifer water temperature of 11° C, PEITC may have a half-life of 90 hours.

1.6 Aims and rationale

Past literature on the impacts of watercress farming on chalk streams have largely focussed on abiotic factors such as alterations to hydrological regimes, increased sediment loading and changes in physicochemistry, while studies of biotic impacts have primarily focussed on assessing changes in macroinvertebrate assemblages. There is evidence of macroinvertebrate assemblages shifting to from pollution-sensitive to pollution-tolerant taxa downstream of watercress farm discharges, and the release of phenethyl isothiocyanate and inputs of fine sediments are considered key drivers of these changes. Very little research has been conducted on the impact of watercress farm discharges on fish population structures, and that which has remains unpublished and limited to electric fishing surveys of a single watercress farm only. There is anecdotal evidence from chalk stream fishery managers suggesting that the intensification of watercress production over recent decades is reducing the abundance and sizes of *S. trutta* in their waters. Speculation as to the cause has focussed on the availability of macroinvertebrate prey, in particular a reduction in gammarid shrimp abundances which form a key component of winter forage for a range of chalk stream species. A reduction in the abundance of macroinvertebrate prey downstream of watercress discharges may render foraging suboptimal for *S. trutta* and other chalk stream fish species. As salmonids spawn in the perennial headwaters where watercress farming typically occurs, it is possible that elevated phenethyl isothiocyanate levels associated with discharge water may be having a deleterious impact on the toxicant-sensitive early life stages of chalk stream fish, and by extension, fish recruitment. The embryotoxicity of phenethyl isothiocyanate and its impact on embryonic development of fish has yet to be quantified, and this forms a key element of the thesis. The aims of the research are as follows:

I. To determine the extent to which watercress farm discharges are impacting habitat and macroinvertebrate assemblages in chalk streams (chapter three)

II. To determine whether discharges from watercress farms are having a population-level impact on fish (chapter four)

III. To determine the concentrations of PEITC that result in lethal and sub-lethal impacts on developing fish embryos (chapter five)

IV. To consolidate the results of physicochemical, habitat, macroinvertebrate and fish surveys. If fish populations are impacted, then identify the causative factor or factors (chapter six)

1.7 Thesis overview

Chapter two: *Study sites and fieldwork schedule* describes the study sites used in the fieldwork components of the research. The chapter starts with the site selection criteria, then provides descriptions of the three rivers under study and the four sites connected with the watercress farm surveyed on each river. The rivers are then compared in terms of key characteristics, both biotic and abiotic. The watercress farming practices and discharges from the farms situated each are described. The general surveying approach and survey schedule common to all fieldwork elements are described.

Chapter three: *The impact of watercress farm discharges on stream habitat and macroinvertebrates* presents results from physicochemical, habitat and macroinvertebrate surveys of the sites introduced in chapter two. Differences in macroinvertebrate assemblages between sites are explored, highlighting differences in composition downstream of discharges. Biotic indices are used to assess habitat quality, a novel feature of which is highlighting the differences between sites with a focus on sites receiving salad wash effluent and watercress bed irrigation effluent. Observed biotic index scores are compared to scores predicted by RICT/RIVPACS to assess the ecological health of each site.

Chapter four: *The impact of watercress farm discharges on fish populations in chalk streams* presents data obtained via electric fishing surveys; examining species composition, condition, density and diversity of fish populations at the sites introduced in chapter two. To control for habitat variance between sites, observed salmonid density data is compared to densities predicted by the habitat variables using HABSCORE. Fish condition is correlated with habitat variables and macroinvertebrate abundances to assess if changes in sites downstream of discharges affect *S. trutta* condition. This is the first time a study has surveyed fish populations in relation to watercress farm discharges using multiple farms and over a duration of several years. Spot recognition software is trialled as a mark-recapture technique for *S. trutta*, with growth rates, site fidelity and population estimates obtained using this novel technique for the species.

Chapter five: *Ecotoxicology of PEITC on early life stages of fish* examines the effects of PEITC exposure on developing fish embryos. For the first time, this research exposes developing fish embryos to increasing PEITC concentrations to gain insight into potential environmental PEITC concentrations that may impact on recruitment and survival in natural populations. Using embryos

of *Salmo. trutta*, *Cyprinus carpio* and *Danio rerio*, mortality rates are quantified, along with a suite of sublethal and teratogenic effects.

Chapter six: *Final discussion* synthesises the findings from chapters three to five to reach a conclusion as to the extent of any impact of watercress farm discharges on habitat, macroinvertebrates and ultimately on chalk stream fish populations. Changes in fish populations are discussed in terms of changes in physicochemistry, habitat, prey availability and PEITC exposure. Future challenges are discussed, and potential ameliorative strategies are proposed.

STUDY SITES AND FIELDWORK SCHEDULE

2.1 Study site selection

In order to ensure, as far as possible, a degree of equivalence between the sites, the following criteria were drawn up in shortlisting potential sites:

- All sites to be located on or near perennial headwaters and not encompass winterbournes
- There should be access to survey a 100m reach immediately upstream and 100 m immediately downstream of the watercress farm discharge. In addition, there should also be access to survey two further 100m reaches. One of which should be at least 1 km downstream and a maximum of 2 km downstream, and an intermediate site approximately equidistant between the immediate downstream site and the furthest downstream site.
- As far as possible, there should be an unbroken reach with no major tributaries entering or leaving the channel from the watercress farm outflow until past the last sampling point at least 1km downstream and at most 2km downstream to avoid further dilution of discharge.

Using satellite imagery, potential sites were identified. Contact was made with the relevant stakeholders, such as landowners, fisheries owners and watercress farms in order to obtain consent for access. The shortlist was reduced to three chalk streams with operating watercress farms; the Bourne Rivulet, Hampshire (SU 42892 49182), the River Frome, Dorset (SU 79725 90868) and the River Crane, Dorset (SU 07187 12676) (Figure 8).

Figure 8 locations of the three watercress farms under study; The Bourne Rivulet: blue; The River Crane: yellow; The River Frome: red

2.2 The Bourne Rivulet

The Bourne Rivulet is a headwater chalk stream in the county of Hampshire in southern England. It is one of the most northerly tributaries of The River Test, a classic English chalk stream that discharges into The Solent via Southampton Water. The Bourne Rivulet has its source in chalk springs in the village of Upton and extends for approximately 16 km before its confluence with the Test. The Bourne Rivulet has been given the highest classification for water quality (River Ecosystem level 1 [RE1]) as defined by the UK Environment Agency (EA). The classification means that its waters are suitable for drinking water abstraction and supporting game fisheries. However, there have been incidents of sewerage entering the river from septic tanks (William Daniel, pers. comm. 2016). The hydromorphology of the Bourne Rivulet is described as 'heavily modified' by the Environment Agency (Environment Agency 2017), and this is evident at the study sites which have historically undergone straightening. The reaches surveyed on the Bourne Rivulet fall within a managed and well-regarded wild *S. trutta* fishery, which has not been stocked with farmed fish since 1996 (William Daniel, pers. comm. 2016). As part of the management practices, grayling (*Thymallus thymallus*) have been excluded from these reaches and are unable to repopulate from downstream due to the placement of a sluice 3.1km downstream of the watercress farm. Weed cutting of *Ranunculus spp*. stands is typically carried out during June/July under the direction of the fishery manager.

2.2.1 Sampling sites on the Bourne Rivulet

Upstream of the watercress farm, the Bourne Rivulet is a winterbourne, which precluded the inclusion of an upstream site due its ephemeral flow drying out in summer. The west channel (BRWC) has a small proportion of its flow from the 1.4 hectares of watercress beds situated to the west of the Bourne Rivulet, while the east channel (BREC) is entirely comprised of abstracted water used for irrigation of 5.5 hectares of watercress beds and salad washing (Figure 9), allowing comparison of the impacts of salad wash effluent and watercress bed irrigation discharge. Two further downstream 100 m reaches on the Bourne Rivulet were surveyed, BRDS1 which lies 380 m from the watercress farm, and BRDS2 which likes approximately 1km downstream of the watercress farm (Figure 9).

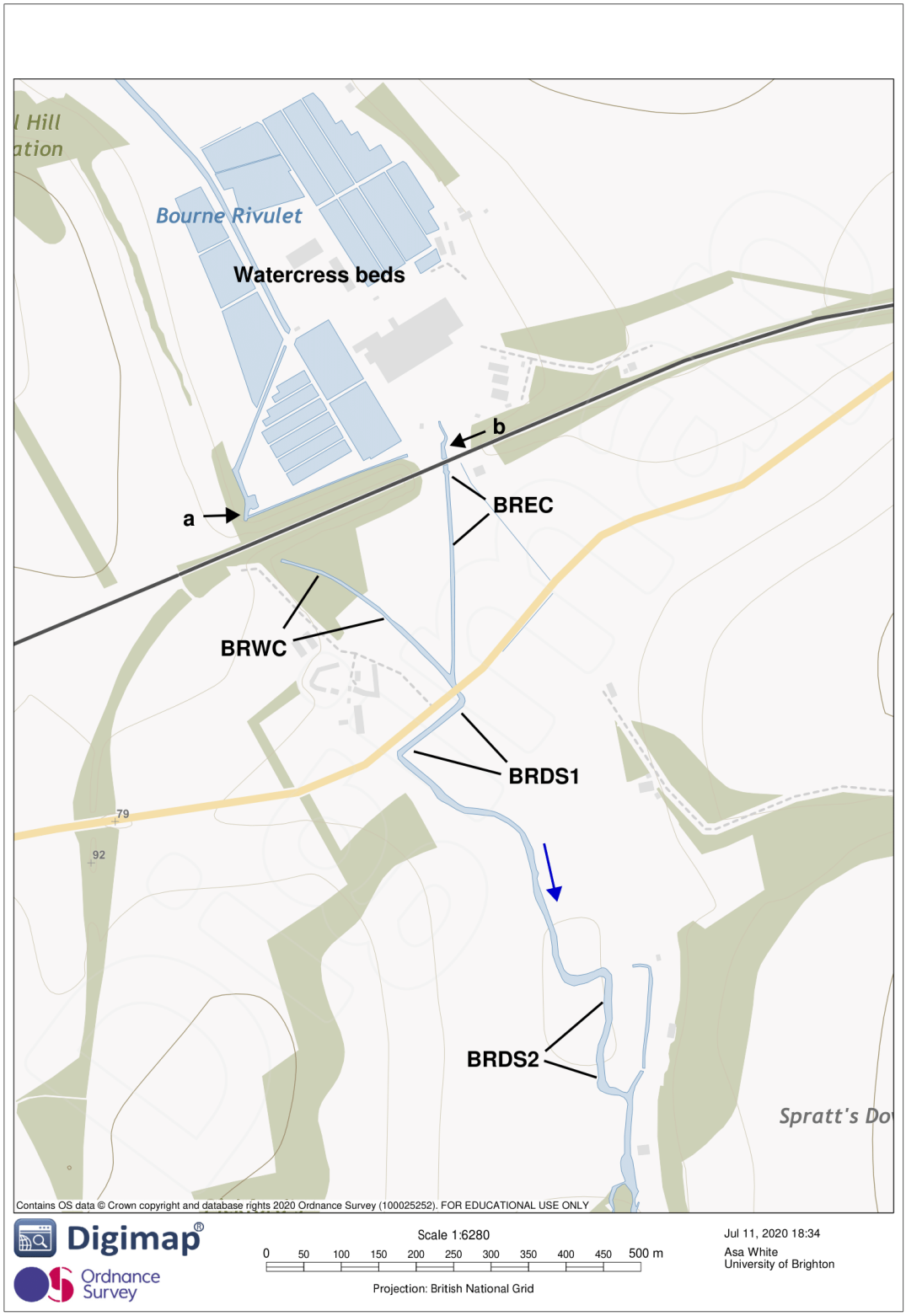

Figure 9 Map of survey sites on The Bourne Rivulet, showing the location of the watercress farm, the west channel discharge point (a) which drains from the watercress beds on the west of into the Bourne Rivulet, the east channel discharge point (b) which drains watercress beds on the east of the farm and contains salad wash effluent from salad washing and packing facility. The 100 m reaches surveyed; BRWC (Bourne Rivulet West Channel), BREC (Bourne Rivulet East Channel, BRDS1 (Bourne Rivulet downstream one) and BRDS2 (Bourne Rivulet downstream two) are displayed

2.2.1.1 Bourne Rivulet West Channel (BRWC)

The Bourne Rivulet west channel (Figure 10) extends from its downstream location of SU 42953 48750 upstream 100 metres to SU 42867 48814. The reach receives water from the irrigation of watercress beds, but unlike the east channel (BREC), it does not receive any salad wash effluent. The irrigated beds that feed into the west channel lie on the western side of the farm and are of a smaller area than those that feed into the BREC. The channel is intermittently shaded by tree, but largely receives direct sunlight promoting the growth of water crowfoot (*Ranunculus spp*.).

Figure 10 BRWC facing upstream from bottom of reach (a) and looking downstream from top of reach (b) in June 2018. Looking upstream from mid reach in October 2017 (c) and looking upstream from below the confluence of BRWC (on the left) and BREC (on the right) soon after annual weedcutting in April 2018 (d)

2.2.1.2 Bourne Rivulet East Channel (BREC)

The Bourne Rivulet east channel (BREC) (Figure 11) extends from SU 43027 48874 upstream 100 m to a point a short distance below where it emerges from under a railway viaduct at SU 43021 48961, some 65 metres from the watercress farm. The east channel was cut off by the construction of the watercress farm early in the 20th century and is now fed entirely from water abstracted by the

watercress farm for irrigation of the watercress beds and salad washing. Prior to 2009, the channel was straight, before gentle meanders were added to create a more natural topography (Figure 11a). In surveys in the present study, the land either side of the channel was dominated by tall grasses and other herbaceous vegetation (Figure 11b), which is occasionally cut by the landowner (Figure 11c). During summer surveys, the tall grasses effectively shaded much of the channel except when the sun was overhead. During the autumn surveys, the channel was completely infilled with *Phragmites australis* (Figure 11d) rendering it unsuitable for electric fishing surveys. Annual weedcutting by the fishery owner in the early spring each year opens up the channel once more.

Figure 11 BREC facing downstream while undergoing channel reprofiling in 2009, the straight and wider profile of the channel can be seen beyond the mechanical digger, and the newly contoured profile in the foreground (Image: Simon Cain, reproduced with permission) (a), looking upstream reach from the furthest downstream point in June 2018 (b), looking upstream from below the confluence with BRWC (on the left) following annual weedcutting in April 2018 (c) and having become infilled with emergent macrophytes in November 2018 (d)

2.2.1.3 Bourne Rivulet downstream one (BRDS1)

Bourne Rivulet downstream one (BRDS1) (Figure 12) has its upstream location 36 metres after the confluence of BRWC and BREC at SU 43041 48648 which is in turn 380m downstream from the watercress farm. It extends for 100 m downstream to SU 42962 48582. The majority of the reach is open to direct sunlight allowing vigorous growth of *Ranunculus*, which is annually cut by the fishery owner.

Figure 12 BRDS1 from the furthest downstream point facing upstream in Oct 2017 (a) and the approximate top 20 metres of the reach in June 2016 (b)

2.2.1.4 Bourne Rivulet downstream two (BRDS2)

Bourne Rivulet downstream two (BRDS2) extends from SU 43232 48133 for 100 metres to SU 43239 48232 (Figure 13). The top of this reach is 952 metres from the watercress farm. Approximately 20% of the reach is shaded by deciduous trees and shrubs. The channel has undergone habitat improvement works, with large woody debris and faggotting (Figure 13c) placed into the channel at various points.

Figure 13 BRDS2 facing upstream from the downstream extent of the reach in November 2018 (a), the downstream part of the reach netted off for electric fishing in June 2017 (b), mid-reach showing faggoting (c) and the upstream extent of the reach facing downstream in November 2018 (d)

2.3 The River Crane

The River Crane in Dorset is located on the upper reaches of the Moors River, which is itself a tributary of the River Stour. The Crane rises on the South Wessex Downs as a winterbourne, becoming a perennial SSSI chalk stream below the village of Cranbourne. The Crane exemplifies a classic chalk stream, with strongly calcareous waters rich in nutrients and of a high water quality (Natural England 1999). Unlike many chalk streams, the River Crane maintains a natural channel topography along much of its length, little modified for agriculture. The valley bottom wetland includes swamp, tall-herb and woodland fen and meadow fen. While many chalk streams have had their riparian vegetation modified with extensive removal of bankside trees, the River Crane maintains extensive bank edge trees, especially alder *Alnus glutinosa* (Natural England 1999). These riparian tree canopies provide shade to reduce in-stream primary productivity, while the occasional breaks in the canopy allow the growth of macrophytes, providing a mosaic of habitats. In-stream tree root and brash tangles which provide cover for fish and invertebrates are an important feature of natural stream ecosystems (Raven et al. 1998). These are abundant on the River Crane.

The River Crane represents the most unspoiled and natural reaches in the present study, though there are some structural modifications of channels outlined in section 2.3.2. The Environment Agency awarded the Crane a grade 'a' for biology between 2007 and 2009 on the basis of biotic index assessments of macroinvertebrates that found the taxonomic composition was greater than expected for a river of its type. The Crane also scored an 'a' for water chemistry every year between 1990 and 2009 except in 1993 and 1994 where it scored a 'b' (Environment Agency 2017).

The fish community of the River Crane is typical of a chalk stream headwater and not particularly diverse. The dominant species is brown trout (*Salmo trutta*), with the gravel beds of the River Crane being the primary spawning ground of this species in the Stour catchment (Natural England 1999). There is limited angling on surveyed reaches of the River Crane, with the estate allowing only one fisher per week. There is no stocking of salmonids for sport, subsequently the *S. trutta* population is an entirely a wild population. Anadromous sea trout are a common feature of the River Crane, and they have been regularly seen on annual spawning migrations along some of the survey reaches. Other species common to the river include bullhead *Cottus gobio* and eel *Anguilla anguilla*, two species of conservation concern, and the brook lamprey *Lampetra planeri* (Natural England 1999).

2.3.1 Sampling sites on The River Crane

Four sites in proximity to discharges from the watercress farm were surveyed (Figure 14), heading downstream they were CRUS (Crane upstream), CRDS1 (Crane downstream one), CRDS2 (Crane downstream two) and CRDS3 (Crane downstream three). There is a small water treatment works (Cranbourne STW) located 1.26 km upstream of the CRUS site. Locating suitable survey sites downstream of the watercress farm on the Crane proved to be challenging due to the unaccommodating terrain. The Crane flows through a woodland river valley featuring swampy fen along most of its length, severely restricting potential survey sites for electric fishing with a boat. This can be observed in woodland around the stream channel downstream of CRDS2 in Figure 14. Consequently, CRDS2 was only 50 m in length, and the farthest downstream site (CRDS3) is 2.4 km from the watercress farm where access to the river with electric fishing gear was possible. This was at a greater distance downstream than the other two surveyed watercress farm outfalls.

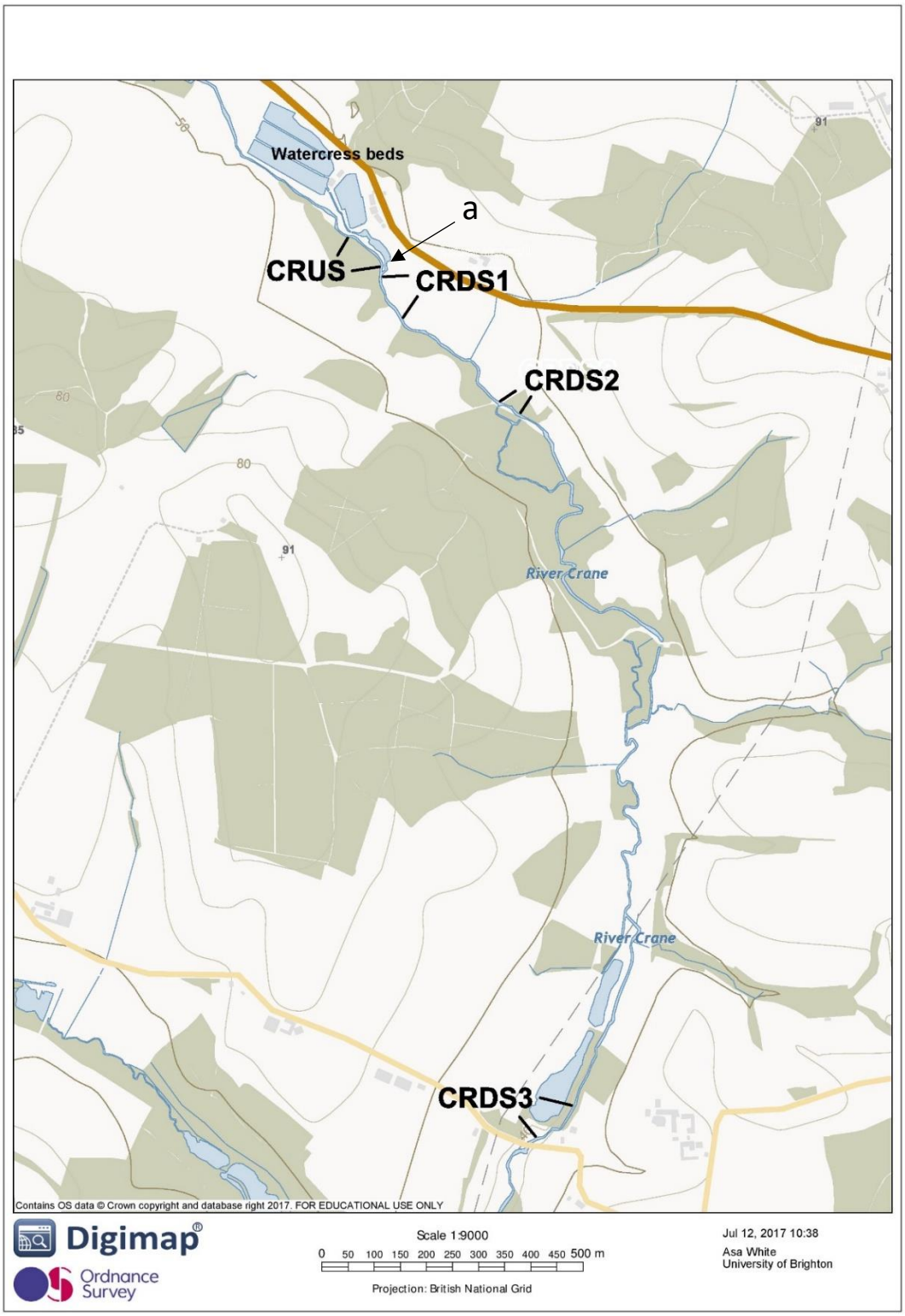

Figure 14. Map of the River Crane showing the location of the four survey sites and the watercress beds. Water leaves the watercress beds through a settlement lagoon, first passing the Environment Agency sampling point (a) before entering the River Crane. The Crane upstream (CRUS), Crane downstream one (CRDS1) and Crane downstream three (CRDS3) are 100 m reaches. Crane downstream two (CRDS2) is a 50 m reach

2.3.1.1 Crane upstream (CRUS)

The discharge from the watercress farm enters the River Crane at SU 07301 12493, after running a short length along a channel leading from a settlement lagoon. The upstream site (CRUS) starts from a point approximately 5 metres upstream from the discharge channel at SU 07301 12502. It extends 100 metres upstream to SU 07233 12565 in a canalised channel with dense riparian vegetation along the length of its right bank, while the left bank is steep sided and faces the watercress farm (Figure 15). The channel, though straightened and steep-sided, has a mix of riffle and pools, and abundant emergent and instream macrophytes.

Figure 15 CRUS channel facing upstream from the far downstream extent of the reach in September 2017 (a) and looking back towards the downstream (b) in June 2018. Thick macrophyte growth in the middle section (c) and (d) both in June 2018

2.3.1.2 Crane downstream one (CRDS1)

The Crane downstream one (CRDS1) (Figure 16) extends from a point roughly 3 to 4 meters below the watercress farm discharge channel at SU 07299 12489, 100 metres downstream to SU 07340 12403. The reach starts just after its emergence from a wood, and for the first the first 40 metres,

this channel is wide and shallow, and banked by grasses which are occasionally grazed by cattle. The cattle have access to the stream here which can occasionally lead to some areas of poaching. The top 60 metres are characterised by a deciduous and herbaceous canopy, with naturally fallen branches and roots providing large quantities large woody debris in the channel.

Figure 16 CRDS1 showing the lower 40 metres of the reach (a) and part of the upper section (b) which is largely under canopy. Both images taken in June 2018

2.3.1.3 Crane downstream two (CRDS2)

The farthest downstream point of CRDS2 (Figure 17) lies 460 m from the watercress discharge point at SU 07567 12221 and extends for 50 metres upstream to SU 07524 12246. Unlike all other sites surveyed which extend for 100 metres, dense impenetrable vegetation upstream and a tunnel and weir at the downstream limit the extent of the reach available to survey. Water is slow flowing, owing largely to two tunnelled outflows at the top and bottom of the reach. Due to the low water velocity, much of the reach is dominated with deep silt substrates, with just the top 20 m faster flowing over gravels and cobbles. Dense tree canopy blocks direct sunlight for much of the reach, with just the top 10-20 metres with more open canopy.

Figure 17 CRDS2 downstream extent looking upstream showing the extensive tree canopy in October 2017 (a) and the top of the reach where the canopy opens (b) in October 2016

2.3.1.4 Crane downstream three (CRDS3)

The furthest downstream site CRDS3 (Figure 18) begins at Pinnock's Moor Bridge 2.4 km downstream from the watercress farm effluent discharge point at SU 07600 10831 and extends 100 metres upstream to SU 07661 10884. This site is located close to an angling club lake, which stocks a range of coarse fish species. There is an overflow channel from the lake which occasionally discharges into the Crane above CRDS3. This reach maintains the appearance of an unmodified chalk stream, with a mosaic of habitats, facilitated by occasional breaks in tree canopy allowing for lush growth of *Ranunculus spp*., well defined pool and riffle habitats and tree root systems and overhang providing extensive cover for fish. This site remains the most natural, physically unmodified chalk stream habitat of any site on any river surveyed in the present study.

Figure 18 CRDS3 downstream extent of the reach at Pinnock's Moor Bridge (a), facing upstream from the downstream extent (b) and middle reaches showing tree canopy shading open channel (c) and open canopy with dense *Ranunculus spp*. growth in July 2017 (d)

2.4 The River Frome

Of all the major chalk streams in Great Britain, the River Frome is the most westerly with a catchment area of 454 km^2. The river rises at Evershot in the Dorset Downs and flows into Poole Harbour via the Wareham Channel. The River Frome is one of the most intensively studied rivers in the UK, with the Freshwater Biological Association's River Laboratory on the lower reaches of the river in East Stoke (Bowes et al. 2011). The River Frome is a long and extensively braided river, with the section under study called variously the North Channel, Waddock Reach and Snelling Farm Reach. This section has been denoted as part of the of the River Frome SSSI and extends from its divergence from the main channel at Pallington Tilting Weir (SY 78748 90953) to its re-joining the main channel 5.1 km downstream at SY 80840 88378. Its channel morphology is heavily modified along much of its length, having historically been affected by digging and dredging and channelization. The hydrology of the North Channel is artificially maintained, with discharge from the watercress farm and water from the main river channel apportioned to the North Channel via the Pallington Tilting Weir. Upstream of the upmost survey site at a distance of 3.7km in Tincleton

lie a further two small watercress farms, though their influence in the North Channel are likely to be extremely marginal, with their discharges diluted by several tributaries before joining the main channel of the Frome. Further, as the North Channel is apportioned from the main channel, the majority of flow bearing the discharge waters from the upstream farms will bypass the North Channel altogether.

Of the three chalk streams surveyed in the present study, the sites on the North Channel of the Frome are the most impacted by anthropogenic activity. A Riverine SSSI unit condition assessment of the Frome by Natural England in 2010 (Brunner et al. 2010) found that water quality was unfavourable along the length of the SSSI designated reach, with the North Channel being no exception. The reason stated was agricultural practices and sewage effluent. Historically, a persistent problem in the North Channel has been the growth of filamentous algae on the substrates due to nutrient enrichment. However, the problem appears to have improved in recent years, possibly due to the closure in the mid-2000s of a fish farm, whose abandoned ponds can be seen above the sampling points in Figure 19. Atlantic Salmon (*Salmo salar*) have historically used the North Channel but have been absent from the reach for many years (Richard Slocock personal comm. 2016). During high rainfall events, field drains contribute significant sediment loading to the North Channel (Brunner et al. 2010).

2.4.1 Sampling sites on the North Channel of the River Frome

There are four 100 m reach study sites on the North Channel of the River Frome (Figure 19). A site upstream of the watercress farm discharge, Frome upstream (FRUS), then one immediately downstream, Frome downstream one (FRDS1), followed by two further sites heading downstream, Frome downstream two (FRDS2) and Frome downstream three (FRDS3). The main channel of the River Frome can be seen to the south of the North Channel in Figure 19, and it is notable how the North Channel's course has been modified by straightening compared to the natural meanders of the southerly half of the main channel.

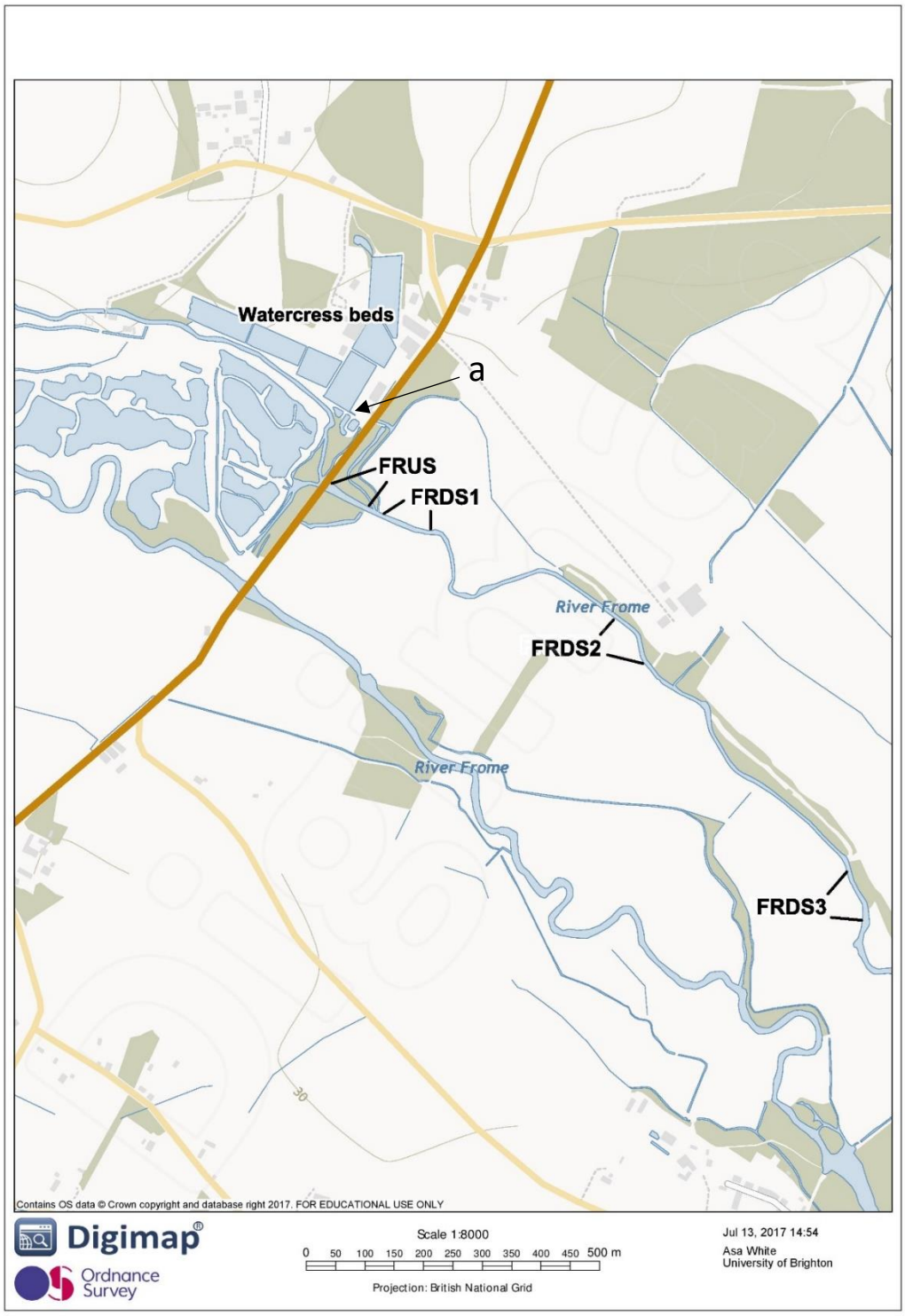

Figure 19. Map of the River Frome showing the watercress farm and the Environment Agency sampling point (a) where the watercress bed irrigation water is sampled. From (a) the water then flows through a tunnel under a road and through a south heading channel before entering the North Cannel of the River Frome. The four 100 m survey reaches on the North Channel, Frome upstream (FRUS) and downstream sites one (FRDS1), two (FRDS2) and three (FRDS3) are marked out

2.4.1.1 Frome upstream (FRUS)

The Frome upstream site FRUS starts approximately 10 metres upstream of the drainage channel from the watercress farm (Figure 20). It extends from SY 79749 90621 for 80 metres to the Hurst Bridge at SY 79679 90661. Heading upstream, the character of the river changes abruptly after 20 metres from one typical of FRDS1 (see below) to a more natural unmodified channel, which is wider, shallower and faster flowing. As a result, the substrate contains a lower percentage area of fine sediments and more gravel and cobble than the lower velocity downstream sites. There are also riffle and pool systems and riparian tree canopy that are lacking in the more modified downstream reaches. Despite there being no agricultural activity on the banks of FRUS, in times of heavy rainfall the reach carries suspended sediment from agricultural activity upstream (Figure 20b).

Figure 20 The River Crane upstream site (FRUS) viewed looking upstream towards its termination at Hurst Bridge (a) in August 2016, and from Hurst Bridge looking downstream in November 2018 following heavy rainfall when the water can be seen to be carrying suspended sediment

2.4.1.2 Frome downstream one (FRDS1)

The site immediately downstream of the watercress farm drainage channel is FRDS1 (Figure 21), which extends from a metre downstream of the discharge at SY 79764 90613 for 100 metres downstream to SY 79859 90582. This reach is highly canalised, being relatively uniform in width, depth, flow and consequently substrate composition. There is no riparian tree canopy to provide shade, and the banks are steep and lined with long grasses. Dairy cattle are grazed on pastures on either side of the reach, but unlike FRDS2 and FRDS3, there are no cattle drinks along its length or upstream, with electric fencing on both banks blocking access to cattle.

Figure 21 FRDS1 looking downstream from the top of the reach in August 2016 (a) and viewed from the downstream point of the reach looking upstream in October 2016 (b)

2.4.1.3 Frome downstream two (FRDS2)

The upstream extent of FRDS2 (Figure 22) lies at SY 80164 90438 which lies 526 metres from the watercress drainage channel and extends 100 m downstream to SY 80218 90358. Similar to FRDS1, this channel is modified by artificial straightening, but unlike FRDS1 it has riparian tree canopy along the length of its right bank and is shallower and wider. The left bank is open and backs onto pasture for a dairy farm. There is a cattle drink 250 m above FRDS2 where the ground is lightly poached which may introduce fine sediments.

Figure 22 FRDS2 facing upstream from the lowest extent of the reach (a) and downstream from the upmost extent of the reach (b) in November 2018. The images were taken following heavy rainfall when and the water darkened with suspended sediments. Typically, suspended sediments are low and the river runs clear

2.4.1.4 Frome downstream three (FRDS3)

The upper extent of FRDS3 (Figure 23) at SY 80567 89994 lies 1.17 km from the watercress drainage channel and extends for 100 metres to SY 80588 89900. Like the other two downstream reaches it is channelized and is more similar to FRDS2 than FRDS1 in width and flow. The last 40 meters have little canopy cover and unlike the other downstream reaches it is more heterogeneous in its depth profile, with pool and riffle habitat. The top 60 metres is similar to FRDS2 in having riparian tree canopy on the right bank while the left is open. There is a major cattle drink 575 m above FRDS3, where the ground is heavily poached (Figure 24).

Figure 23 FRDS3 from the downstream extent facing upstream (a) and from the upstream extent looking downstream (b) in November 2018, following heavy rain when the river had higher than usual river levels and turbidity.

Figure 24 cattle poaching 575 m above site FRDS3 in September 2017 (a) and following heavy rainfall in November 2018 (b). The images highlight the potential for cattle to mobilize fine sediments into the river

2.5 River characteristics

2.5.1 Data collection

Site characteristics were obtained using Ordnance Survey (OS) maps accessed *via* the Digimap website (https://digimap.edina.ac.uk/). The 'measurement tools' function in Digimap was used to calculate the distance from source and tidal limits of sites using an OS map at 1:40 000 scale. Catchment gradients and site gradients were calculated in accordance with guidelines in the UK Invertebrate Sampling and Analysis Procedure for Star Project (EU STAR 2004), as these variables are used to generate predicted macroinvertebrate biotic index scores in chapter three. Discharge categories for the Crane and Frome were obtained using figures quoted in Smith (1992). Discharge categories for the Bourne were estimated from long-term datasets from a gauging weir in close proximity to the sites. Mean widths and depths were calculated from data collected during all HABSCORE surveys as outlined in section 3.3.3.

2.5.2 Comparative summary of key river characteristics and morphology

The distance from source and tidal limit indicates where sites are situated longitudinally on the river, which has implications on channel morphology, physicochemistry, discharge and ultimately the biota present. All three rivers are in lowland settings, ranging from 25-73 m elevation (Table 3). The Crane and Bourne sites are considered upper reaches, being relatively close to their sources at c. 6.4 km and c. 10.7 km respectively, and are first order streams with a Strahler (1957) link number of one. The Frome sites are c. 33 km from the source and with a Strahler link number of ten - having ten first order streams upstream of the sites - can be considered middle reaches.

Table 3 shows that the mean cross section follows the longitudinal location, with the Crane closest to source having the smallest mean cross section, the Bourne being intermediate and the middle reach Frome sites having the largest. The discharge category follows the same pattern, with the smallest mean discharge category on the Crane and largest on the Frome. In Table 4 mean width, depth, cross section and discharge for individual sites are presented. The mean cross sections of each site are similar for each river, with the exception of the Bourne Rivulet east channel (BREC), which as discussed in section 2.2.1 is fed solely from the watercress farm. This reach is approximately one quarter of the size in cross section of the other Bourne sites and has the lowest discharge category.

Table 4 presents classifications obtained from the Environment Agency Catchment Data Explorer (Environment Agency 2019a) which categorises rivers using a five-point classification running from bad, poor, moderate, good and high quality. These classifications are used to distinguish the

environmental condition or status of a waterbody in the EU Water Framework Directive, with the objective of all waterbodies reaching at least ‘good’ status (Visser et al. 2019).

The river morphology of all three rivers was classified as supporting good ecology (Table 4). In terms of macroinvertebrates, all three rivers score the highest classification, indicating high water quality. All three rivers scored ‘high’ in terms of the absence of specific pollutants; those identified as potential pollutants and introduced into legislation by the UK government, and priority substances; a pollutant, or group of pollutants including 'priority hazardous substances', presenting a significant risk to the aquatic environment under Article 16 of the EU Water Framework Directive. In general terms, the EA categorisation suggests that in terms of general physicochemistry, the Crane has the highest quality, though all sites were rated as at least good. Key physicochemical determinands are examined independently in the subsequent section (2.5.3).

Table 3 River characteristics of the Bourne Rivulet, River Crane and River Frome, including the means of all four survey sites of each river for elevation, source distance, tidal limit, catchment gradient, site gradient, discharge category, channel width and depth and channel cross section and the Strahler link number; ordinal variables to indicate location of sites longitudinally on river (reach), the extent of channalisation, the presence of weedcutting, SSSI status and management of fisheries. Water Framework Directive classifications (bad, poor, moderate, good or high) for river morphology, macroinvertebrates, the absence of specific pollutants and priority substances and general physicochemical quality as determined by the Environment Agency (2019b)

	Bourne Rivulet	*River Crane*	*River Frome*
Elevation (m)	72.5	44	25
Source distance (km)	10.75	6.45	33.1
Tidal limit (km)	31.9	25.75	16.4
Stream order (Strahler link number)	1	1	10
Reach (upper, middle or lower)	upper	upper	middle
Catchment gradient (m/km)	3.23	4.66	3.23
Site gradient (m/km)	2.9	3.05	2.63
Discharge category (mean of all sites $n = 4$)	2.25 ± 1	1.75 ± 0.5	3 ± 0
Channel width (m)	5.83 ± 2.36 ($n = 196$)	4.24 ± 1.11 ($n = 179$)	7.03 ± 1.96 ($n = 126$)
Channel depth (cm)	55.32 ± 15.94 ($n = 588$)	29.09 ± 12.69 ($n = 537$)	45.33 ± 18.29 ($n = 380$)
Mean cross section (w x d) (m^2)	2.58	1.23	3.19
Channalisation	high	low	high
Weedcutting	yes	no	yes
SSSI status	yes	yes	yes
Managed fishery	yes	no	yes
Morphology (to support biology)	good	good	good
Macroinvertebrate quality	high	high	high
Specific pollutants and priority substances	high	high	high
Physicochemistry quality	good	good to high	good

Table 4 Site specific elevation, source distance, tidal limits, locations, mean widths, depths and cross section and discharge categories for sites on the Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), the River Crane (CRUS, CRDS1, CRDS2, CRDS3) and the River Frome (FRUS, FRDS1, FRDS2, FRDS3)

Site	*Elevation (m)*	*Source distance (km)*	*Tidal limit (km)*	*Mean width (m)*	*Mean depth (cm)*	*Mean cross section (w x d) (m^2)*	*Discharge category*
BRWC	73	10.7	32	5.52 ± 1.89 (*n* = 55)	46.4 ± 13.7 (*n* = 165)	2.54	2
BREC	73	10.7	32	2.23 ± 0.70 (*n* = 33)	34.2 ± 7.9 (*n* = 99)	0.76	1
BRDS1	72	10.8	31.8	7.04 ± 1.70 (*n* = 53)	47.2 ± 18.1 (*n* = 159)	3.32	3
BRDS2	71	11.4	31.4	7.15 ± 1.47 (*n* = 55)	45.6 ± 17.1 (*n* = 165)	3.26	3
CRUS	46	6.2	26	3.79 ± 0.64 (*n* = 53)	28.8 ± 13.3 (*n* = 159)	1.09	1
CRDS1	44	6.3	25.9	5.12 ± 1.24 (*n* = 54)	24.7 ± 11.4 (*n* = 162)	1.26	2
CRDS2	44	6.6	25.6	4.54 ± 0.92 (*n* = 24)	39 ± 9.7 (*n* = 72)	1.77	2
CRDS3	38	7.3	24.9	3.62 ± 0.69 (*n* = 58)	30.5 ± 12.1 (*n* = 144)	1.10	2
FRUS	26	29.9	17.1	9.58 ± 1.72 (*n* = 27)	27.7 ± 14.8 (*n* = 84)	2.65	3
FRDS1	25	33	16.1	5.73 ± 1.04 (*n* = 33)	51.8 ± 17.1 (*n* = 98)	2.97	3
FRDS2	25	33.2	16.7	6.13 ± 1.19 (*n* = 33)	47.0 ± 15.3 (*n* = 99)	2.88	3
FRDS3	25	34.8	15.1	7.15 ± 1.44 (*n* = 33)	52.2 ± 14.5 (*n* = 99)	3.73	3

2.5.3 Physicochemical

Mean physicochemical determinands of the three rivers are presented in Table 5. Water quality measurements were obtained from Environment Agency (2019) datasets, which contains public sector information licensed under the Open Government Licence v3.0. Readings were chosen from sampling points that were nearest the furthest downstream site of each river. For consistency, sites upstream of watercress farms were not considered as the Bourne Rivulet is a winterbourne upstream of the watercress farm with no dataset available. The sampling station on the Bourne Rivulet, Ironbridge (SU 43463 47382) lies 900m below the furthest downstream site BRDS2. On the Crane the sampling station Romford Bridge was used, which lies 1.6km below CRDS3 at SU 07467 09397. The sampling station Waddock Cross at SY 80739 89693 which lies 330m below the furthest downstream site FRDS3 was use for the Frome.

Categorisation of pH, ammonia, dissolved oxygen and temperature by the Environment Agency (2019b) places all three rivers at the highest status. However, compared to a pristine chalk stream, all three rivers had phosphate levels slightly higher than the <0.02 mg/L for an upper reach and <0.04 mg/L for a middle reach considered ideal for a pristine river (Mainstone 1999). Phosphates were lowest on the Crane, which was categorised as good to high, while both the Bourne and Frome were classified as good. Phosphates are sometimes added to watercress beds, but they may also arise from agricultural run-off (Senthil Kumar et al. 2018).

Unlike ammonia and phosphates, there are no EA guidelines nor classifications for nitrates in surface waters, only for groundwaters. Over the last 100 years, nitrate levels in the chalk aquifers feeding chalk streams have risen due largely to the increased application of inorganic fertilizers and livestock wastes from more intensive farming practices (Limbrick 2003). For example, the aquifer feeding the River Frome between 1894 and 1946 had a mean nitrate level of 1.04 mg/L, which rose between 1976 and 2001 to a mean reading of 6.13 mg/L (Limbrick 2003). This would suggest that the mean levels of 6.25 mg/L between 2010 and 2019 recorded by the EA readings in Table 5 reflect typical contemporary groundwater levels. The Crane had the highest nitrate levels at 8.77 mg/L, which is higher than historical background levels, but still puts the river in the top 25% lowest nitrate surface waters in England, with 75% of English surface waters recording over 10 mg/L and 6% reaching over 50 mg/L (House of Commons Environmental Audit Committee 2018).

Suspended solids were not classified by the EA, but as presented in Table 1 (section 1.2.3), in an ideal near-pristine chalk stream, upper reaches such as the Bourne and Crane would have <2 mg/L. Both rivers slightly exceeded this benchmark, with the Crane most negatively impacted exceeding

it by 4.3 mg/L and the Bourne the least by 1.75 mg/L. Middle reaches of chalk rivers such as the Frome would ideally have <4mg/L, and as it exceeded this measure by just 1.71 mg/L, it appears to be the highest quality in terms of expected suspended solids. However, as noted in section 2.4, the Frome can carry high sediment loads following heavy precipitation. Such sporadic events may have been missed during intermittent sampling by the EA.

Table 5 Mean (±SD) water quality determinands obtained from Environment Agency sampling points downstream of the furthest downstream surveyed sites on the Bourne Rivulet, the River Crane and the River Frome. Means were generated from measurements taken between 2010 and 2019. Target values for an upper chalk stream reach such as the Bourne Rivulet and River Crane and middle reach such as the River Frome are expected values for a near pristine chalk stream (Mainstone 1999). River quality status on a five-point scale (bad, poor, moderate, good and high) as determined by the Environment Agency (2019b

	Target values		*Bourne Rivulet*			*River Crane*			*River Frome*		
	Upper	Middle	*n*	mean	Status	*n*	mean	Status	*n*	mean	Status
pH	7.8-8	7.8	90	8.02 ± 0.21	high	35	8.06 ± 0.13	high	65	8.06 ± 0.16	high
Phosphate* (mg/L)	0.02	0.04	48	0.08 ± 0.07	good	35	0.05 ± 0.04	good - high	38	0.06 ± 0.02	good
Ammonia (mg/L)	0.01	0.03	90	0.03 ± 0.01	high	35	0.03 ± 0.01	high	35	0.04 ± 0.03	high
Nitrate (mg/l)	0.2	0.5	81	6.06 ± 0.62	n/a	35	8.77 ± 1.55	n/a	35	6.25 ± 0.58	n/a
Nitrite (mg/L)	n/a	n/a	90	0.01 ± 0.01	n/a	35	0.02 ± 0.01	n/a	35	0.02 ± 0.01	n/a
Dissolved oxygen (DO) (mg/L)	n/a	n/a	89	11.02 ± 1.06	high	35	10.91 ± 1	high	36	11.09 ± 0.91	high
Suspended solids (SS) (mg/L)	<2	<4	60	3.75 ± 1.39	n/a	17	6.3 ± 3.49	n/a	9	5.71 ± 2.16	n/a
Temperature (°C)	n/a	n/a	65	10.82 ± 2.63	high	36	10.79 ± 3.15	high	36	11.12 ± 3.25	high

** Bourne Rivulet; total inorganic phosphate, River Crane and River Frome; Orthophosphate P reactive; target value, soluble reactive phosphate*

2.6 Watercress production

All three watercress farms under study are conventional producers, using modern techniques allowing for year-round production with peak production occurring in the summer months. There are some differences in the scale of operation, both in terms of the watercress bed area and salad washing. These key differences have been summarised in Table 6, with further details on the particular practices at each farm discussed later in the subsequent sections.

As described in section 2.2.1, the watercress farm on the Bourne has two discrete discharge points, one feeding into the west channel (BRWC) and the other into the east channel (BREC). In contrast, the Crane and Frome farms have just a single discharge point each. The area of watercress beds feeding the Bourne west channel is the smallest of all studied sites at just 1.4 Ha, while the east channel drains the largest area of watercress beds of any site at 5.5 Ha. Between these two in size, the Crane farm drains 3.2 Ha, and the Frome at 2.4 Ha of watercress beds.

The maximum permitted volume of water consented to discharge gives an indication of the typical volume of water discharged, though the actual volume of water discharged will vary temporarily depending on the stage of crop growth. The consented discharge on the Bourne west channel reflects the small area of watercress beds, being the lowest of the four discharges, while the east channel is the highest. Similar to the area covered, the Crane and Frome fall somewhere in between in terms of discharge.

Salad wash effluent is discharged into the Bourne east channel and the Crane only. A volume of 2500 m^3 daily is known to be discharged into the east channel daily. Data for salad wash effluent discharge was not available for the Crane, but it is known that the washing facility operates only sporadically and is much smaller in scale than on the Bourne.

As conventional farms, the discharges from the watercress farms are spot sampled for analysis by the Environment Agency to monitor for any breech of the licenced discharge permits. Data presented in Table 6 was obtained from Environment Agency (2019) fixed sampling points in the outflow/discharge channels of the watercress beds over the last decade. The range of determinands measured were not consistent between sites, with a larger number of determinands recorded on the Bourne than the Crane and Frome. Presented in Table 6 are the determinands that were recorded for all farms, which was suspended solids and pH. Temperature was recorded on the Bourne only, but has been included to highlight that despite water in the east channel running through a much larger area of watercress beds and including salad wash effluent, the mean, minimum and maximum temperatures are very similar to the west channel discharge.

Mean suspended solids were within the 20 mg/L permitted discharge for all three farms. However, on occasion, each farm was found to be discharging above the permitted concentration. This is most apparent on the Crane, for which a concentration of 279 mg/L was once recorded.

Table 6 Comparison of watercress farm discharges into the Bourne Rivulet west and east channels, the River Crane and the River Frome in terms of watercress bed area irrigated, the maximum discharge consented and the quantity of salad wash effluent discharged. Data obtained from monthly sampling of discharge by the Environment Agency is also presented in terms of mean, minimum and maximum levels of suspended solids, pH and temperature. Monthly sampling data for the Bourne Rivulet and River Crane covers the period of 2009 and to 2019, while data for the River Frome was only available for 2009 and 2013

	Bourne Rivulet		*River Crane*	*River Frome*
Discharge point	West Channel	East Channel	-	-
Watercress bed area (Ha)	1.4	5.5	3.2	2.4
Max discharge consent	1140	14438	8727	9400
Salad wash effluent	none	2500 m^3 per day	Intermittent and low volume	none
Environment Agency sampling				
n	127	135	122	46
Mean suspended solids (mg/L)	4.19 ± 5.64	4.7 ± 5.99	6.4 ± 24.99	4.57 ± 5.79
Min Suspended solids (mg/L)	3	3	3	3
Max Suspended solids (mg/L)	58.2	67	279	40.8
Mean pH	7.9 ± 0.39	7.83 ± 0.25	7.72 ± 0.13	7.7 ± 0.22
Min pH	6.98	7.41	7.38	7.2
Max pH	9.3	8.67	8.06	8.21
Mean temp °C (*n* = 65)	11.23 ± 3.03	11.12 ± 2.86	n/a	n/a
Min temp °C	4.87	3.33	n/a	n/a
Max temp °C	18.3	16.9	n/a	n/a

2.6.1 The Bourne Rivulet watercress production

The watercress farm on the Bourne Rivulet is the largest in Europe, with 6.9 hectares of watercress beds. The farm is located on the perennial head of the Bourne Rivulet, and has been producing watercress since it was established in 1904. In recent decades, concern about a decline in *Gammarus pulex* abundances downstream of the farm effluent have resulted in research into the ecological impact of watercress production (Medgett 1998; Worgan and Tyrell 2005; Marsden 2008; Medgett 2008; Cox 2009; Dixon and Shaw 2011; Cotter 2012), and as such, it is arguably one of the most studied watercress farms in the UK.

Water used for irrigation of the watercress beds and salad washing is continuously pumped from 30 boreholes sunk into the underlying chalk aquifer. Peak production occurs through the summer, when watercress is routinely harvested using a self-propelled mechanical harvester. During this period, the watercress beds are cleaned after each harvest, and new crops sown from seedlings raised in modular plugs in a poly tunnel. During this peak growing season (May – September), planting to harvest takes under 30 days. In late summer, autumn and winter, the crops are typically left to re-grow from cut stubbles. To obtain an even growth, the stubbles are mown following harvest – a process known as 'chipping'. During harvest and chipping, water flow through the watercress beds is reduced or temporarily stopped.

Fertilisers are applied in slow-release pellet form comprising a mix of clay, bone-charcoal, poultry ash and rock phosphate. The pellets were once supplemented with liquid fertiliser applied directly to the borehole water at rates appropriate to crop demand, but this practice was discontinued in 2009 in response to concerns about excessive nutrient inputs into chalk streams. At around the same time that liquid fertiliser was discontinued, the application of pesticides and zinc was also discontinued in response to concerns over macroinvertebrate declines (Steve Rothwell, pers. comm. 2018).

Alongside watercress grown onsite, the facility washes and packs watercress grown on other UK watercress farms owned by the operating company. Watercress and rocket make up roughly 40% of the salad washed, both of which may release PEITC. In addition, imported salad leaves from Portugal, Spain, USA and Kenya are washed and packed. Up to 30 metric tonnes of salad is washed per day, using borehole water, which produces up to 2,500 m^3 of salad wash effluent per day during working hours seven days a week, 365 days a year (Steve Rothwell, pers. comm. 2018).

The salad leaves undergo a primary wash in borehole water to remove dirt and foreign material. The salad wash effluent is pumped to a parabolic screen which removes particles >2mm, then to a sediment trap prior to being pumped up to the top of the farm to flow though watercress beds before being released into east channel of the Bourne Rivulet. The salad wash process on the Bourne aims to wash 1kg leaf in 50 L water. An approximately 5% maximum of the leaf tissue becomes loose and is macerated by water pumps and enters the discharge (Steve Rothwell, pers. comm. 2018). Prior to July 2005, the screened and settled salad wash effluent was released directly into the eastern channel. Concerns about low abundances of gammarids in the east channel led to a project whereby watercress beds were trialled for bioremediation of the salad wash effluent. Since July 2005, salad wash effluent has been permanently re-routed through the watercress beds, so that the action of bacteria in the root systems, phytodegradation, phytolysis and exposure to elevated temperatures might degrade PEITC in the discharge effluent (McEldowney et al. 1993;

Cotter 2012). The abundances gammarids in the east channel improved significantly following this action (Medgett and Court 2008; Cotter 2012)

2.6.2 The River Crane watercress production

Information on watercress production on the River Crane site was relatively limited compared to the level of detail the author was able to obtain for production practices on the Bourne Rivulet and River Frome. The watercress farm on The River Crane covers 3.2 hectares of conventionally farmed watercress beds (Cox 2009). Watercress is harvested approximately 2-3 times per year, and no harvesting occurs over winter months. The farm is consented to discharge up to 8727 m^3 of water in any 24hr period.

A packhouse was built in 2014, where watercress grown onsite and occasional watercress crops imported from Spain are washed in borehole water. The spent water from the packhouse is sent directly to a settlement lagoon before discharging into the Crane. Data pertaining to absolute frequency of salad washing and leaf/water volume wash ratios were not available as they were for the salad wash on the Bourne Rivulet. It was advised that salad washing was not a daily occurrence but was performed on an ad-hoc basis when crops were harvested or imported.

2.6.3 The River Frome watercress production

Watercress is produced on the North Channel of the River Frome on the Waddock Cross Farm. This farm has been in existence since the 1930s, and today 22 watercress beds cover 2.4 hectares of conventionally farmed watercress. The discharge limit has been set to 9400 m^3 in 24hrs, and not to exceed 109.4 L/s.

Harvesting typically occurs between the months of May and November, with the frequency of harvest increasing towards the end of the growing season. In total, each bed is harvested between 4-6 times per year. On average, two beds are harvested on any one day using mechanical harvesters on tracks to reduce damage to bed bases. This is always conducted in drained beds. Beds are cleaned twice yearly, and settlement lagoons are in place to allow sediment to settle out before discharging into the North Channel (Ollie Bedford pers. comm. 2019).

Surveying for soluble reactive phosphorus (SRP) discharges by the EA between Feb 2003 and Dec 2004 at the outfall of the settlement lagoon found a mean level of 0.491 mg/L, with a minimum of 0.106 and maximum of 0.491 mg/L, which were well above the minimum SRP target of 0.04 mg/L

(Cox 2009). However, since 2006, the application of phosphate fertilizer has been reduced by the watercress producers by 88%. In addition to the reduction in the use of phosphate fertilisers, the company has also eliminated the use of nitrogen fertilizers in the watercress growing process, with none having been used since 2015 (Ollie Bedford pers. comm. 2019).

Unlike the watercress farms on the Bourne Rivulet and the River Crane, there is no salad washing on the Frome. The watercress crop is packed into crates and sent offsite for washing and packing.

The application of zinc to control crook root was discontinued at the site in 2004. Bti (*Bacillus thuringiensis israelensis*) has been used in the past to control chironomids, but the application of all pesticides has long been discontinued at the farm (Ollie Bedford pers. comm. 2019).

2.7 Surveying schedule

Fieldwork comprised of four main elements; physicochemistry, fish surveys, habitat surveys and macroinvertebrate sampling. The methodology for the techniques used and the rationale behind each can be found in the relevant thesis chapters. The four main elements were conducted in the order listed above to limit the effects of disturbance from one element influencing the next. For example, collection of water samples and deployment of probes were conducted from the riverbank prior to entering the river and disturbing sediments. Fish surveys were conducted before entering the channel to complete habitat surveys which may cause the fish to flee or seek cover. Macroinvertebrate collection was left until last as the kick-sample collection method used causes the greatest sediment disturbance. Where more than one site on a river was surveyed in a single day, the site furthest downstream was surveyed first so that fine sediment released during surveying was not transported downstream to affect subsequently surveyed sites.

Effort was made to survey each site biannually in spring/early summer and in autumn as close to the same dates each year. However, due to the availability of volunteers, access permission and equipment availability, it was not possible to do this precisely. The actual survey dates are presented in Table 7. For ease of interpretation in subsequent chapters, survey dates are referred to by season and year rather than actual dates. In figures and tables, the seasons are described as spring (S) or autumn (A) followed by the last two digits of the year; i.e. S16 for the first surveys in spring 2016 and S18 for the final surveys in spring 2018.

It was possible to survey two sites per day, so each river with four sites was surveyed over two consecutive days where possible. An exception to this was the Bourne Rivulet where access was sometimes limited by the fishery owner during the angling season to one day a week. Therefore,

on this river, surveying in spring 2017 and 2018 was carried out with a week's gap between pairs of sites. CRDS2 was not surveyed in spring 2016 as the site had not been identified at this time. In addition, no sites were surveyed on the Frome in spring 2016 as permission to survey was not obtained until after the spring 2016 season had passed. On the Frome, permission to survey in the spring was only granted before the opening of the fishing season in May. This led to spring surveys on the Frome being earlier in the year than the Bourne Rivulet and River Frome. Following a period of sustained heavy rainfall, river levels and current velocities on the River Frome in spring 2018 were too high to safely enter the river and so all surveying had to be abandoned (Table 8).

Table 7 Survey dates for electric fishing, macroinvertebrate collection and habitat surveys on the Bourne Rivulet; West channel (BRWC), East Channel (BREC) Downstream one (BRDS1) and Downstream two (BRDS2); the River Crane upstream (CRUS) and the three downstream sites (CRDS1, CRDS2 and CRDS3); and the River Frome upstream (FRUS) and the three downstream sites (FRDS1, FRDS2 and FRDS3)

River	*Site*	*Season*				
		S16	*A16*	*S17*	*A17*	*S18*
The Bourne Rivulet	BRWC	7/6/16	5/10/16	20/6/17	8/10/17	26/6/18
	BREC	7/6/16	5/10/16	20/6/17	8/10/17	26/6/18
	BRDS1	8/6/16	4/10/16	27/6/17	8/10/17	19/6/18
	BRDS2	8/6/16	4/10/16	27/6/17	7/10/17	19/6/18
The River Crane	CRUS	14/6/16	13/10/16	17/6/17	30/9/17	24/6/18
	CRDS1	14/6/16	13/10/16	17/6/17	30/9/17	24/6/18
	CRDS2	-	14/10/16	18/6/17	1/10/17	23/6/18
	CRDS3	5/7/16	14/10/16	18/6/17	1/10/17	23/6/18
The River Frome	FRUS	-	19/10/16	30/4/17	15/10/17	-
	FRDS1	-	19/10/16	30/4/17	15/10/17	-
	FRDS2	-	18/10/16	29/4/17	14/10/17	-
	FRDS3	-	18/10/16	29/4/17	14/10/17	-

2.8 Discharge regime

Recruitment of salmonids has been shown to be linked to river discharge levels, particularly around the time of emergence from redds (Solomon and Paterson 1980; Mann et al. 1989; Jensen and Johnsen 1999; Cattanéo et al. 2002; Lobón-Cerviá and Rincón 2004). To ascertain if the surveys during the present study occurred during typical discharge, mean daily discharge data were obtained from gauging weirs spanning the ten years between January 2009 and December 2018. Gauging weirs were chosen that were those closest to the watercress farms on each of the three rivers. On the Bourne Rivulet, this was the gauging weir at Hurstbourne Priors (NGR SU 44100

46290), a site 2.3 km downstream of the furthest downstream site BRDS2. The location of this gauging weir was close to the sites, and not influenced by any tributaries entering the Bourne Rivulet between the sites and the gauging weir. As the Bourne Rivulet sites were close to the perennial head and the flow is groundwater dominated so the discharge recorded at the weir closely reflects the hydrology of the sites. On the River Crane, the station 'Hurn' (NGR SZ 12623 96894) on the Moors River was used, which lies 19.5 km downstream of CRDS3. There are many tributaries entering The Crane and Moors River between the watercress farm sites and the gauging weir, which would increase discharge and will be affected by surface water run-off in times of rainfall. Similarly, there are many tributaries entering the River Frome after the gauging weir at Loudsmill (NGR SY 70810 90350), which lies 11.1 km upstream of the watercress farm sites.

The data from the gauging weirs was obtained through the Environment Agency using a request under the Freedom of Information Act 2000 and Environmental Information Regulations 2004, which contains public sector information licenced under the Open Government Licence v3.0. The data consisted of mean daily discharge in m^3/s. When plotted over time, the Bourne Rivulet had a smooth hydrograph, while the Crane and Frome was considerably more erratic, reflecting the influence of spates generated by surface water run-off. In order to better visualise discharges and even out spates on the latter rivers, the daily means were used to generate average monthly means. For the ten-year period, monthly mean flow, minimum flow and maximum flow was averaged to produce an average annual hydrograph. The monthly mean discharge data for the ten-year period was then overlaid on the mean annual hydrograph in order to visualise how discharges compared with mean values over the ten-year timespan (Figure 25). The black line traces mean discharges over a ten-year period which encompasses the surveys. The gauging weir on the Bourne Rivulet is within the perennial head and is predominantly recording spring water from chalk aquifers, while the River Crane and River Frome have a larger component of run-off influence. Even so, the general patterns are similar across the years for all three rivers, with discharge in 2009 and 2010 on par with the ten-year average, followed by two dry years. The spring of 2013 and 2014 were exceptionally wet years, and these were followed by an average year in 2015, the year preceding the surveys, and in 2016 when the first survey took place. There was below average discharge in 2017 during the surveying, and discharges returned to average in 2018.

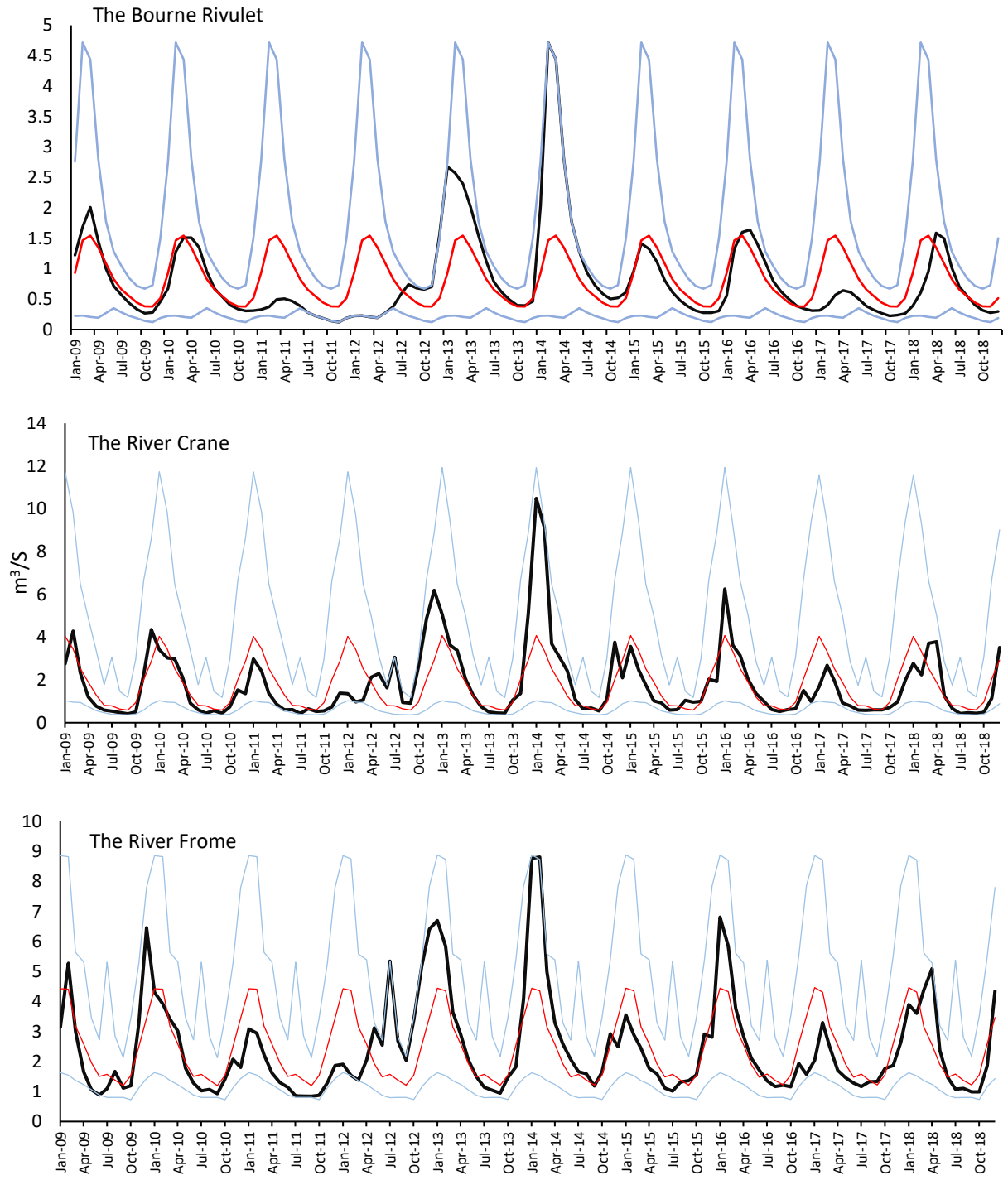

Figure 25 Hydrographs of mean monthly discharge (m^3/S) recorded at gauging weirs on the Bourne Rivulet, the River Crane and the River Frome between January 1st 2009 and December 31st 2018. The thick black line is the mean monthly discharge of the whole ten-year period. This is overlaid onto the mean annual hydrograph for the ten-year period so that deviations from typical flow can be observed. The red line is the average annual discharge and the blue lines the minimum and maximum annual flows over the ten-year period.

THE IMPACT OF WATERCRESS FARM DISCHARGES ON STREAM HABITAT AND MACROINVERTEBRATES

3.1 Introduction

Pollutants emanating from a range of anthropogenic activities exert stressors on riverine ecosystems (Dudgeon et al. 2006). It has been widely-recognised that effective monitoring of stream health is vital to determine the extent of impact of anthropogenic activities (Herman and Nejadhashemi 2015). The health of streams can be broadly examined with three components; chemical, physical and biological integrity (Butcher et al. 2003). Chemical monitoring may be employed, whereby spot water samples are taken for analysis of chemical composition to identify pollutants. Field measurements for water quality evaluation typically involve the deployment of probes and collection of water samples followed by transportation of samples to laboratories for subsequent analysis (Glasgow et al. 2004). As such sampling is costly and labour intensive, it can only be feasibly be carried out on an intermittent basis. For example, the Environment Agency gather water quality data from over 7000 monitoring sites across England, but typically on monthly basis, or at best a fortnightly interval (Bowes et al. 2009). The intermittent nature of spot sampling is therefore very prone to missing pulsed or one-off releases of pollutants in riverine systems (Glasgow et al. 2004; Norris and Barbour 2009).

More recently, in-situ water quality analysis stations are becoming available which can provide continuous data on the current chemical status of rivers accessed in real-time online (Glasgow et al. 2004; Meyer et al. 2019). Such measuring stations overcome the issues of intermittency affecting spot testing regimes (Bowes et al. 2009). However, to be reliable and account for instrumentation drift, such devices need regular calibration, some of which may be automated, but this can result in large and costly instillations (Meyer et al. 2019). In-situ stations are therefore best situated strategically in locations that are important for water quality and known hot spots for fish kills, harmful algal blooms and oxygen deficits (Glasgow et al. 2004). Real-time online analysis of river water chemistry is therefore not yet able to compete with spot testing of water quality in terms of widespread national coverage.

The monitoring of fauna has some distinct advantages over spot testing of water chemistry parameters, and the use of biological monitoring programmes to evaluate the health of fluvial systems is now widely employed by researchers, consultancies and environment agencies (Sharma and Rawat 2009; Pelletier et al. 2012; Clarke and Davey-Bowker 2014; Herman and Nejadhashemi

2015; Fierro et al. 2017). As biological factors are influenced by both chemical and physical characteristics, and both will interact, the fauna at a given site will reflect accumulative and additive effects of chemicals and pollutants (Herman and Nejadhashemi 2015; Fierro et al. 2017). Moreover, the use of fauna as indicators of habitat health provides a direct assessment of the ecological state of a system, rather than inferring perturbations based on physicochemistry (Kalogianni et al. 2017; Meyer et al. 2019).

Of all fauna, macroinvertebrates are the most widely used for assessing habitat quality in streams and rivers (Herman and Nejadhashemi 2015). Responding rapidly to environmental stress they are often the first ecological indicator to react to changes in the environment (Metcalfe 1989; Feeley et al. 2012; Clarke and Davy-Bowker 2014). Moreover, the limited longitudinal movement of macroinvertebrates within the stream channel aids in pin-pointing localised sources of degradation (Kerans and Karr 1994). Macroinvertebrate communities respond to pollutants or habitat degradation with an increase in the abundance of pollution-tolerant taxa, which typically include chironomids and oligochaetes, and a concomitant decrease in abundance and diversity of pollution-sensitive taxa such as Ephemeroptera, Plecoptera and Trichoptera (EPT) (Gücker et al. 2006; Berger et al. 2018; Mezgebu et al. 2019). The relative abundances of the range of taxa present at a given site can therefore be used to assess habitat quality and signal the presence of pollutants (Norris and Barbour 2009; Herman and Nejadhashemi 2015). As macroinvertebrate assemblages within a stream channel are subjected to and react to both pulsed and chronic releases of pollutants, the use of macroinvertebrates as bioindicators circumvents the issues of intermittency associated with chemical spot testing (Fierro et al. 2017). For example, a chemical spill or intermittently discharging outflow pipe into a river will only be picked up in a spot test if they happen to coincide. However, the legacy of such an event may be detected in macroinvertebrate assemblages if it results in mortality to pollution-sensitive taxa. Unlike chemical sampling, the use macroinvertebrates as bioindicators is not well suited to ascertain the identity of a pollutant (Berger et al. 2018). However, the use of macroinvertebrate surveying allows environmental resource managers to readily identify degraded areas to allocate resources to identify sources of degradation and to put in action to address them (Butcher et al. 2003; Walters et al. 2009; Einheuser et al. 2012; Pelletier et al. 2012).

3.1.1 Macroinvertebrate biotic indices

Biotic indices are widely used to evaluate and track changes in the environment and to define the ecological health of biological systems (Friberg 2010; Berger et al. 2018). By condensing the large quantities of data gathered during biological surveys into numerical form (Smith 1992), biotic indices simplify both temporal and spatial comparisons of the ecological health of a habitat.

Before macroinvertebrate biotic indices can be calculated, the organisms must first be collected in a standardised manner to ensure data consistency. Standardised protocols have been developed which may vary between global regions and habitat type (Herman and Nejadhashemi 2015). In Europe, the standard protocol for wadeable waterways takes the form of timed kick sampling (see section 3.3.4). Samples are typically collected over two or more seasons to determine the year round condition of a river (Neumann et al. 2003).

Due to the utility and frequent use of macroinvertebrates in biotic assessments, many biotic indices have been developed to monitor stream health (Flinders et al. 2008; Sharma and Rawat 2009; Pelletier et al. 2012; Herman and Nejadhashemi 2015). Herman and Nejadhashemi (2015) review a total of 41 macroinvertebrate biotic indices, all of which are modifications of four common indices; the Benthic Index of Biotic Integrity (B-IBI), Hilsenhoff Biotic Index (HBI), Ephemeroptera, Plecoptera, Trichoptera (EPT) Index, and the Biological Monitoring Working Party Index (BMWP). Of these, the most commonly used base index is the EPT index. Due to their sensitivity to a range of stressors, such as organic loading from WWTP discharges (Quinn and Hickey 1993) and heavy metals from mining activities (Wright and Ryan 2016), EPT have been widely used in bioassessment. The EPT index is calculated as sum of unique EPT taxa in a sample (Barbour et al. 1996). In addition to EPT taxon richness, the abundance of all EPT taxa are often quantified. However, this does not give indication of the proportion of EPT in a sample. To address this, the percentage abundance of EPT taxa in composite samples have often been employed (Weigel et al. 2002; Klemm et al. 2003). EPT indexes are effective and are often used due to their simplicity, but they naturally take no account of the full range of taxa in samples, nor have sample collection techniques been standardised to allow robust comparisons between studies. However, when used in standalone studies, they are a powerful tool to compare degradation between sites (Barbour et al. 1996).

The two most commonly employed and nationally-recognised standardised biotic indices in use in UK waters are BWMP and its more recent derivation, Walley, Hawkes Paisley Trigg (WHPT) (Clarke and Davy-Bowker 2014). The BMWP score system was developed by the UK Biological Monitoring Working Party in 1978 to evaluate stream health in England and Wales (Chesters 1980; Paisley et al. 2014). To develop the model, the general organic pollution tolerances of macroinvertebrate taxa were determined through questionnaires, surveys and discussion of a panel of experts (Chesters 1980; Hawkes 1998). The BMWP provides pollution tolerance rankings for 82 different macroinvertebrate taxa from 0 to 10 with 10 being the most pollution-sensitive, and 0 being the most pollution-tolerant taxa (Chesters 1980; Hawkes 1998). The scores were chiefly based on the dissolved oxygen tolerances of macroinvertebrate taxa, as the release of a broad range of organic effluents typically results in oxygen depletion via increased O_2 uptake by microbial communities (Hawkes 1998; Dang et al. 2009).

In order to calculate BMWP, macroinvertebrate samples are identified to family level (Paisley et al. 2014). The BMWP score is calculated as the sum of the tolerance scores of all macroinvertebrate families in the sample, with higher scores indicating higher quality sites. The BMWP score is sometimes further categorised into classes to provide easy categorisation of sites, such as poor, moderate, good and excellent (Herman and Nejadhashemi 2015). In addition to the BMWP score, the BMWP index is typically calculated, being the average score per taxon (ASPT), generated by dividing the sum of scores of all taxa present by the number of taxa present (NTAXA). ASPT is the preferred metric by many biologists as it removes the dependence on sample size and limits variability due to seasonal factors and sampling effort and sample processing (Walley and Hawkes 1996). This score will range from 0-10, with higher scores equating to higher quality sites. As the worker needs only identify the presence of macroinvertebrate families in a sample, with experience, running BMWP is a relatively straightforward and rapid process. Moreover, the BMWP values assigned to each family are a useful metric when discussing the relative tolerance of macroinvertebrates to general organic pollution.

A drawback of the BMWP method is that it only considers the presence of invertebrates in a sample. Walley, Hawkes Paisley Trigg (WHPT) is a successor to BMWP and improves the precision by providing scores based on the abundance of each taxon, rather than just its presence. It is therefore able to respond to perturbations that affect the abundances of different taxa, which improves its ability to track smaller degradations in river quality (Paisley et al. 2014). Moreover, WHPT is derived from large sets of field data (in excess of 100,000 kick samples) rather than a reliance of expert opinion (Paisley et al. 2014). In addition, the number of scored taxa were expanded for WHPT, where BMWP grouped some families together into composite taxa. As such, WHPT metrics are considered more robust and accurate than BMWP (Clarke and Davy-Bowker 2014). WHPT is considered one of the most well-developed biomonitoring tools in the world, and is now the standard biotic index for European Union Water Framework (WFD) river classification (Clarke and Davy-Bowker 2014; Wilkes et al. 2017).

Multivariate indices such as BMWP and WHPT provide good evidence of general degradation, but they have limited capacity in identifying the stressor or stressors causing the degradation (Böhmer et al. 2004; Berger et al. 2018). In many rivers, multiple stressors may be acting on a single habitat (Friberg 2010; Schäfer et al. 2016). Few stressor-specific indices have been developed to attempt to monitor specific stressor gradients and there have been instances of stressor-specific indices responding to non-target stressors (Rasmussen et al. 2012; Mondy et al. 2012). However, a stressor-specific index which is regularly used in the UK by regulatory authorities and considered robust is the Proportion of Sediment-sensitive Index (PSI) (Everall et al. 2017; Extence et al. 2017). Macroinvertebrates have a varying range of tolerance for fine sediments, with some taxa

responding negatively through clogging of sensitive gills, increases in drift and reductions in oxygen and food availability and quality (Jones et al. 2012; Graeber et al. 2017). Conversely, fine sediments are favoured habitats of some taxa, with abundances positively impacted (Kreutzweiser et al. 2005; Jones et al. 2012). The PSI index quantifies the percentage of sediment-sensitive taxa in a sample and has been used as a proxy for the extent of fine sediment deposition at a given site (Seeney et al. 2019; Aspin et al. 2020). To the authors knowledge it has not yet been applied to sites receiving watercress farm effluent and may prove informative when used in conjunction with WHPT to disentangle the effects of fine sediment from organic pollution more broadly.

3.1.2 Contextualising biotic indices: RIVPACS and RICT

There is a need to take into account a rivers location, channel morphology, substrate and velocity when interpreting water quality attributes from macroinvertebrate assemblages using biotic indices. For example, pristine rivers of low velocity will often have fewer sensitive macroinvertebrates and therefore generate lower biotic index scores than similarly pristine high velocity sites (Hawkes 1998). Without reference to a rivers' hydrology and geographical location, there is a danger, for example, of erroneously characterising a sluggish river as being impacted from biotic index scores alone. The River Invertebrate Prediction and Classification System (RIVPACS) is a multivariate model which addresses this issue. RIVPACS relates macroinvertebrate diversity to 30 physical and chemical features within 685 reference streams in the UK. Reference sites are assigned to 43 sites types in the UK, with inter-relationships between macroinvertebrates and habitat characteristics summarised using cluster analysis (Clarke and Davy-Bowker 2014). Based on known physical and chemical features, the RIVPACS model can be used to predict the composition of organisms that would be expected to appear in a stream in the absence of environmental stress (Wright et al. 1998). Due to the complexity of the model, and to allow continued development, RIVPACS has been incorporated into a web-based application accessed online via the Scottish Environmental Protection Agency (SEPA) called the River Invertebrate Classification Tool (RICT) (Clarke and Davy-Bowker 2014). RICT is able to generate expected scores for a range of common biotic indices such as BMWP, WHPT and PSI. Division of the Observed score with the Expected score (O/E) generated by RICT can then be used to evaluate stream condition; with a score of one indicating parity, scores <1 indicating habitat degradation and scores >1 indicating the macroinvertebrate assemblages reflect high quality habitat (Herman and Nejadhashemi 2015).

3.1.3 Macroinvertebrates and watercress farm discharges

Discharges from watercress farms may be considered a point source of pollution into chalk streams. Studies have shown that discharges from watercress farms may increase levels of suspended solids

(Cox 2009), alter the hydrology (Casey and Smith 1994), increase nutrient levels (Casey et al. 1993; Cox 2009), introduce pesticides (Cox 2009) and introduce phenethyl isothiocyanate (PEITC) (Newman et al. 1990; Kerfoot et al. 1998; Dixon and Shaw 2011; Ntalli at al. 2017). The extent and direction to which these stressors may impact on the habitat and biota of chalk streams is discussed in section 1.5.1.

There is evidence suggesting that discharges from watercress farms have led to altered macroinvertebrate assemblages in chalk streams (Roddie et al. 1992; Mainstone 1999; Medgett and Court 2008; Cox 2009; Dixon and Shaw 2011). The most comprehensive study on British chalk streams was undertaken by Smith (1992) which encompassed surveys up and downstream of 15 watercress farms. The study found macroinvertebrate fauna at sites immediately below farm discharges generally changed from one associated with eroding substratum to depositing substratum and so pointed to sedimentation of substrates as the primary causative agent. It also highlighted a decrease of, and in a few cases, complete absence of Gammaridae and Elmidae and a number of Trichoptera families below watercress farms. However, Smith's (1992) study predates the full implementation of The Water Resources Act (1991) bought in by the National Rivers Authority, which set stricter limits on sediment, fertiliser, zinc and chlorine in discharges. More recently, the Environment Agency handles and have further strengthened discharge consents.

Medgett and Court (2008) studied the macroinvertebrate assemblages on the Bourne Rivulet between May 2004 and November 2007. On this farm, the east channel receives salad wash effluent and is comprised entirely by discharge water from watercress production, while the west channel receives a small proportion of its flow from watercress beds. In the eastern channel, taxa tolerant of organic pollution such as Oligochaetes, Hirudinea and Asellidae were found in high abundances, while taxa indicating good water quality such EPT and gammarids were extremely low or often absent. There may be many causative agents altering macroinvertebrate assemblages, such as increases in siltation, which creates unfavourable habitat for many pollution-sensitive taxa (Jones et al. 2012). However, a number of studies on the effect of PEITC on gammarids indicate its toxicity to this ecologically important macroinvertebrate (Newman et al. 1990; Newman et al. 1996; Kerfoot et al. 1998; Dixon and Shaw 2011). Dixon (2010) sited caged *G. pulex* at various locations around a watercress farm on The Bourne Rivulet and found *G. pulex* mortality was significantly higher in salad wash effluent than in reference borehole water. Dixon (2010) noted a strong smell of PEITC in the salad wash effluent and concluded that PEITC was the most likely causative agent for the Gammarid mortality. A study by Marsden (2008) found that the effect extended to the Bourne Rivulet east channel where salad wash was ultimately discharged, as caged gammarid mortality was significantly increased compared to those placed in control reaches. While research into PEITC toxicity to macroinvertebrates has tended to focus on gammarids, they may be more tolerant to PEITC than

other macroinvertebrates. For example, Newman et al. (1990) calculated the 48 hr LC50 for *Gammarus pseudolimnaeus* between 0.96 and 3.62 mg/L, which is considerably higher than the published 48 hr LC50 for *Daphnia magna* of 0.13 mg/L (Sigma Aldrich). This suggests that PEITC release into chalk streams may have a wider impact on biota than previously considered.

Macroinvertebrates play a key role in nutrient cycling in streams and in aquatic/terrestrial energy subsidies (Wallace and Webster 1996; Marcarelli et al. 2011). The abundance and species richness of many insects, including those in the aquatic orders EPT and Odonata have been in decline in Europe since the 1950s (Wagner 2020). Being consumers at intermediate trophic levels, macroinvertebrates consume primary producers, and in turn constitute a vital source of food for numerous fish, bird, mammal and amphibian species (Wallace and Webster 1996). Therefore, maintaining macroinvertebrate diversity and individual species populations contributes to the integrity of stream ecosystems (Spänhoff and Arle 2007). It is therefore essential that chalk streams are monitored to ensure that they have adequate diversity and abundance of macroinvertebrates to sustain ecosystem function of these globally important habitats.

3.2 Aims and objectives

The aim of this study was to assess the impact of watercress bed irrigation and salad wash effluents emanating from watercress farms on physicochemistry, river habitat and macroinvertebrate assemblages. The breadth of available analysis to assess such impacts is great. Therefore, the present study primarily focused on factors that have the potential to impact on the capacity of sites to support fish populations through changes in physicochemistry, habitat and macroinvertebrate abundances.

Physicochemical, habitat and macroinvertebrate surveys were conducted around the discharges of three watercress farms. Each downstream site either had watercress bed irrigation effluent only, or a combination of watercress bed irrigation and salad wash effluent. Sites upstream acted as reference sites, while sites further downstream were used to assess the extent or recovery of any perturbation. Impacts from discharges was examined in terms of the following:

i) Changes in dissolved oxygen, pH, conductivity and suspended solids
ii) Habitat variables known to predict and support fish populations, to be related to observed fish populations in chapter four
iii) Macroinvertebrate abundance, with focus on gammarid shrimp, an important dietary component of chalk stream fish
iv) Composition and diversity of macroinvertebrate assemblages, with focus of the abundances of pollution-sensitive taxa and pollution tolerant taxa

v) The presence of organic pollution stress, evaluated by comparing observed biotic index scores to those predicted using RICT/RIVPACS methodology

3.3 Methods

3.3.1 Fieldwork

Surveying was carried out on three river systems described in chapter two; the Bourne Rivulet, the River Crane and the River Frome (hereon referred to in text as the Bourne, the Crane and the Frome respectively). Surveys were conducted twice yearly, with exact dates described in chapter two section 2.6. For convenience, heron the biannual surveys are described season (spring or autumn) and year. For example, surveys in spring 2016 become 'S16'.

3.3.2 Physicochemistry

Water quality readings were taken from the stream bank at each site prior to the start of surveying to avoid disturbing the stream substrate. A single measurement per site per survey of dissolved oxygen, conductivity, temperature and pH were recorded using electronic probes. Dissolved oxygen levels were recorded in mg/L to an accuracy of 0.01 mg/L using a Hanna H19142 probe (USA). Conductivity was recorded in µS/cm to an accuracy of 0.1 µS/cm using a Mettler Toledo Seven Go conductivity meter (Columbus, USA). Temperature was recorded in Celsius to an accuracy of 0.1 °C using the Mettler Toledo Seven Go conductivity meter. A Camlab CW/6110 pH meter (Cambridge, UK) was used to record pH to an accuracy of 0.01.

Following deployment of the probes, three replicate 1L PET bottles of stream water were collected from the bank of each site. These were returned to the laboratory for suspended solids analysis.

Water velocities were recorded at three random points in the channel of each site using a Valeport 801 Electromagnetic Open Channel flow meter (Totnes, UK) set to produce a mean velocity in m/sec from 30 seconds recording.

3.3.3 Habitat

Instream habitat surveys were conducted using the HABSCORE methodology (Milner et al. 1998) HABSCORE is an empirical habitat-fish model developed to aid the interpretation of salmonid data and is frequently used for environmental impact assessments (Armstrong et al. 2003; Cowx et al. 2009). The system is based on a series of statistical models relating populations of salmonids to observed habitat variables. The use of HABSCORE served a dual purpose in the present study. The

survey data was used to gather general habitat variables to investigate differences between sites and relate them to macroinvertebrate assemblages in the present chapter. In chapter four, the use of HABSCORE software outputs is used to compare expected salmonid densities with the observed densities.

Habitat variables were recorded for the whole length of each site following electric fishing surveys using a HAB*form* (example presented in appendix 1). The habitat variables presented and/or analysed in the present study are listed below, together with a brief description of the method of data collection.

3.3.3.1 Reach dimensions

Reach length was recorded in a series of 10m sections starting at the downstream extent of the site and moving to its upstream termination, which in practice was where stop nets were set. A 100m reach length was aimed for, but in a small number of instances this was not possible due to physical obstructions. At each 10m section, the wetted stream width and three equally spaced depth measurements at ¼, ½ and ¾ of the channel width were made.

3.3.3.2 Substrate

Six substrate categories are recorded on the HAB*form*; bedrock/artificial, boulders >25.6cm, cobbles 6.4-25.6cm, gravel/coarse sand 0.2-6.4cm, fine sand/silt <0.2cm. A visual estimate was made for each 10m section of river, with an abundance category recorded using the ASCFD method (Table 8)

Table 8. ASCFD coding for abundance categories

	Estimated % of bed area *within the section*				
	zero	>0% : <5%	≥5% : <20%	≥20% : <50%	≥50%
Classification	Absent	Scarce	Common	Frequent	Dominant
Coding	*A*	*S*	*C*	*F*	*D*

To obtain percentage cover data for subsequent analysis, the median value of each abundance category recorded by HABSCORE was used as the input value (e.g. absent (0%), scarce (2.5%), common (12.5%), frequent (35%) or dominant (75%), after Angelopoulos et al. (2018). Where only 'dominant' was recorded then that category was the only present, so a value of 100% was input. Categories other than 'dominant' when 'dominant' was present were considered as full median

value and the value for 'dominant' was the value to make up 100%. For example, where 'dominant' and 'frequent' were recorded, 'frequent was considered 35% and 'dominant' 65%. A mean of the input values for the 10 m sections was generated for each substrate type at each site.

3.3.3.3 Instream features

Instream features are categorised as sources of cover for >10cm trout in the HABSCORE survey methodology. In practice, this equated to the presence of such submerged features, which were estimated as the percentage cover in each 10 m section of river. The total percentage of cover available for >10cm trout was recorded for the following categories; submerged vegetation, boulders and cobbles, tree root systems, branches and logs, undercut banks and areas of deep water.

Large Woody Debris (LWD) is an important habitat for macroinvertebrates (Gustafsson et al. 2014) and was analysed by adding together percentage coverage of 'tree root systems' and 'branches and logs' gathered during HABSCORE surveys and recording as LWD. As boulders and cobbles as a category are included for analysis under substrate, and areas of deep water were analysed under channel depth, these variables were not included twice to avoid repetition and autocorrelation in further analysis.

3.3.3.4 Riparian shading of the reach

Riparian vegetation was recorded as the percentage area of the reach's water surface which would be obscured if viewed from directly above. Such overhanging vegetation is an important source of terrestrial invertebrate prey for adult salmonids (Kawaguchi and Nakano 2001; Dineen et al. 2007). Two forms of riparian shading were analysed. Firstly, overhang, which was any vegetation overhanging the channel within 0.5m of the water surface. This was estimated as a percentage for each 10 m section. Secondly, the percentage of deciduous tree and shrub canopy cover was estimated for the whole reach as per the HABSCORE methodology.

3.3.4 Macroinvertebrate collection

There are a wide range of available methods for macroinvertebrate collection in rivers. The three most commonly applied are kick-nets, Surber and Hess samplers (Buss et al. 2015). Surber (1937) and Hess (1941) samplers are quantitative, extracting benthic macroinvertebrates from a fixed area of streambed. The fixed area of streambed is demarcated by the apparatus, with the substrate agitated by the operator, suspending the invertebrates into the water column to be transported by the water current into an attached net. The advantage of such quantitative methods is that they allow density estimates of captured macroinvertebrates. However, as Surber and Hess samplers

sample just 0.09 m^2 of streambed at a time, extrapolating sample data to a square metre, and further to the river scale can lead to overestimation of species abundance and diversity due to the patchy distribution of benthic macroinvertebrates in streams (Ghani et al. 2016).

Kick sampling involves disturbing the substrate with the foot upstream of a collection net, which collects the dislodged and drifting benthic macroinvertebrates. A standardised technique has been developed for use in a range of biotic index assessments (Buss et al. 2015). The operator 'kicks' for 15-20 seconds in one spot before moving diagonally upstream to another spot close by to repeat the process, thereby covering a wide area of the streambed for a timed three minutes. A one-minute manual search collects macroinvertebrates attached to rocks, tree roots, macrophytes and other submerged objects (EU STAR 2004). In contrast to Hess and Surber sampling, kick sampling is considered qualitative or semi-quantitative. However, an advantage is that it covers a greater area of stream bed and incorporates collection from instream features. Studies have found that kick sampling collects more taxa and enables more accurate biotic index values to be calculated than Surber and Hess samplers (Mackey et al. 1984; Buss and Borges 2008; Ghani et al. 2016).

The standardised kick sample method described was chosen for the present study for the following reasons:

- The macroinvertebrate data could be used to generate biotic index scores which could be compared between upstream and downstream sites. Moreover, biotic index scores could be compared with estimated biotic index scores generated using RIVPACS (see section 3.1.2) to assess if macroinvertebrate assemblages reflected degradation from organic pollution
- As Surber and Hess samples extract only benthic macroinvertebrates and the present study will assess macroinvertebrates in terms of potential prey availability to fish, the one-minute manual collection technique incorporated in the kick sample protocol would better sample the range of macroinvertebrates available to insectivorous fish

Macroinvertebrate collection followed guidelines set out in the RIVPACS macroinvertebrate sampling protocol (EU STAR 2004) and used nationally by the Environment Agency (Buss et al. 2015). Using an industry standard EFE&GB Professional hand net (EFE&GB, Lostwithiel, UK), the time spent kick sampling various substrate types was proportional to their occurrence within the site. For example, if the site had an estimated 75% gravel and 25% silt, then 135 seconds were spent kick sampling gravel and 45 seconds kick sampling the silt. Similarly, the proportion of time spent manually searching macrophytes and other submerged objects was proportional to their occurrence within the channel. A single kick and manual sweep sample was taken at each site on

each survey date. To obtain consistency in sampling efficiency, the author undertook every kick and sweep sample, with a volunteer on the riverbank using a stopwatch to ensure accurate time allocation.

The net contents were transferred into 1L wide-necked plastic bottles, and the net rinsed through with water until the bottle was approximately 90% full. 100 ml of 38% formalin was then added, the bottle sealed, and the contents inverted a few times to distribute the formalin and produce an approximate 4% formalin fixative for the contents. The samples were allowed to fix for at least a week to harden macroinvertebrate cuticles before the contents were transferred to 70% alcohol.

3.3.5 Laboratory

3.3.5.1 Suspended solids

The 1L bottles of stream water were filtered through pre-dried and weighed 1.2 µm pore filter papers (Fisherbrand™ Microglass Fibre Filters, Grade 261). The samples were then reweighed following drying to a constant weight in an oven at 70 °C for 48 hrs, with the original filter paper mass subtracted from the results. Weighing of the filter papers before and after filtering was performed on a Sartorius AZ124 Analytical Balance (Germany) to four decimal places.

The suspended solid data for the Crane and the Bourne for the first surveys in Spring 2016 were unusable. During this first survey the samples were frozen immediately on return to the laboratory to avoid microbial breakdown of particulate organic matter before filtering. However, due to the high calcium content of these samples, the freezing had precipitated calcium carbonate which became trapped in the filter paper, giving inaccurately high measurements. All further samples were kept cool and returned to the lab to be filtered upon arrival.

3.3.5.2 Macroinvertebrate processing and identification

The macroinvertebrate samples were gently rinsed through a 500 µm sieve with tap water to remove sediment and formalin. Cleaned samples were sorted by eye in a white plastic tray, and individual animals identified under a stereo zoom microscope (Motic SMZ-168, China). All macroinvertebrates with the exception of Oligochaeta were identified at a minimum to family level using keys within Dobson et al. (2013). Oligochaete taxonomy using preserved specimens is extremely difficult (Ladle and Bird 1980) and so are commonly identified to order level only (Walley and Hawkes 1996). The high abundances of macroinvertebrates coupled with the large number of samples necessitated the use of family-level (TL2) identification. TL2 is considered the best resolution for assessing macroinvertebrate assemblages to assess biological integrity based on a cost /benefit analysis (Marshall et al. 2006). For the purposes of calculating WHPT and PSI biotic

indices for each site, TL2 fulfilled the requirements. However, although never incorporated into any analysis such as biotic indices, in some instances where there were only one or few representative species in a family, species or genus level was achieved.

3.3.6 Data analysis

The present study used a range of methods to compare macroinvertebrate assemblages between sites and river and biotic indices to examine and compare ecological health based on macroinvertebrate assemblages. An overview of the tests and indices used are presented in Table 9, while methodological detail is provided in the sections referenced.

Table 9 Summary table of the tests used to assess differences in macroinvertebrate assemblages and biotic indices to assess site-specific stream health

Test	*Analysis type*	*Specific characteristics*	*Metrics*	*Section*
Ephemeroptera Plecoptera Trichoptera (EPT) (Lenat 1988)	Species abundance metric	Uses metrics describing Ephemeroptera, Plecoptera, and Trichoptera populations to determine stream health. Higher scores indicate healthier streams	EPT richness, EPT % abundance	3.3.6.1
Shannon-Weiner Index (*H'*) (Shannon 1948)	Species richness	Diversity score to describe biodiversity. High scores indicate high biodiversity	Proportional abundances of each species within a sample	3.3.6.1
Biological Monitoring Working Party Index (BMWP) (Chesters 1980)	Biotic index	Uses macroinvertebrate pollution tolerances of individual families to evaluate stream health. Higher scores indicate healthier streams	Organism tolerance to organic pollutants	3.3.6.2
Walley Hawkes Paisley Trigg (WHPT) (Walley and Hawkes 1996)	Biotic index	Uses macroinvertebrate pollution tolerances of families and their abundance within samples to evaluate stream health. Higher scores indicate healthier streams	Organism tolerance to organic pollutants	3.3.6.2
Proportion of Sediment Sensitive Invertebrates (PSI) (Extence et al. 2013)	Biotic index	Uses sediment sensitivity characteristics of macroinvertebrate families to evaluate levels of sedimentation in a stream. High values indicate a high presence of sediment sensitive taxa, indicating low levels of fine sediment	Organism tolerance to fine sediments	3.3.6.3
River Invertebrate Classification System (RICT) incorporating the River Invertebrate Prediction and Classification System (RIVPACS) (Clarke and Davy-Bowker 2014)	Multivariate model	Predicts organism occurrence and biotic index scores for a stream based on environmental features	Date of sampling (spring and autumn), site location (NGR), altitude, distance from source, gradient, discharge category, alkalinity, mean channel width and depth and substrate composition in terms of % boulders/cobbles, % pebbles/gravel, % sand and % silt/clay	3.3.6.4
Analysis of Similarity (ANOSIM) (Clarke 1993)	Non-parametric statistical test	Compares similarity between sets of samples, testing if similarity between groups is greater or equal to similarity within groups	Bray-Curtis dissimilarity matrice of macroinvertebrate abundance data	3.3.6.5
Similarity Percentage (SIMPER) (Clarke 1993)	Method	Identifies the percentage contribution of each taxon to overall average dissimilarity observed between samples	Bray-Curtis dissimilarity matrice of macroinvertebrate abundance data	3.3.6.6

3.3.6.1 Macroinvertebrate diversity and abundance

The life-cycles of many macroinvertebrate species mean that it is normal to find different abundances in chalk streams at various periods in the year (Wright 1992). In addition, seasons with high flows may support different abundances of certain macroinvertebrates than seasons of low flow (Wright and Symes 1999). Total macroinvertebrate abundances, the number of different families (NTAXA), the abundance of EPT, %ETP and EPT family richness (EPT NTAXA) were calculated for each site for each survey. In addition, in order to even out seasonal fluctuations and provide an overview, the aforementioned metrics were averaged for each site across all surveys.

Macroinvertebrate diversity was quantified using the Shannon Index. The Shannon Index (*H'*) is an information index and is the most commonly used diversity index in ecology (Shannon 1948). The Shannon Index quantifies the uncertainty associated with predicting the identity of a new taxa given number of taxa and evenness in abundances of individuals within each taxa.

$$H' = -\sum\left(\frac{n_i}{N}\ x\ ln\ \frac{n_i}{N}\right)$$

where n_i is the number of individuals of amount (biomass) of each of the *i* species and *N* is the total number of individuals for the site.

Approximate confidence intervals for *H'* were computed using a bootstrap procedure using 9999 random samples. 95% confidence intervals were calculated for each site/survey and non-overlapping CIs considered as significant differences. *H'* values and bootstrapping was performed using PAST v3.26 (Hammer et al. 2001).

3.3.6.2 Walley Hawkes Paisley Trigg (WHPT)

The Biological Monitoring Working Party (BMWP) provides single values based on organic pollution tolerance for each macroinvertebrate family. A requirement to allocate single values to specific families arose in section 3.4.9, so here BMWP values were used. However, to calculate biotic index scores for each site, the Walley Hawkes Paisley Trigg (WHPT) method, which uses abundance data, was chosen for it its greater fidelity (Walley and Hawkes 1996). WHPT uses sensitivity scores based on the abundance of macroinvertebrate families (with the exception of Oligochaeta). Each invertebrate family is allocated scores (ranging from 12.2 for the most pollution-sensitive to -0.8 for the most pollution-tolerant) based on four logarithmic abundance categories 1-9, 10-99, 100-999 and >1000 individuals in a sample. For example, the pollution-tolerant Asellidae scores 4 if there are 1-9 individuals, and -1.6 if there are in excess of 1000. Conversely, the pollution-sensitive

mayflies Heptageniidae score 8.5 for between 1-9 individuals, and 11.1 for >1000. WHPT scores were generated for every sample, covering every site and survey occasion.

The WHPT score is expressed as the average score per taxon as follows:

$$WHPT_{ASPT} = \sum \frac{AB}{WHPT_{NTAXA}}$$

Where AB = the value for each taxon according to its abundance. NTAXA is the number of taxa contributing to the assessment

3.3.6.3 Proportion of Sediment-sensitive Invertebrates (PSI)

Discharges from watercress farms have been observed to result in increased deposits of fine sediments downstream of discharges (Smith 1992; Mainstone 1999). In addition to surveying the quantity of visible fine sediments during habitat surveys, the response in the composition of macroinvertebrate taxa to fine sediments was assessed using the Proportion of Sediment-sensitive Invertebrates (PSI) in each sample (Extence et al. 2013). PSI scores were generated following the methodology of Extence et al. (2013), whereby macroinvertebrate families are assigned to one of four Fine Sediment Sensitivity Ratings (FSSR) based on extensive literature reviews and assessment of anatomical, physiological and behavioural traits exhibited by individual taxa (Extence et al. 2013). The PSI score describes the percentage of fine sediment sensitive taxa in samples, with high scores denoting a high presence of sediment sensitive taxa, indicating low levels of fine sediments. Conversely, low scores indicate a low presence of sediment sensitive taxa, indicating high levels of fine sediments. PSI scores were generated for every sample, covering every site and survey occasion. The PSI score was calculated using the matrix in Table 10 and then applying the following formula:

$$PSI = \frac{\sum Scores\ for\ sediment\ sensivity\ groups\ A\&B}{\sum Scores\ for\ all\ sediment\ sensitivity\ groups\ A;B;C\&D}\ x\ 100$$

Table 10 Fine Sediment Sensitivity Rating definitions and abundance weighted scores for PSI calculation (after Extence et al. 2013)

Group	Fine Sediment Sensitivity Rating (FSSR)	Log abundance			
		1-9	10-99	100-999	>1000
A	Highly sensitive	2	3	4	5
B	Moderately sensitive	1	2	3	4
C	Moderately insensitive	1	2	3	4
D	Highly insensitive	2	3	4	5

3.3.6.4 RICT

The River Invertebrate Classification Tool (RICT) was used to contextualise WHPT and PSI scores. The RICT software is accessed online via the Scottish Environmental Protection Agency (SEPA) at rict.sepa.org.uk. The software system incorporates RIVPACS (River Invertebrate Prediction and Classification System) using a database of reference pristine or near-pristine UK river sites to generate predicted biotic indices based on a range of invariant input data; NGR, slope, discharge category, distance from source and altitude, and variant input data; alkalinity, mean width and depth, % boulders/cobbles, % pebbles/gravel, % sand and % silt/clay. For this study, WHPT and PSI estimates were extracted for all surveys at all sites, enabling comparison with observed WHPT ASPT and PSI scores. An Observed-to-Expected value (O/E-ratio) was generated from the observed values and expected values.

The RICT assessment is only appropriate for UK rivers provided suitable analogue sites exist in the RICT reference database. A suitability code is generated by RICT from 1-4 which indicates how closely abiotic variables match reference sites, with one being the best match and four indicating that no suitable matches were found. The suitability code indicates the level of trust than can be placed in the RICT outputs (Clarke and Davy-Bowker 2014).

3.3.6.5 ANOSIM

To discriminate global differences in macroinvertebrate assemblages between sites, Analysis of Similarity (ANOSIM) tests were performed. ANOSIM is a non-parametric test of significant difference between two or more groups based on distance measures converted into ranks (Clarke 1993). Differences between sites based on Bray-Curtis similarity metric are represented by *R* which ranges from -1 to +1, with lower values indicating similarity and higher values indicating dissimilarity. ANOSIM was run using a Bray-Curtis similarity matrix incorporating data from all

surveys at each site. It was first performed using data across all sites on each river, followed by post-hoc pairwise testing between sites on each river to compare sites. ANOSIM was performed using PAST v3.26 (Hammer et al. 2001). Significance testing for *P* was set at α 0.05.

3.3.6.6 SIMPER

SIMPER analysis is a distance-based procedure allowing for the computation of the relative contribution of each taxon to the overall average dissimilarity observed between two or more groups of taxonomic assemblages (Gibert and Escarguel 2019). Similarity Percentage (SIMPER) analysis was used to investigate which taxa were primarily responsible for differences in macroinvertebrate assemblages between sites following ANOSIM. The Bray-Curtis similarity measure (multiplied by 100) was used to calculate the percentage contribution of individual taxa to dissimilarity between sites. SIMPER was run using a Bray-Curtis similarity matrix incorporating data from all surveys at each site. SIMPER was performed on all sites on each river system in addition to pairwise analysis between sites on each river. Taxa that contributed to the greatest differences on each river were individually plotted for each river to examine the key differences in macroinvertebrate identity between sites. These were examined as either pollution sensitive (BMWP => 5) or pollution tolerant (BMWP <5). As abundances varied between sites on each river, BMWP scoring was used rather than WHPT as BMWP scores provide single values for each taxon based on their presence. SIMPER was performed using PAST v3.26 (Hammer et al. 2001).

3.3.6.7 Univariate analysis

Univariate analysis of observed data between sites on the same river was avoided due to the pseudoreplicaton inherent in comparing riverine sites which are not spatially independent of one another at the time of sampling (Hickey and Golding 2002). However, univariate analysis was used to look for significant differences between expected and observed biotic index scores on a site-by-site basis. At each site, the biotic index scores for repeated surveys were considered replicates, while treatments were the observed and expected scores. The data was tested for homogeneity of variance and normality of distribution and fulfilled the requirements of t-tests. The observed and expected scores for each site were compared using two-way paired t-tests to identify any significant differences (α 0.05) using Minitab 19 software (Minitab, Inc, USA).

3.3.6.8 Multivariate analysis

Principal Component Analysis (PCA) was used to visualise differences between sites and their habitat variables. PCA is a useful technique to assess patterns in complex data sets by effectively reducing the dimensionality of multivariate data into linear combinations of the original variables

(components) (Peres-Neto et al. 2003). Prior to running PCA, percentage variables were arcsin square root transformed. PCA was performed using Minitab 19 software (Minitab, Inc, USA).

Because PCA uses Euclidean distances, it is not suitable for datasets containing many null variables (Paliy and Shankar 2016). The macroinvertebrate data matrix contained many null variables where taxa were absent. Because Non-metric multidimensional scaling (NMDS) uses only rank information and maps ranks non-linearly onto ordination space, it can accommodate non-linear species responses of any shape and robustly find underlying gradients (Oksanen 2015). As NMDS makes few assumptions about data distribution, it was used to investigate the relationships between macroinvertebrate assemblages and site identity.

NMDS is an ordination technique that allows qualitative assessments of species assemblages based on the relative positioning of species and site on an NMDS plot. Species that are close to a site on an NMDS plot have a greater presence at that particular site than those at a greater distance. In addition, sites that are closer together on an NMDS plot have more similar species assemblages than those further apart.

NMDS analysis used a Bray-Curtis similarity matrice of physical data and macroinvertebrate abundance. To investigate patterns of relatedness of sites in terms of macroinvertebrate composition and abundance, NMDS was performed on all 77 recorded families and ordinated with site identity. The extent of the disagreement between measured values and their position in NMDS is quantified as stress. The lower the stress, the better the goodness-of-fit, with stress <0.20 considered acceptable. NMDS were performed using PAST v3.26 (Hammer et al. 2001).

3.4 Results

3.4.1 Principal component analysis of habitat variables

Three components were produced that together explained 62.8% of the variance in the data and each had an eigenvalue >1 (Table 11). The first component corresponding to the largest eigenvalue (6.16) accounts for 41.1% of the total variance and was was negatively correlated with silt, sand and overhanging vegetation, and positively correlated with gravel and cobbles and boulders. Therefore, the PC1 axis primarily denotes a gradient of substratum, with negative scores denoting increased depositing substratum and positive scores denoting increased eroding substratum. The second component corresponding to the second eigenvalue (3.07) accounts for 20.5% of the total variance The second PC was positively correlated with higher macrophyte abundance and negatively correlated with pH, LWD and channel width. The immediate downstream sites (BREC, CRDS1 and FRDS1) all fall to the left of the respective upstream sites (BRWC, CRUS and FRUS) on

the PC1 axis, indicating a stronger association with depositing substrates downstream of watercress farm discharges (Figure 26). The sites on each river are clustered with no overlap in demensional space, indicating river-wide differences in environmental variables. The Bourne sites are characterised by eroding substatum such as gravel and cobbles, deep water and high macrophyte coverage. High levels of riparian canopy vegetation, fine sediments and high presence of large woody debris in the channel characterise the physical habitat of Crane sites. The Frome sites are associated with high water velocities and dissolved oxygen levels, wide channel width and higher pH. As there were distinct differences between rivers, in futher analysis, each river system will be analysed seperately in terms of habitat variables and macroinvertebrate assemblage rather than the data being aggregated.

Table 11 Principal component loadings, eigenvalues and percentage of variation explained by the first three components of a PCA on the habitat variables for all sites on the Bourne Rivulet, the River Crane and the River Frome

Variable	*PC1*	*PC2*	*PC3*
Silt	-0.344	-0.003	0.145
Sand	-0.211	-0.008	0.063
Gravel	0.302	0.159	-0.027
Cobbles & boulders	0.312	-0.12	-0.22
Overhang	-0.304	0.151	-0.075
Tree canopy	-0.309	0.05	-0.162
LWD	-0.267	-0.358	0.088
Width	0.216	-0.414	0.079
Depth	0.145	0	0.68
Flow	0.191	-0.246	-0.539
pH	0.089	-0.454	0.298
DO	0.308	-0.271	-0.046
Conductivity	-0.251	-0.32	-0.06
SS	-0.294	-0.215	-0.181
Macrophytes	0.18	0.384	-0.016
Eigenvalue	6.1611	3.0717	1.7991
Percentage of variance explained	41.11	20.5	1.2

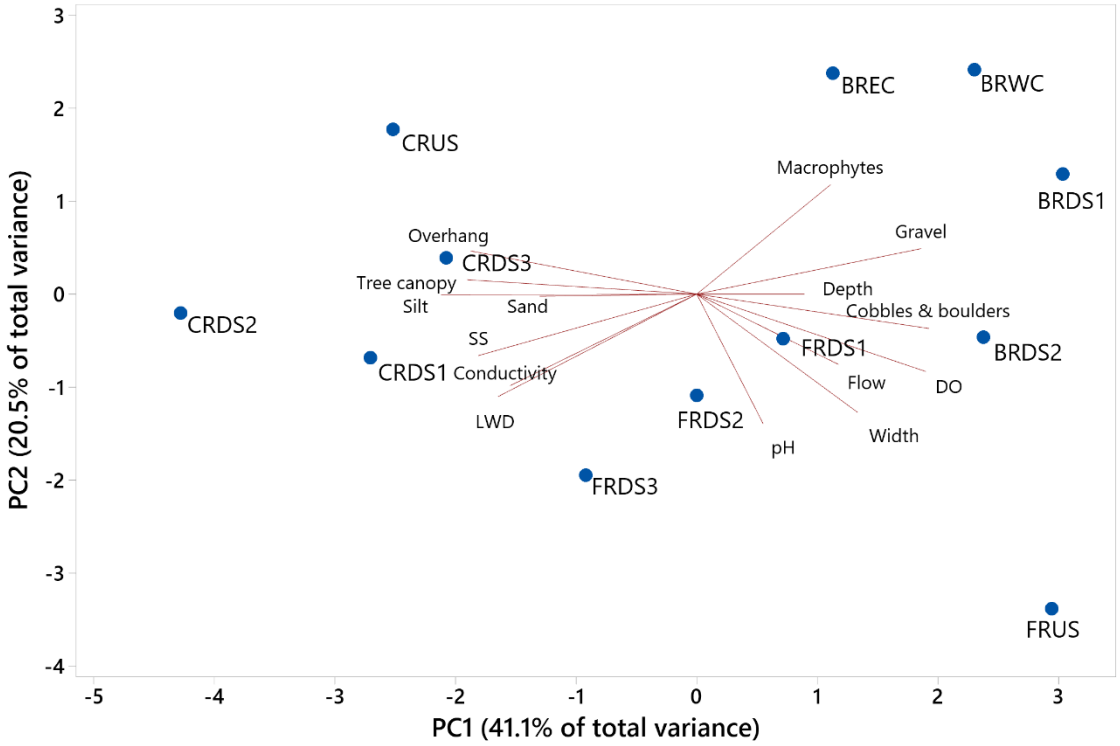

Figure 26 Biplot for principal component analysis of environmental variables with site score plots projected for all sites on the Bourne Rivulet (BRWC, BREC, BRDS2 and BRDS1), the River Crane (CRUS, CRDS1, CRDS2 and CRDS3) and the River Frome (FRUS, FRDS1, FRDS2 and FRDS3)

3.4.2 Water quality

On the Bourne, mean DO was lowest on BREC (Table 12) which was apparent for all surveys except Autumn 2017 when DO values were broadly similar (Figure 27). The mean pH increased with increasing distance from the discharge and the furthest downstream reach BRDS2 had consistently higher pH than all other Bourne sites. BRWC had the lowest mean pH which remained relatively unchanged in Spring 2017 when all other Bourne sites showed an elevated pH. Concurrent with the spike in pH in Spring 2017, there was a notable drop in conductivity at all Bourne sites. In contrast to DO and pH, all Bourne sites had relatively similar conductivity readings across surveys.

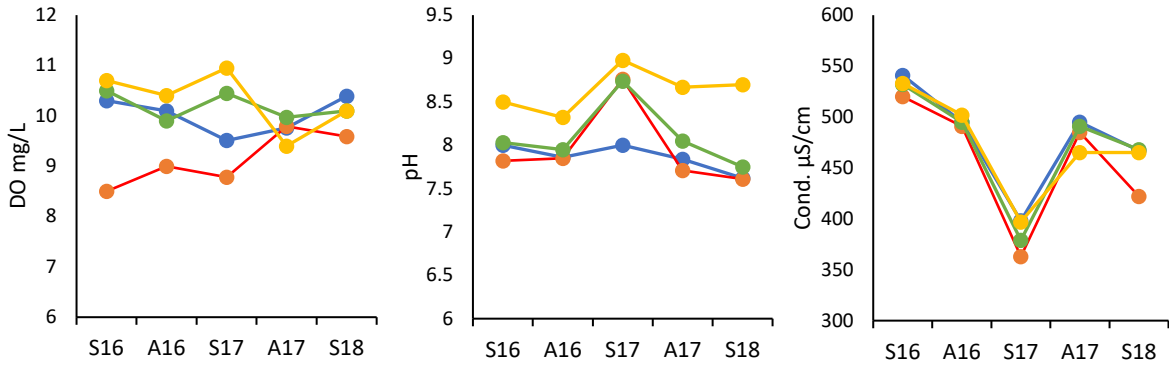

Figure 27 The Bourne Rivulet water quality parameters; dissolved oxygen (DO mg/L), pH and conductivity (µS/cm) recorded during surveys (S = spring, A = autumn + year) at BRWC; blue, BREC; orange, BRDS1; green, BRDS2; yellow

On the Crane, the sites up and downstream of the discharge recorded the greatest differences in mean DO, with the upstream site CRUS having the lowest and the immediate downstream site CRDS1 having the highest (Table 12). The mean increased DO downstream compared to upstream of the discharge on the are most likely to be a result of a combination of oxygenation from the agitation of water falling from the weir conveying the discharge water and the wider and shallower channel of the reach. No data exists for the Crane in spring 2018 for any water quality determinand due to equipment failure. Similar to the Bourne, mean pH was lowest upstream at CRUS and steadily increased with distance from the discharge, and spring 2017 saw a spike in pH and a concomitant drop in conductivity (Figure 28).

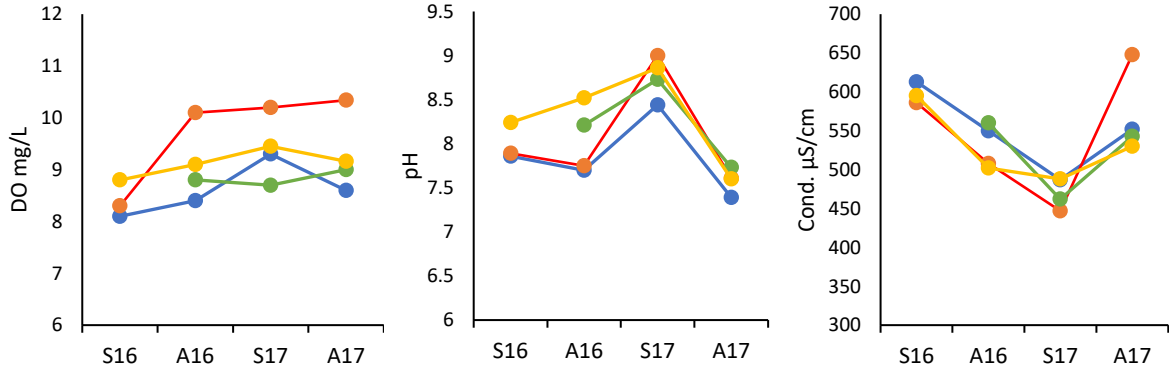

Figure 28 The River Crane water quality parameters; dissolved oxygen (DO mg/L), pH and conductivity (µS/cm) recorded during surveys (S = spring, A = autumn + year) at CRUS; blue, CRDS1; orange, CRDS2; green, CRDS3; yellow

On the Frome, mean DO was highest upstream in FRUS, with the value progressively decreasing with distance from the site (Table 12). DO values for FRUS were particularly high during spring 2017 (Figure 29). Higher DO concentrations upstream compared to downstream on the Frome may again be attributed to differences in channel morphology and water velocity, with the upstream site having a wide shallow profile and a high velocity, while downstream was deeper and narrow with approximately 25% slower velocity. Across all sites, pH was broadly similar, and all sites showed a peak in the spring of 2017. The immediate downstream site FRDS1 had the lowest mean pH and conductivity, both of which gradually increased downstream to CRDS3.

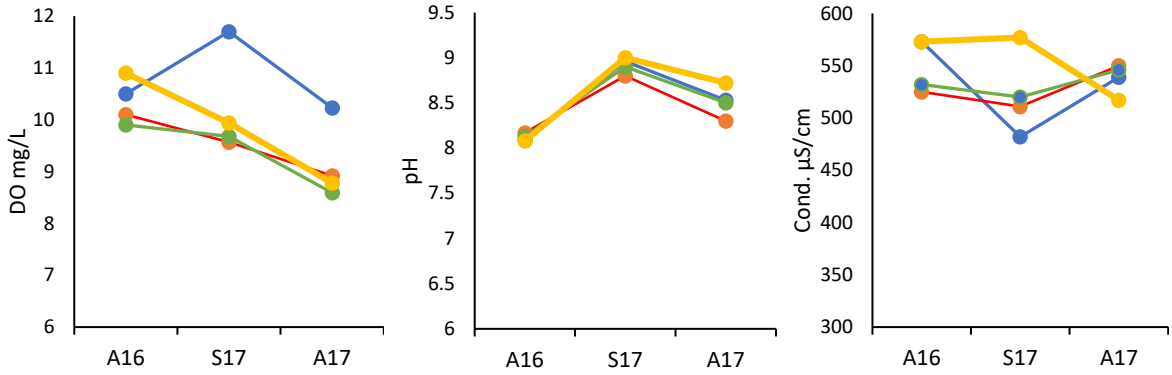

Figure 29 The River Frome water quality parameters; dissolved oxygen (DO mg/L), pH and conductivity (µS/cm) recorded during surveys (S = spring, A = autumn + year) at FRUS; blue, FRDS1; orange, FRDS2; green, FRDS3; yellow

Table 12 Mean dissolved oxygen (DO mg/L), pH and conductivity (µS/cm) recorded during surveys between spring 2016 and spring 2018 on The Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), The River Crane (CRUS, CRDS1, CRDS2, CRDS3) and The River Frome (FRUS, FRDS1, FRDS2, FRDS3

Site	*n*	*Mean DO*	*SD*	*Mean pH*	*SD*	*Mean Cond.*	*SD*
BRWC	5	10.01	0.37	7.86	0.16	479.2	52.6
BREC	5	9.13	0.54	7.95	0.46	456.2	63.2
BRDS1	5	10.18	0.28	8.18	0.51	473	57.3
BRDS2	5	10.31	0.60	8.70	0.38	472.4	50.9
CRUS	4	8.60	0.51	7.85	0.44	550.5	51.4
CRDS1	4	9.74	0.96	8.06	0.64	547.3	88.0
CRDS2	3	8.83	0.15	8.22	0.50	521.7	52.4
CDRS3	4	9.13	0.27	8.31	0.53	528.8	47.5
FRUS	3	10.81	0.78	8.53	0.44	531.3	46.0
FRDS1	3	9.53	0.59	8.42	0.33	528.7	19.8
FRDS2	3	9.39	0.70	8.51	0.39	532.67	13.0
FRDS3	3	9.87	1.06	8.60	0.47	555.7	33.5

3.4.3 Suspended solids

Mean suspended solid (SS) concentrations were generally found to be higher in the sites below watercress farm discharges (Figure 30). Concentrations of suspended solids (SS) in chalk stream water are typically very low at 2-5mg/L (Casey and Smith 1994), with annual means under near-pristine conditions expected to be below 2 mg/L in the upper reaches of a chalk stream, and below 4mg/L in middle reaches (Mainstone 1999). The sites on the Bourne and Crane were upper reaches close to the perennial head (c. 11 and 6km from source respectively) where SS concentrations would be expected to be <2 mg/L. Both of these rivers had elevated SS concentrations downstream of discharges. The Bourne east channel had mean SS concentrations just over the expected levels for a pristine site, but after joining the main river channel this dropped to 1.16 mg/L, suggesting that SS from the discharge had fallen out of suspension rapidly. At the Crane upstream site CRUS had the lowest levels of SS (1.87 mg/L) and the immediate downstream site, CRDS1, had the highest at 3.74 mg/L and the further downstream sites were intermediate. The high mean SS levels in CRDS1 were partially caused by cress bed cleaning in August 2017, when the stream water was visibly clouded with sediment (Figure 31). Mean SS concentrations in the Frome were all between 2 mg/L and 3 mg/L, as would be expected for a middle reach chalk stream. Once again, the site immediately downstream had higher SS than immediate upstream, but the FRDS2 had the highest levels of all, which is likely to be a result of bank erosion by livestock using a cattle drink upstream of the site, a process known to contribute sedimentation to rivers (Myers and Swanson 1992; Stevens and Cummins 1999; Sovell et al. 2000).

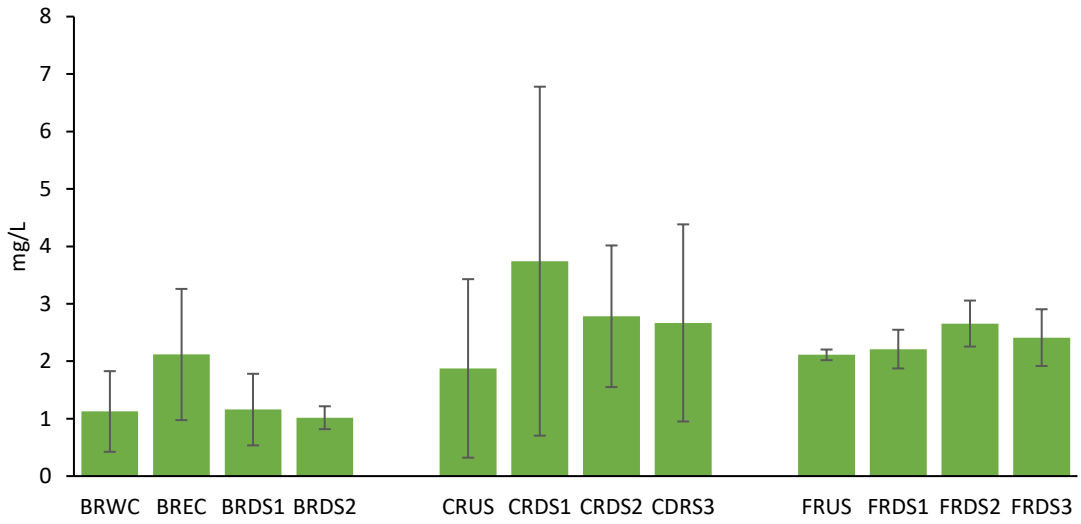

Figure 30 Mean suspended solid concentrations in mg/L (±SD) taken as three replicates at each site on the Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), the River Crane (CRUS, CRDS1, CRDS2, CRDS3) and the River Frome (FRUS, FRDS1, FRDS2, FRDS3) during spring and autumn surveys between May 2016 to June 2018 (*n* = 12 for each Bourne Rivulet site; *n* = 12 for each River Crane sites except CRDS2 where *n* = 9; *n* = 9 for each River Frome site)

Figure 31 Turbid water below the discharge weir on The River Crane in autumn 2017 following watercress bed cleaning operations. The outflow is much reduced to limit sediment release, but suspended solids still entered the river at CRDS1, where levels at 8.5mg/L were recorded downstream

3.4.4 Water velocity

Water velocity was measured at three randomly selected but representative points in the channel on each survey and presented as the mean ± SD of all surveys (Figure 32). Mean velocity on the Bourne was broadly similar across sites, but there appeared to be a split between faster flowing sites BREC and BRDS2 and the slower flowing BRWC and BRDS1.

Velocities on the Crane showed more variation, with both CRUS and CRDS2 being slower than CRDS1 and CRDS3. CRDS2 had the lowest velocity of all sites under study.

The Frome site FRUS has the highest velocity of any site, which was approximately fourfold that of the three downstream sites; FRDS1, FRDS2 and FRDS3.

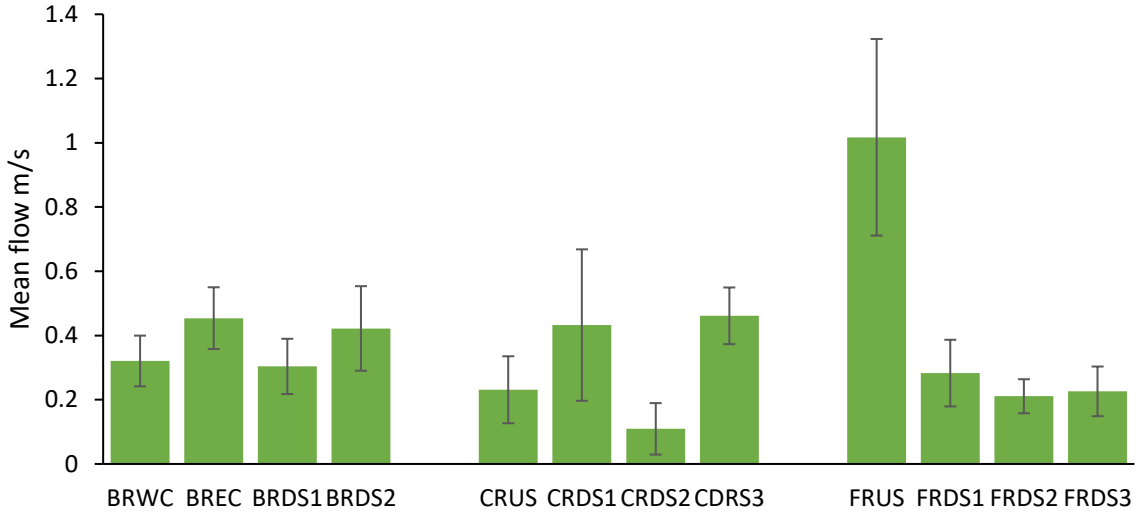

Figure 32 Mean (±SD) flow velocities (m/s) of sites on the Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), the River Crane (CRUS, CRDS1, CRDS2, CRDS3) and the River Frome (FRUS, FRDS1, FRDS2, FRDS3). Means were generated from three flow readings taken at randomly selected points in the main river channels in spring and autumn between May 2016 to June 2018 (*n* = 15 for each Bourne Rivulet site except BREC where *n* = 6; *n* = 15 for each River Crane site except CRDS2 where *n* = 12; *n* = 9 for each River Frome site

3.4.5 Substrate

Of all substrate types, all but one site was dominated by eroding substrates such as gravel and cobbles, as would be expected of chalk stream habitat (Figure 33). The exception was CRDS2, the site with the lowest water velocity, with a mean silt substrate of 79.4%. The site with the highest velocity, FRUS, had the lowest coverage of silt (1.2%) and highest occurrence of cobbles. There was a trend for higher levels of silt in the sites immediately downstream of the watercress farm discharges.

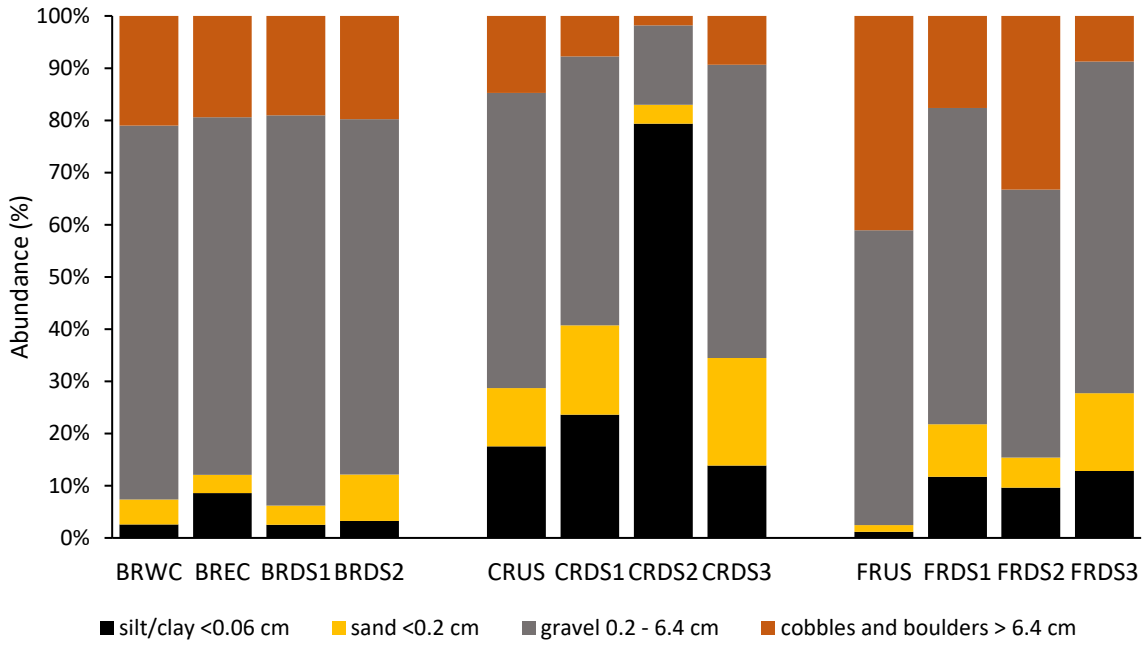

Figure 33 Percentage abundance of substrate categories following HABSCORE surveys on the Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), the River Crane (CRUS, CRDS1, CRDS2, CRDS3) and the River Frome (FRUS, FRDS1, FRDS2, FRDS3). Percentage abundances are means of estimates taken of successive 10 m sections along the length of each site during spring and autumn surveys between May 2016 to June 2018

3.4.6 Instream macrophytes

Instream macrophyte growth is largely dependent on light intensity. The dominant chalk stream macrophyte, *Ranunculus*, has requirements for high light intensities, high water velocities and clean gravel substrates. *Ranunculus* is often annually cut by fisheries managers where lack of shading from limited riparian canopy would otherwise cause the channel to become clogged with the macrophyte. As such, the degree of shading and artificial management will have a greater baring on macrophyte coverage at a given site than discharges from watercress farms.

Macrophyte coverage was highest on the Bourne, with a mean coverage of 37.05% for all sites combined. From low to high, BRWC had the highest mean coverage, followed by BRDS1, BRDS2 and finally BREC (Figure 34).

The Crane had the next highest mean coverage for combined sites at 12.24%. Of the Crane sites, CRDS3 had the highest coverage, followed by CRUS, CRDS1 and finally CRDS2.

The Frome had the lowest mean coverage for combined sites at 2.61%, and here there was less variation between sites than at the Bourne and Crane sites. Of these, FRUS had the highest mean coverage, FRDS1 and FRDS2 were very similar and the lowest coverage was in FRDS3.

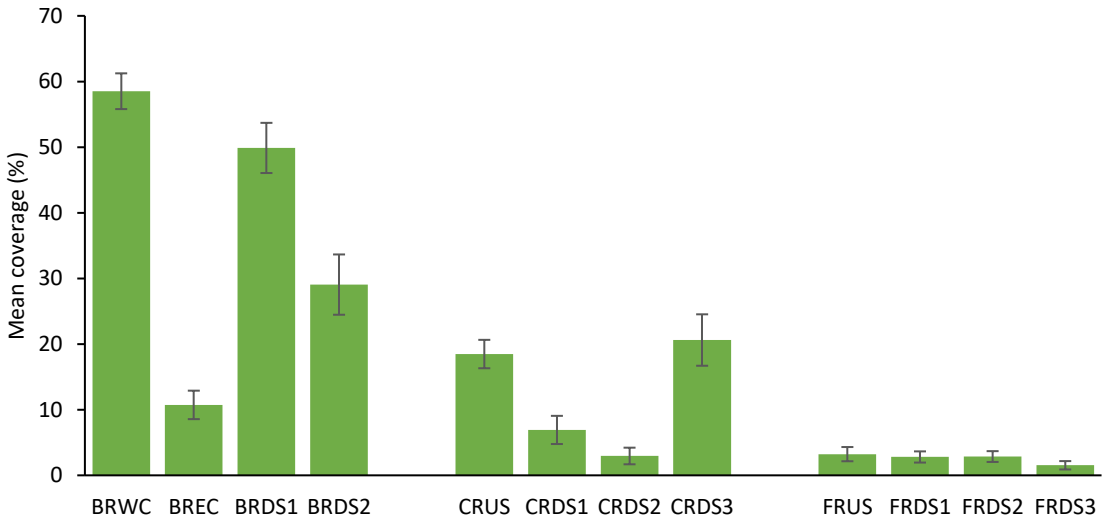

Figure 34 Mean submerged macrophyte cover % (±SE) estimates following HABSCORE surveys on the Bourne Rivulet; BRWC (*n* = 50), BREC (*n* = 30), BRDS1 (*n* = 50), BRDS2 (*n* = 50); the River Crane (CRUS (*n* = 50), CRDS1 (*n* = 50), CRDS2 (*n* = 20), CRDS3 (*n* = 50); the River Frome; FRUS (*n* = 30), FRDS1 (*n* = 30), FRDS2 (*n* = 30), FRDS3 (*n* = 30). Percentage abundances are means of estimates taken of successive 10 m sections along the length of each site during spring and autumn surveys between May 2016 to June 2018

3.4.7 Riparian vegetation

The extent of riparian vegetation at each site is down to management practices and would not be influenced by watercress farm discharges. Riparian vegetation is regularly removed or cut back on some reaches to maximise access for leisure activities or the agricultural potential of adjacent land, while others flow through unmanaged woodland and fen.

The Crane has the least artificial management, with much the river course studied flowing through woodland, fen and dense vegetation. As such, it had both the highest combined tree canopy cover at 73.73% and overhanging vegetation at 26.92%. The site CRDS2 had the highest mean tree canopy coverage at 91.67% (Figure 35). Concurrently, of all Crane sites it had the least overhanging vegetation, likely a result of poorer growth under the low irradiance levels under the extensive tree canopy.

The Bourne had the next greatest a percentage of overhead tree canopy with all sites combined at 15.35%. BRWC had the greatest coverage, followed by BRDS2, BRDS1 and finally BREC where there was just a single tree. The Bourne is maintained for its wild trout fishery, with riparian vegetation annually cut to enhance access for anglers. It had the lowest riparian vegetation overhang at 11.88%. BRWC and BREC had very similar levels at 14.14% and 14% respectively. BRDS1 and BRDS2 similarly were near identical at 9.67% and 9.72% respectively

The Frome had the lowest combined tree coverage at 14.78%. Of these sites, FRDS2 had the greatest coverage followed by FRUS and then FRDS3. FRDS1 runs through open pasture and had no tree canopy. The River Frome had a mean overhang of 14.78% for all sites combined. Here the overhanging vegetation increased from up to downstream.

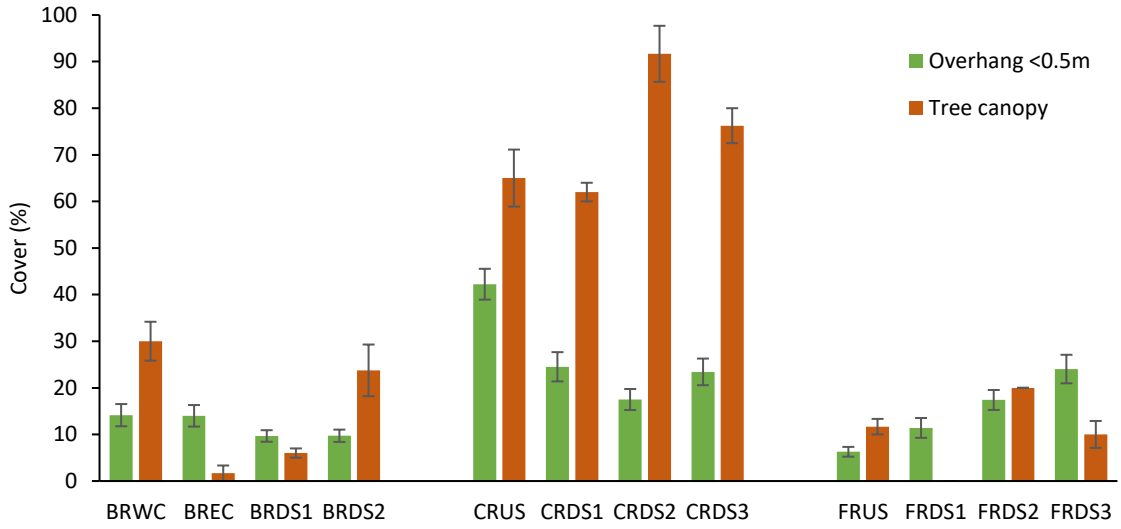

Figure 35 Mean (±SE) riparian vegetation overhanging channel estimates following HABSCORE surveys on the Bourne Rivulet; BRWC (*n* = 50), BREC (*n* = 30), BRDS1 (*n* = 50), BRDS2 (*n* = 50); the River Crane (CRUS (*n* = 50), CRDS1 (*n* = 50), CRDS2 (*n* = 20), CRDS3 (*n* = 50); the River Frome; FRUS (*n* = 30), FRDS1 (*n* = 30), FRDS2 (*n* = 30), FRDS3 (*n* = 30). Percentage abundances of overhang are means of estimates taken of successive 10 m sections along the length of each site. Tree canopy was estimated for the whole site at each survey. Mean coverage was generated for both variables encompassing spring and autumn surveys between May 2016 to June 2018

3.4.8 Large woody debris

The relative occurrence of Large woody debris (LWD) in chalk stream river channels is largely a result of riparian land and river channel management practices rather than watercress farm discharges. For example, where riparian tree canopy has historically been cleared, there is an absence of natural treefall events and root systems in the channel. Channels may be cleared of LWD for navigation purposes, while more recently, efforts have been made to 're-snag' some river reaches by introducing LWD to increase habitat heterogeneity in an effort to increase biodiversity (Lyon et al. 2019).

On the Bourne, LWD was absent from BREC, and scarce on BRWC and BRDS1 (Figure 36). As BREC runs through open grassland, no natural treefall would not occur, while BRWC and BRDS1 are managed for fisheries and do not have extensive tree canopies. BRDS2 had the highest percentage of the Bourne Rivulet sites, due in part to deliberate re-snagging operations to improve habitat.

The River Crane has undergone little in the way of management for leisure activities or agriculture, and here instream woody debris is relatively abundant and derived from natural treefall events and roots. The dense tree canopy cover over CRDS2 is concurrent with the abundant LWD in the

channel. The next highest occurrence of LWD on the Crane are in CDRS1, followed by CRDS3 and finally CRUS.

The upstream reach of the Frome has a natural morphology, and an LWD component of 4.8%. Downstream at FRDS1, the river is canalised and runs through open pasture, and concurrently the LWD drops to the lowest of all Frome sites. FRDS2 has a bank of trees, and the LWD rises. Furthest downstream, at FRDS3, trees bank both sides, and there is considerable treefall and root systems, giving the site the highest LWD fraction.

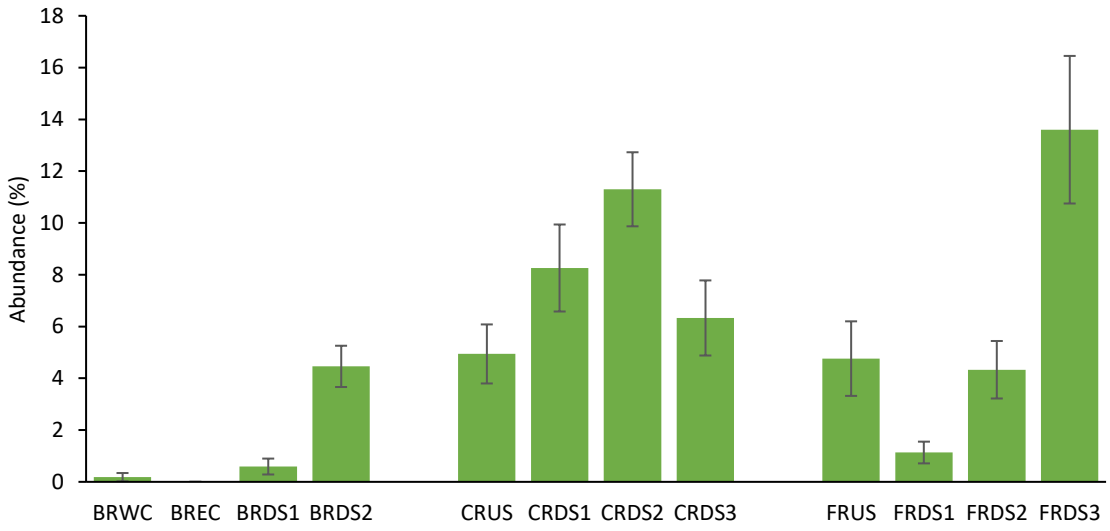

Figure 36 Mean percentage (±SE) of large woody debris (LWD) estimates following HABSCORE surveys on the Bourne Rivulet; BRWC (*n* = 50), BREC (*n* = 30), BRDS1 (*n* = 50), BRDS2 (*n* = 50); the River Crane (CRUS (*n* = 50), CRDS1 (*n* = 50), CRDS2 (*n* = 20), CRDS3 (*n* = 50); the River Frome; FRUS (*n* = 30), FRDS1 (*n* = 30), FRDS2 (*n* = 30), FRDS3 (*n* = 30). Percentage abundances of LWD are means of estimates taken of successive 10 m sections along the length of each site from spring and autumn surveys between May 2016 to June 2018

3.4.9 Macroinvertebrates

A total of 77 macroinvertebrate families were captured and identified across all sites over the duration of the study, in addition to oligochaetes that remained unidentified to family level. The full list of identified macroinvertebrate families and their abundances for the Bourne, Crane and Frome are listed in appendices two, three and four respectively.

3.4.9.1 Non-Metric Multidemensional Scaling (NMDS)

An NMDS plot ordinating all macroinvertebrate families with site identity (Figure 37) reveals similarities and dissimilarities in macroinvertebrate composition at each site. The grouping of sites indicates clear river-wide differences in macroinvertebrate assemblages (stress value 0.1204). The

Bourne has the greatest variability with the points most widely scattered. The site BREC stands out as being the most distant from the other sites on the river suggesting macroinvertebrate assemblages on BREC strongly differ from other Bourne sites. On the Crane, there is an apparent split, with CRUS and CRDS1 more closely ordinated together than CRDS2 and CRDS3. This suggests that macroinvertebrate assemblages at CRUS and CRDS1 are together more similar than both CRDS2 and CRDS3. Sites on the Frome are the most tightly clustered, indicating that macroinvertebrate composition is more consistent between sites than on other rivers. However, of the Frome sites, FRDS1 stands out as being the most dissimilar.

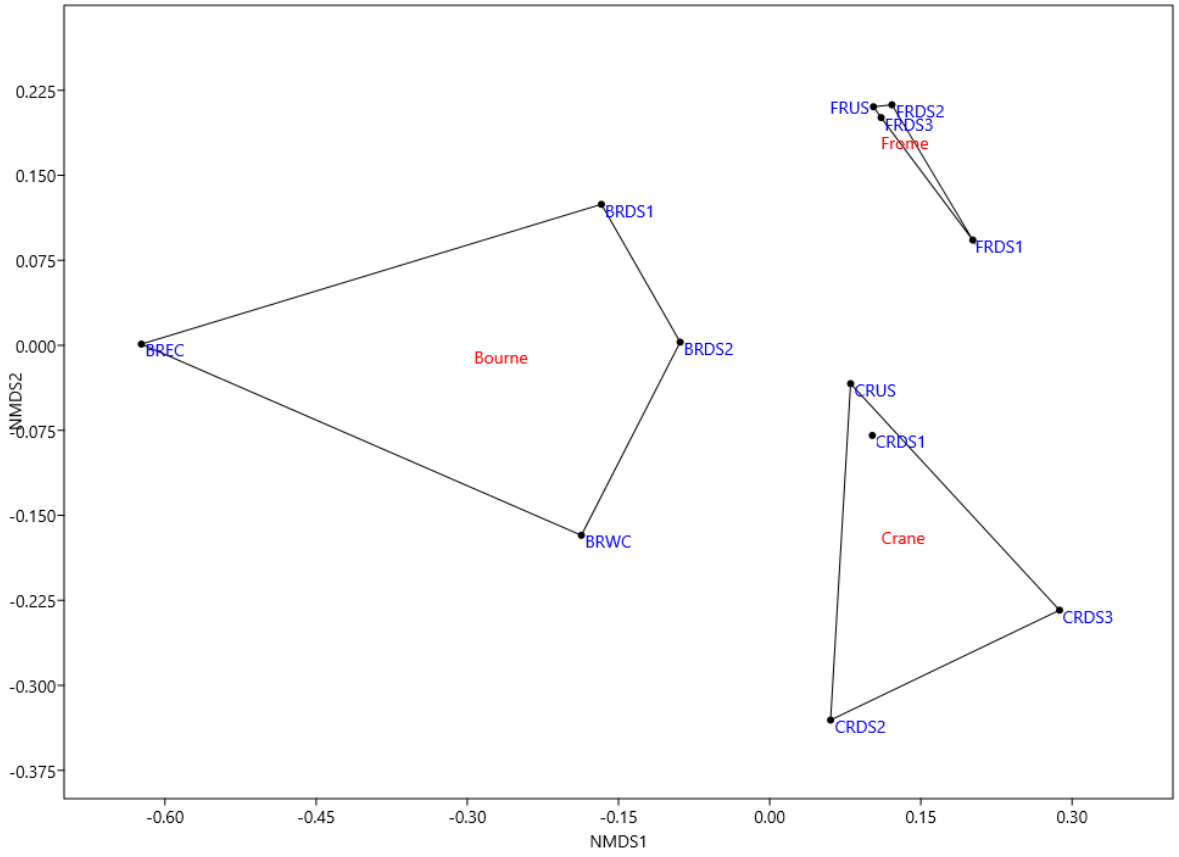

Figure 37 Non-Metric Multidimensional Scaling (NMDS) ordination of 77 macroinvertebrate families against site identity. Sites in closer proximity to one another have a more closely matched macroinvertebrate assemblage than those further apart. Macroinvertebrate samples were taken as standard three-minute kick and one-minute manual search samples between spring 2016 and spring 2018 on the Bourne Rivulet (BRWC, BREC, BRDS1, BRDS2), the River Crane (CRUS, CRDS1, CRDS2, CRDS3) and the River Frome (FRUS, FRDS1, FRDS2, FRDS3). NMDS stress = 0.1204

3.4.9.2 ANOSIM

One-way nested ANOSIM yielded significant differences between macroinvertebrate assemblages across Bourne sites (R = 0.336, P < 0.001). Pairwise ANOSIM testing (Table 13) indicated that significant differences existed between BREC and all other sites and no significant differences existed between any other pairwise combination.

There were significant differences between Crane sites (R = 0.2004, P = 0.0253). Pairwise ANOSIM indicated the differences were caused by CRDS3 being significantly different to both CRDS1 and CRDS2.

One-way nested ANOSIM yielded no significant differences between macroinvertebrate assemblages across Frome sites (R = -0.0216, P = 0.5122), nor any pairwise combination.

Table 13 Pairwise ANOSIM of macroinvertebrate assemblages on Bourne Rivulet sites (BRWC, BREC, BRDS1 and BRDS2), River Crane sites (CRUS, CRDS1, CRDS2 and CRDS3) and River Frome sites (FRUS, FRDS1, FRDS2 and FRDS3) showing *R* values and significant differences in assemblage similarity (α 0.05) highlighted in bold

	BRWC		BREC		BRDS1	
	R	*P*	*R*	*P*	*R*	*P*
BREC	**0.528**	**0.0098**				
BRDS1	0.364	0.064	**0.504**	**0.0072**		
BRDS2	0.188	0.136	**0.484**	**0.0079**	0.112	0.1745

	CRUS		CRDS1		CRDS2	
	R	*P*	*R*	*P*	*R*	*P*
CRDS1	0.012	0.3848				
CRDS2	0.125	0.2045	0.2437	0.1194		
CRDS3	0.124	0.191	**0.312**	**0.0154**	**0.3312**	**0.0348**

	FRUS		FRDS1		FRDS2	
	R	*P*	*R*	*P*	*R*	*P*
FRDS1	0.2963	0.3974				
FRDS2	-0.1852	0.6957	-0.0741	0.5989		
FRDS3	-0.0370	0.5062	0.0741	0.396	-0.444	0.903

3.4.9.3 SIMPER

In order to compare the results obtained by NMDS and to identify the degree to which individual macroinvertebrate taxa differed between study sites, the SIMPER test was applied. The SIMPER test revealed a dissimilarity of 64.42% across all rivers and sites.

The river system with the greatest dissimilarity between sites was the Bourne, which had an overall dissimilarity of 66.43%. On the Bourne, pairwise SIMPER found the greatest dissimilarity between

BREC and BRWC (Table 14). BREC was most dissimilar to all other sites. The least dissimilarity existed between the furthest downstream reaches BRDS1 and BRDS2.

The Crane had the second greatest dissimilarity in macroinvertebrate assemblages between all sites at 59.58%. The sites with the most similar assemblage were CRUS and CRDS1, and the greatest dissimilarity existed between CRDS2 and CRDS3.

The Frome had the least dissimilarity overall, at 44.96% across all sites. Pairwise analysis showed that the greatest dissimilarity existed between FRUS and FRDS and the most similar sites were FRDS2 and FRDS3 (Table 14).

Table 14 Pairwise SIMPER analysis of macroinvertebrate assemblages on Bourne Rivulet sites (BRWC, BREC, BRDS1 and BRDS2) River Crane sites (CRUS, CRDS1, CRDS2 and CRDS3) and River Frome sites (FRUS, FRDS1, FRDS2 and FRDS3) showing percentage dissimilarity between sites

	BRWC	BREC	BRDS1
BREC	78.69		
BRDS1	67.27	72.52	
BRDS2	59.05	70.81	50.23

	CRUS	CRDS1	CRDS2
CRDS1	53.05		
CRDS2	60.1	58.78	
CRDS3	59.08	60.09	66.15

	FRUS	FRDS1	FRDS2
FRDS1	54.46		
FRDS2	47.46	43.86	
FRDS3	44.43	40.31	39.25

Macroinvertebrate families that cumulatively contributed to 95% of dissimilarity between sites are presented in Table 15. The table orders families according to the contribution to dissimilarity, with the family contributing the greatest dissimilarity, Gammaridae, at the top. Full lists of all captured macroinvertebrates, their abundances and their contributions to dissimilarity for each river can be found in appendices 2b for the Bourne Rivulet, 3b for the River Crane and 4b for the River Frome.

Using BMWP scoring as a guide for pollution tolerances, the two sections that follow discuss relative abundances at each site of indicator pollution-sensitive (section 3.4.9.4) and pollution-tolerant taxa (section 3.4.9.5). Families with a BMWP <5 were considered pollution-tolerant and those with a BMWP >5 considered pollution-sensitive. The taxa that were important in distinguishing differences between sites as highlighted by SIMPER in Table 15 have been prioritised as indicator taxa in the

discussions. In instances where a single family had a large contribution to dissimilarity, that family was examined. In other instances, families have been aggregated into order where the pollution tolerances are similar and/or contribution of individual families to dissimilarity is low.

Table 15 Macroinvertebrate families responsible for over 95% cumulative (Cum.%) dissimilarity between all sites on all rivers as calculated by SIMPER analysis, presented with those with the highest contribution to dissimilarities (Cont.%) first. Biological Monitoring Working Party (BMWP) provides a score from 1-10 of the pollution sensitivities of each family, with a score of one for the most pollution-tolerant and ten for the most pollution-sensitive. Mean abundances are from *n* = 5 kick samples from Bourne Rivulet sites BRWC, BREC, BRDS1 and BRDS2; *n* = 5 kick samples from River Crane sites CRUS, CRDS1, CRDS3 and *n* = 4 from CRDS2; *n* = 3 kick samples from River Frome sites FRUS, FRDS1, FRDS2 and FRDS3. A full list of mean abundances (±SE) of all macroinvertebrate families captured and their contributions to dissimilarity are presented in the appendices 2b for the Bourne Rivulet, 3b for the River Crane and 4b for the River Frome

					Mean abundance											
Order	*Family*	*BMWP*	*Cont.%*	*Cum.%*	*BRWC*	*BREC*	*BRDS1*	*BRDS2*	*CRUS*	*CRDS1*	*CRDS2*	*CRDS3*	*FRUS*	*FRDS1*	*FRDS2*	*FRDS3*
Amphipoda	Gammaridae	6	26.33	26.33	520	228	1610	995	967	693	360	403	1790	850	1510	1450
Ephemeroptera	Ephemerellidae	10	16.53	42.86	972	470	1380	666	242	112	291	144	1	2	4	3
Ephemeroptera	Baetidae	4	9.12	51.98	137	585	408	387	166	312	60	99	211	203	210	274
Diptera	Chironomidae	2	6.83	58.81	138	1230	133	57	85	22	78	42	62	34	29	107
Diptera	Simuliidae	5	5.79	64.59	74	235	118	126	125	76	20	368	86	60	21	28
Isopoda	Asellidae	3	4.88	69.48	2	709	14	1	45	68	25	0	1	5	3	12
Coleoptera	Elmidae	5	4.14	73.62	94	0	9	7	49	28	29	51	155	241	229	226
Oligochaeta	Oligochaeta	1	3.6	77.22	34	255	290	116	25	34	30	25	71	37	15	65
Ephemeroptera	Caenidae	7	3.51	80.73	406	5	117	221	0	0	1	0	6	6	2	15
Trichoptera	Hydropsychidae	5	2.3	83.03	14	0	5	33	0	0	1	21	60	109	247	94
Ephemeroptera	Ephemeridae	10	1.92	84.94	1	0	1	4	2	3	81	6	23	179	87	55
Trichoptera	Glossosomatidae	n/a	1.9	86.85	140	0	1	26	7	15	24	71	9	2	18	14
Mollusca	Sphaeriidae	3	1.74	88.59	1	15	0	5	6	121	122	11	1	1	0	8
Tricladida	Planariidae	5	1.12	89.71	5	176	7	2	1	5	1	0	0	1	1	1
Mollusca	Hydrobiidae	3	0.85	90.57	0	0	0	0	0	0	21	50	34	46	4	26
Mollusca	Ancylidae	6	0.78	91.34	10	0	59	5	38	4	1	16	5	3	1	9
Ephemeroptera	Heptageniidae	10	0.77	92.12	1	0	0	6	18	4	6	52	32	18	28	23
Coleoptera	Dytiscidae	5	0.76	92.88	20	3	77	16	7	2	18	1	0	0	1	0
Trichoptera	Limnephilidae	7	0.58	93.46	13	1	4	8	28	14	21	4	3	18	8	7
Trichoptera	Goeridae	10	0.53	94	1	0	5	3	17	5	3	15	3	5	6	58
Coleoptera	Valvatidae	3	0.53	94.53	0	0	0	0	2	84	1	0	0	0	5	4
Mollusca	Neritidae	6	0.51	95.04	0	0	0	0	0	0	0	0	98	19	17	5

3.4.9.4 Pollution-sensitive indicator taxa

A general pattern emerges in the relative abundances of pollution-sensitive taxa between sites, in that abundances of sensitive macroinvertebrates decrease from sites upstream of discharges to downstream (Figure 38). This is more pronounced on the Bourne where the most salad wash effluent is discharged than on the Crane where the discharge of salad wash effluent is infrequent. In contrast, on the Frome where no salad wash effluent is discharged, there is generally an increase in abundances of pollution-sensitive taxa below the discharge.

Gammaridae were the most abundant of all macroinvertebrate groups and they accounted for the greatest dissimilarity between sites at 26.6% (Table 15). On all three rivers, they were at lower abundances in sites immediately downstream of discharges (Figure 38). However, on the Bourne despite low abundances in the east channel (BREC), immediately downstream of the confluence of the east and west channels at BRDS1, Gammaridae abundances were the highest of all four sites.

In Figure 38, Ephemeroptera abundances are presented with Baetidae excluded. Baetidae are unusual for the order in being considered pollution-tolerant with a BWMP score of four, while all other Ephemeroptera families have a BMWP score ≥ 7. As Baetidae contribute to a large proportion of the dissimilarity between sites, their abundances are presented and discussed under pollution-tolerant taxa in section 3.4.9.5. The most abundant Ephemeroptera was the single species from Ephemerellidae, the blue-winged olive (*Serratella ignata* (Poda)) (Table 15). This mayfly appeared in great abundances in the Bourne and Crane but was scarce on the Frome. This species accounted for the second greatest dissimilarity of all taxa (16.5%), second only to Gammaridae. The lowest abundances of pollution sensitive Ephemeroptera are found in the sites directly below discharges on the Bourne and Crane, while on the Frome the highest abundances were found below discharge.

Plecopterans were responsible for very little dissimilarity between sites using SIMPER analysis due to their relatively very low abundances in samples. However, as they are highly pollution-sensitive, they are considered very useful indicators of habitat quality. Most belonged to the family Perlodidae, but as numbers were low and all Plecopterans are highly sensitive, all families have been aggregated into the order for comparison in Figure 38. Plecoptera were absent in BREC and at a very low mean abundance in BRDS1 rising at the most downstream reach BRDS2, with the highest abundances found in BRWC. On the Crane, there was approximately one tenth of the abundance in CRDS1 as upstream of the discharge in CRUS. Abundances on the Frome were in single figures and increased downstream across all sites.

Trichopterans appeared highly impacted by discharges, particularly on the Bourne. Just three families were present in BREC and mean abundances were in single figures. In BRWC, nine families were present, and abundances were close to 200. There appeared very slow recovery downstream

of BREC, with mean densities showing slow recovery through BRDS1 and BRDS2. On the Crane, mean densities were once again lower downstream of the discharge than above it, but the difference was not as acute as on the Bourne. The Frome had the greatest diversity of Trichoptera families, with 10 each at FRUS and FRDS3, and 11 and 12 at FRDS1 and RRDS2 respectively. The Frome also held the greatest abundances of Trichoptera, and the site below the watercress bed irrigation discharge held greater abundance and diversity than upstream.

Riffle beetles (Elmidae) were the most abundant of all Coleopterans across all sites, with *Oreodytes sanmarkii* belonging to Dytiscidae typically present, though in smaller numbers. Although Elmids have a midpoint BMWP score of five, they are indicative of clean and highly oxygenated water and as such considered excellent indicators of water quality (Elliott 2008). Moreover, as SIMPER analysis categorised them with as having a high contribution to dissimilarity between sites, their abundances are presented in Figure 38. Once again, their abundances are lower below discharges than above for the Bourne and Crane where salad wash effluent is discharged, but in higher abundances below watercress bed irrigation effluent on the Frome.

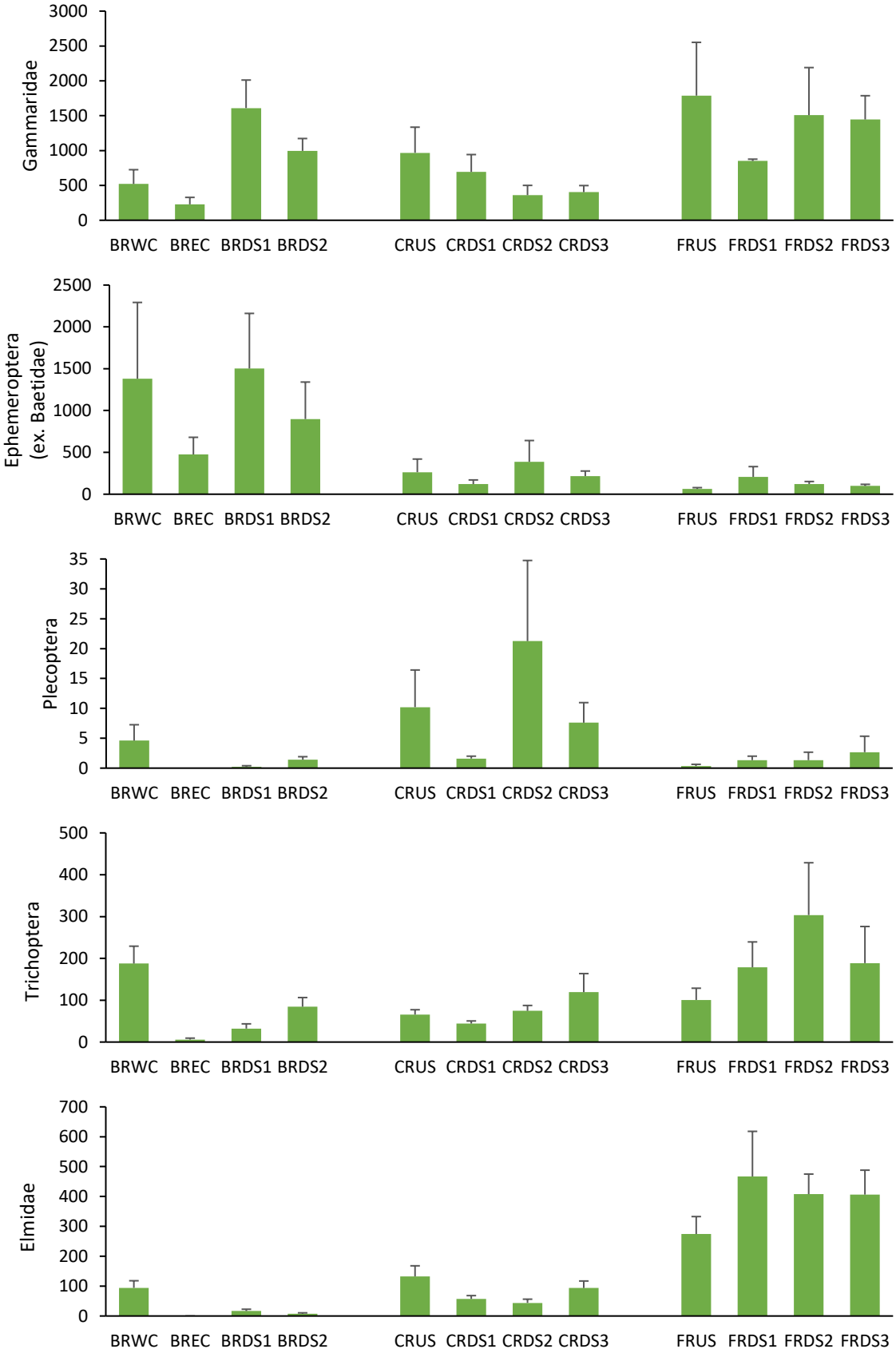

Figure 38 Mean abundances of pollution-sensitive macroinvertebrate taxa; Gammaridae, Ephemeroptera (excluding the family Baetidae), Plecoptera, Trichoptera and Elmidae. Mean abundances (±SE) taken from *n* = 5 kick samples from Bourne Rivulet sites BRWC, BREC, BRDS1 and BRDS2; *n* = 5 kick samples from River Crane sites CRUS, CRDS1, CRDS3 and *n* = 4 from CRDS2; *n* = 3 kick samples from River Frome sites FRUS, FRDS1, FRDS2 and FRDS3

3.4.9.5 Pollution-tolerant indicator taxa

The mean abundances of pollution-tolerant macroinvertebrate taxa are presented in Figure 39. The trend observed for pollution-sensitive taxa in Figure 38 is largely reversed for pollution-tolerant macroinvertebrates, with sites downstream of discharges of salad wash effluent such as the Bourne sites BREC and BRDS1 and the Crane site CRDS1 having the greatest abundances. Once again, on the Frome, where there was no salad wash effluent is discharged, there appeared no consistent pattern between sites upstream and downstream of the watercress bed irrigation discharge.

Baetidae were isolated from the rest of Ephemeroptera, as they have a BMWP score of four. On the Bourne, their abundance is markedly higher in BREC than BRWC (Figure 39), a clear reversal of the trend for pollution-sensitive Ephemeropterans seen in Figure 38. Abundances were higher in CRDS1 than upstream in CRUS, but the difference was not as acute as observed on the Bourne. Baetidae abundances were largely similar across all Frome sites.

The Bourne east channel has exceptionally high abundances of Chironomids, Asellidae, Oligochaetes and Mollusca relative to all other sites. With Chironomids, Asellidae and Mollusca, abundances are largely back to the levels seen in the west channel by BRDS1, suggesting limited downstream effects from the discharges. However, for Oligochaetes, considered the most pollution-tolerant macroinvertebrate with a BMWP score of one, abundances increased in BRDS1 and remained high in BRDS2 (Figure 39). Oligochaetes were also highest in CRDS1 on the Crane, and at relatively consistent abundance at all other Crane sites.

Molluscs are generally considered pollution-tolerant, however in the samples there were two exceptions, the river limpet *Ancylus fluviatilis* and Neritid snails, both of which are considered indicative of high-quality habitat. As such, the mollusc data presented in Figure 39 excludes the families Ancylidae and Neritidae. On the Bourne, molluscs were approximately five times more abundant in BREC than all other Bourne sites. Abundances on the Crane were also highest below the discharge at CRDS1 and were lowest upstream at CRUS. The mollusc assemblage on the Crane mostly consisted on the pea mussel (Sphaeriidae), and these were exceptionally abundant in CRDS1 and CRDS2 (Table 15). On the Frome, the upstream site FRUS held the highest densities of pollution-tolerant molluscs, but this fast flowing site also had an abundance of the pollution-sensitive Neritidae (Table 15).

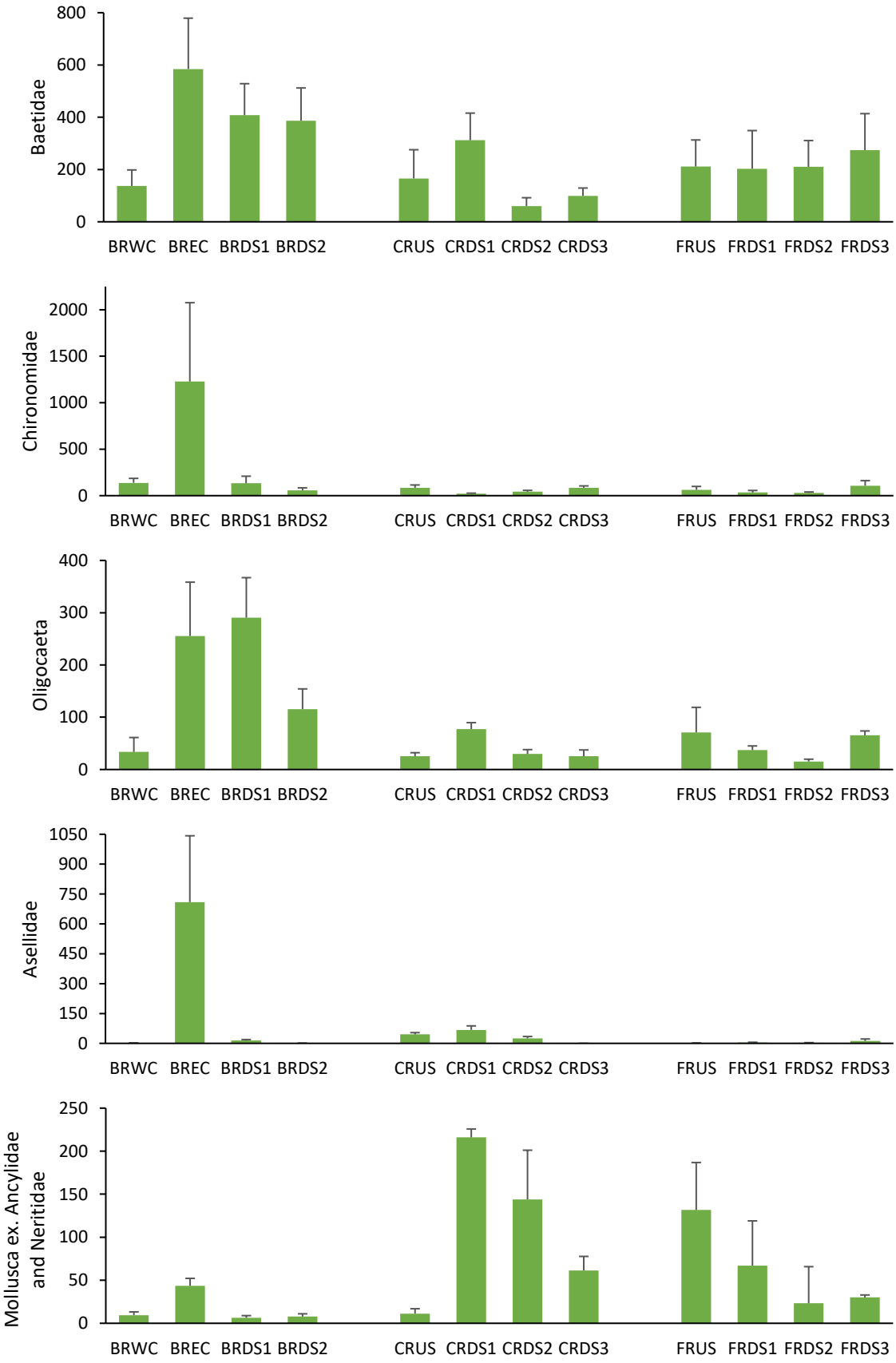

Figure 39 Mean abundances of pollution-tolerant macroinvertebrate taxa; Baetidae, Chironomidae, Oligochaeta, Asellidae, Mollusca (excluding pollution-sensitive Ancylidae and Neritidae) and Hirudinea. Mean abundances (±SE) taken from *n* = 5 kick samples from Bourne Rivulet sites BRWC, BREC, BRDS1 and BRDS2; *n* = 5 kick samples from River Crane sites CRUS, CRDS1, CRDS3 and *n* = 4 from CRDS2; *n* = 3 kick samples from River Frome sites FRUS, FRDS1, FRDS2 and FRDS3

3.4.9.6 Macroinvertebrate abundance and family richness

Patterns of macroinvertebrate abundance and family richness (NTAXA) downstream of watercress farm discharges relative to upstream were not consistent between the three rivers (Figure 40). The discharge on the Bourne displayed more marked differences than both the Crane and Frome and the impact on both metrics was in the opposite direction of those two rivers. Summer abundances tended to be higher than autumn across all sites, though less pronounced on the Frome (Table 16).

On the Bourne, the mean abundances were broadly similar for the west channel (BRWC) which receives watercress bed irrigation water only and the furthest downstream site BRDS2 (Figure 40). Abundances were considerably higher in both the east channel (BREC) which receives 100% of its water from irrigation and salad wash effluent, and after the confluence of BRWC and BREC at BRDS1. The number of different macroinvertebrate families was lowest in BREC and highest in BRWC.

On the Crane, where salad wash effluent in occasionally discharged, the mean macroinvertebrate abundances were highest upstream CRUS, and progressively decreased downstream until there was a slight uptick at CRDS3 (Figure 40). Abundances were greater in the summer than in the autumn at all sites, with the exception of spring 2016 which was relatively low (Table 16). The mean NTAXA peaked in the immediate downstream site (CRDS1) and steadily declined downstream.

On the Frome, total mean macroinvertebrate abundances were highest upstream of the discharge in FRUS and lowest in the site immediately downstream. Mean abundances in FRDS2 and FRDS3 were close to one another. There did not appear to be the seasonal pattern in total macroinvertebrate abundances that were evident on the Bourne and Crane, where abundances were highest in spring (Table 16). On the Frome, abundances were higher in autumn at FRUS and lower in autumn at FRDS1. The total taxonomic richness (NTAXA) increased with increasing distance downstream FRUS to FRDS3.

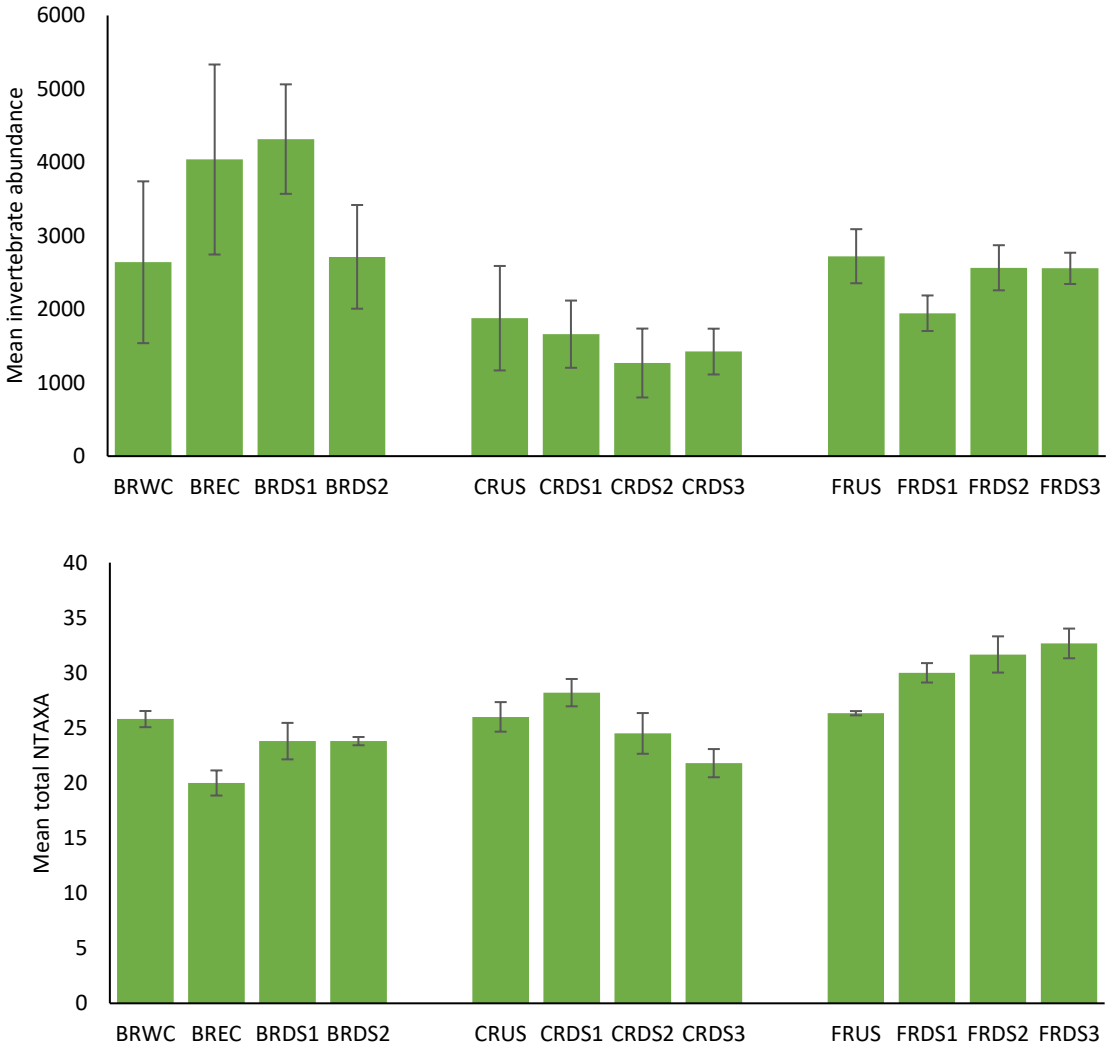

Figure 40 Mean (±SE) macroinvertebrate abundance and family richness (Total NTAXA) from standard three-minute kick and one-minute manual search sampling from five surveys on Bourne Rivulet sites; BRWC, BREC, BRDS1 and BRDS2, River Crane sites; CRUS, CRDS1, CRDS2 and CRDS3, River Frome sites; FRUS, FRDS1, FRDS2 and FRDS3 (see Table 16 for *n* and dates)

Table 16 Macroinvertebrate abundance (Ab.) and family richness (NTAXA) from standard three-minute kick and one-minute manual search sampling during surveying dates (S = spring, A = autumn + year), and the mean and standard error for all surveys on Bourne Rivulet sites; BRWC, BREC, BRDS1 and BRDS2, River Crane sites; CRUS, CRDS1, CRDS2 and CRDS3 and River Frome sites; FRUS, FRDS1, FRDS2 and FRDS3

					Date				
Site	*Measure*	*Mean*	*se*	*n*	*S16*	*A16*	*S17*	*A17*	*S18*
BRWC	Ab.	2639.8	1101.5	5	6819	1395	1050	1014	2921
	NTAXA	25.8	0.7	5	25	28	25	24	27
BREC	Ab.	4038.8	1293.6	5	9017	2693	3488	1543	3453
	NTAXA	20.0	1.1	5	22	16	22	19	21
BRDS1	Ab.	4316.6	745.6	5	4541	2612	4219	3247	6964
	NTAXA	23.8	1.7	5	23	30	23	23	20
BRDS2	Ab.	2713.4	706.0	5	4616	1485	1728	1482	4301
	NTAXA	23.8	0.4	5	24	25	24	23	23
CRUS	Ab.	1878.0	711.2	5	700	929	2179	1049	4533
	NTAXA	26.0	1.3	5	21	27	26	27	29
CRDS1	Ab.	1660.6	457.8	5	955	910	2094	1072	3272
	NTAXA	28.2	1.2	5	25	32	27	27	30
CRDS2	Ab.	1267.8	469.1	4	n/a	499	941	996	2635
	NTAXA	24.5	1.8	4	n/a	24	25	20	29
CRDS3	Ab.	1423.6	311.9	5	384	2181	1351	1267	1935
	NTAXA	21.8	1.3	5	18	22	20	25	24
FRUS	Ab.	2722.0	368.2	3	n/a	3501	1458	3207	n/a
	NTAXA	26.3	0.2	3	n/a	26	27	26	n/a
FRDS1	Ab.	1946.0	242.0	3	n/a	1228	2680	1930	n/a
	NTAXA	30.0	0.9	3	n/a	28	33	29	n/a
FRDS2	Ab.	2564.7	307.2	3	n/a	3628	1997	2069	n/a
	NTAXA	31.7	1.6	3	n/a	26	34	35	n/a
FRDS3	Ab.	2556.7	212.9	3	n/a	1954	2490	3226	n/a
	NTAXA	32.7	1.3	3	n/a	32	37	29	n/a

3.4.9.7 Ephemeroptera, Plecoptera and Trichoptera

Abundances of the orders Ephemeroptera, Plecoptera and Trichoptera (EPT) were higher in summer than in autumn, as would be expected for their typical life cycles, with autumn surveys occurring after summer emergence of adults (Table 17). Averaging across all summer and autumn surveys, the Bourne sites were shown to hold the most abundant EPT populations, approximately double that of the Crane and Frome (Figure 41).

On the Bourne, the percentage of EPT orders in BRWC was approximately double that found in BREC, with the percentage increasing further downstream but never returning to the levels found upstream in BRWC. The EPT family richness (EPT NTAXA) followed a similar pattern, but with the disparity between BRWC and BREC even stronger, with BREC being particularly depauperate in EPT families.

On the Crane, mean EPT abundance was relatively consistent across all four sites (Figure 41). However, as a percentage of EPT in macroinvertebrate samples and number of EPT families in samples, there was greater variation. Both %EPT and EPT NTAXA followed a similar trajectory, increasing downstream from CRUS to CRDS2 before dropping back at CRDS3. As %EPT and EPT abundances were higher across all sites in the summer surveys (Table 17), with one fewer summer survey in CRDS2, it may have been expected that this would generate lower means for these metrics at this site. It is therefore notable that CRDS2 still had the highest mean values for all metrics. Removing the summer 2016 data for all other sites to obtain parity for CRDS2 and regenerating means was tried and found to not change the pattern.

On the Frome, mean EPT abundances increased downstream, with the upstream site FRUS having the lowest abundance. The percentage of EPT in samples peaked and were broadly similar for FRDS1 and FRDS2 and were lowest in FRUS and FRDS3 (Figure 41). The EPT NTAXA was highest on the Frome than either the Bourne or Crane, a broad range of EPT families represented on the river. The mean EPT NTXA clearly increased downstream on the Frome.

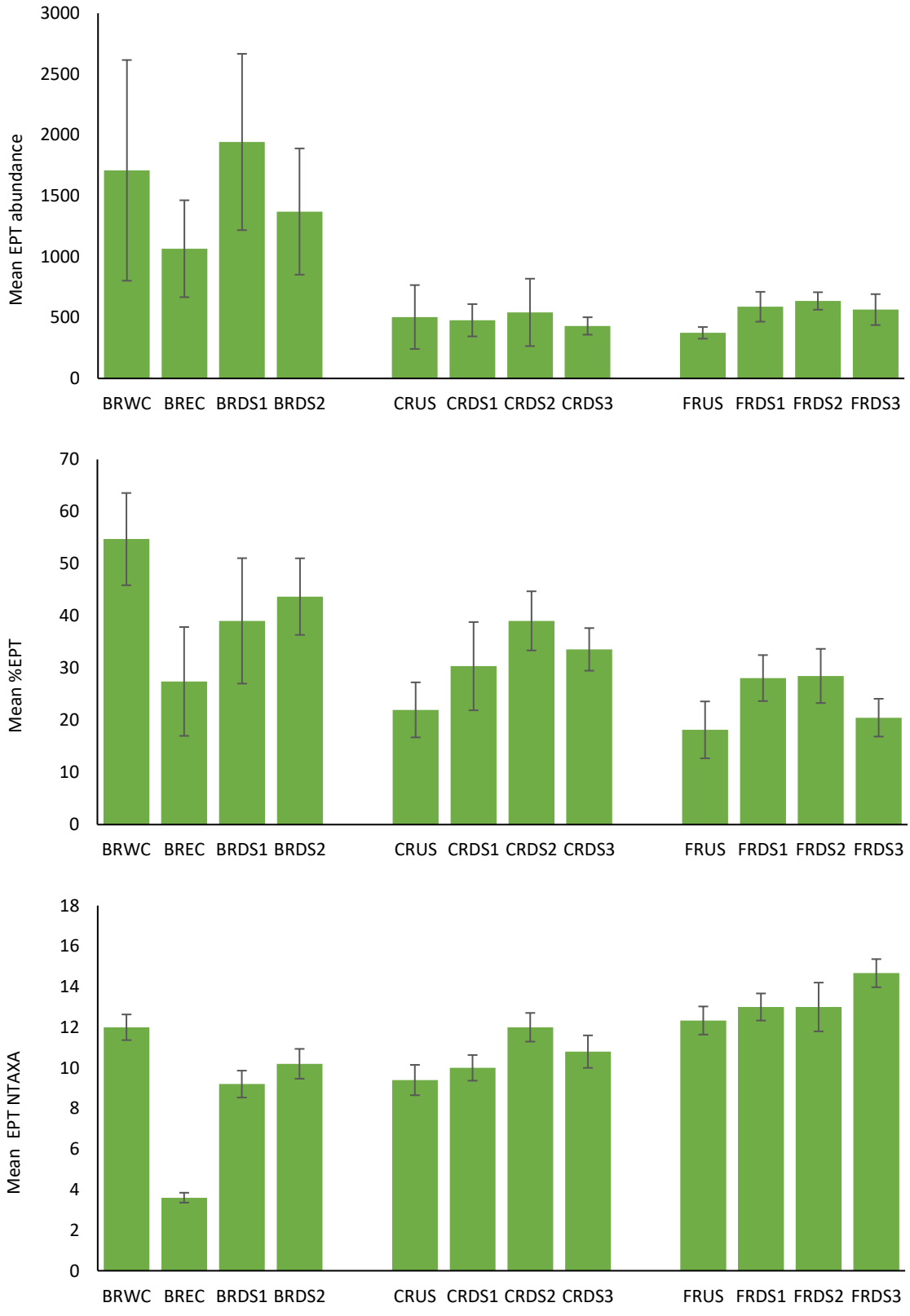

Figure 41 Mean (±SE) Ephemeroptera, Plecoptera and Trichoptera percentage abundance (%EPT), EPT abundance and EPT family richness (EPT NTAXA) from standard three-minute kick and one-minute manual search sampling from five surveys on Bourne Rivulet sites; BRWC, BREC, BRDS1 and BRDS2, River Crane sites; CRUS, CRDS1, CRDS2 and CRDS3 and River Frome sites; FRUS, FRDS1, FRDS2 and FRDS3 (see Table 17 for *n* and dates)

Table 17 Ephemeroptera, Plecoptera and Trichoptera (EPT) abundances (EPT ab.), percentage abundance of EPT taxa (%EPT) and EPT family richness (EPT NTAXA) from standard three-minute kick and one-minute manual search sampling during surveying (S = spring, A = autumn + year), and the mean and standard error for all surveys on the Bourne Rivulet; BRWC, BREC, BRDS1 and BRDS2; the River Crane; CRUS, CRDS1,CRDS2 and CRDS3; the River Frome; FRUS, FRDS1, FRDS2 and FRDS3

					Date				
Site	*Measure*	*Mean*	*se*	*n*	*S16*	*A16*	*S17*	*A17*	*S18*
BRWC	EPT ab.	1709.2	906.7	5	5195	641	692	256	1762
	%EPT	54.7	8.8	5	76	46	66	25	60
	EPT NTAXA	12.0	0.6	5	11	14	13	11	11
BREC	EPT ab.	1065.6	398.0	5	1573	45	1211	306	2193
	%EPT	27.4	10.4	5	17	2	35	20	64
	EPT NTAXA	3.6	0.2	5	4	3	4	3	4
BRDS1	EPT ab.	1942.4	724.4	5	3011	318	2220	265	3898
	%EPT	39.0	12.0	5	66	12	53	8	56
	EPT NTAXA	9.2	0.7	5	7	11	9	10	9
BRDS2	EPT ab.	1370.8	518.3	5	2524	413	804	387	2726
	%EPT	43.7	7.3	5	55	28	47	26	63
	EPT NTAXA	10.2	0.7	5	8	11	9	12	11
CRUS	EPT ab.	504.4	262.4	5	191	105	696	77	1453
	%EPT	22.0	5.3	5	27	11	32	7	32
	EPT NTAXA	9.4	0.7	5	7	9	11	9	11
CRDS1	EPT ab.	477.8	132.6	5	538	60	823	319	649
	%EPT	30.4	8.5	5	56	7	39	30	20
	EPT NTAXA	10.0	0.6	5	8	10	10	10	12
CRDS2	EPT ab.	542.5	276.8	4	n/a	221	314	264	1371
	%EPT	39.0	5.7	4	n/a	44	33	27	52
	EPT NTAXA	12.0	0.7	4	n/a	11	11	12	14
CRDS3	EPT ab.	431.0	71.6	5	172	441	444	488	610
	%EPT	33.6	4.1	5	45	20	33	39	32
	EPT NTAXA	10.8	0.8	5	8	10	12	12	12
FRUS	EPT ab.	374.3	47.8	3	n/a	289	540	294	n/a
	%EPT	18.2	5.5	3	n/a	8	37	9	n/a
	EPT NTAXA	12.3	0.7	3	n/a	13	14	10	n/a
FRDS1	EPT ab.	588.7	122.7	3	n/a	166	840	760	n/a
	%EPT	28.1	4.4	3	n/a	14	31	39	n/a
	EPT NTAXA	13.0	0.7	3	n/a	13	15	11	n/a
FRDS2	EPT ab.	636.3	71.8	3	n/a	395	809	705	n/a
	%EPT	28.5	5.2	3	n/a	11	41	34	n/a
	EPT NTAXA	13.0	1.2	3	n/a	9	16	14	n/a
FRDS3	EPT ab.	565.3	127.1	3	n/a	158	624	914	n/a
	%EPT	20.5	3.6	3	n/a	8	25	28	n/a
	EPT NTAXA	14.7	0.7	3	n/a	14	17	13	n/a

3.4.9.8 Macroinvertebrate assemblage diversity

There was wide variation in Shannon index scores between sites and seasons, with no discernible seasonal patterns emerging on the three rivers surveyed (Figure 42). The data presented have been summarised as averages across all years and seasons to aid comparison between sites and to look for trends between sites upstream and downstream of watercress farm discharges. However, no consistent pattern emerges.

On the Bourne, the Shannon index score is lowest at BRDS1, while at the east channel (BREC) which receives all its water from salad wash effluent and watercress bed irrigation water, the score was not significantly different to the furthest downstream reach, BRDS2 (Figure 42). The west channel (BRWC) which receives a small quantity of watercress bed irrigation water only had the highest mean Shannon index score.

In contrast, on the Crane, the Shannon index score increased downstream of the discharge from CRUS to CRDS1 and increased further at CRDS2.

Mean Shannon index score on the Frome indicated that site FRDS1 was the most diverse and was significantly more diverse than FRUS, the site with the lowest diversity. FRDS1, FRDS2 and FRDS3 did not differ significantly, and all were more diverse than FRUS (Figure 42).

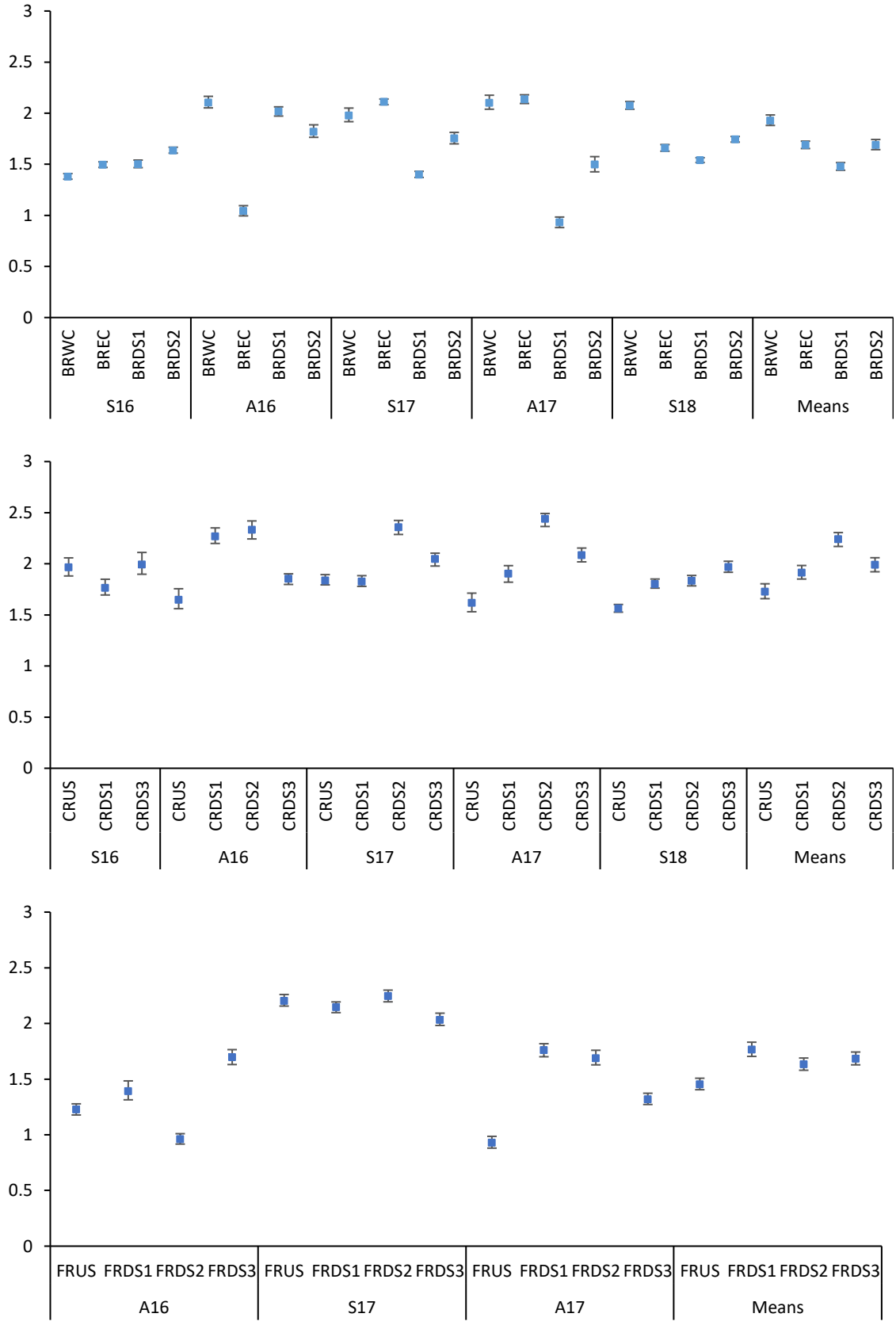

Figure 42 Shannon diversity index of macroinvertebrate families from samples taken with a standard three-minute kick and one-minute manual search sampling during surveying (S = spring, A = autumn + year) and the means of all surveys on Bourne Rivulet sites; BRWC, BREC, BRDS1 and BRDS2 (n = 5); River Crane sites CRUS, CRDS1, CRDS2 and CRDS3 (n = 5 for all sites except CRDS2 where n = 4); River Frome sites FRUS, FRDS1, FRDS2 and FRDS3 (n = 3). Error bars represent bootstrapped 95% CI

3.4.9.9 Walley Hawkes Paisley Trigg biotic index

Sites receiving salad wash effluent had lower observed WHPT scores than upstream or reference sites. This was most notable on the Bourne, which receives the greatest quantity of salad wash effluent. The site receiving the salad wash effluent, BREC, had observed WHPT scores significantly below expected (Table 18). At BRDS1, downstream of the confluence of the west channel (BRWC) and BREC, there appears to be some downstream recovery with the mean Observed/Expected (O/E) WHPT ratio increasing but still <1 (Figure 43). In contrast, the west channel BRWC which receives just a small amount of watercress bed irrigation water and no salad wash effluent has a mean WHPT score significantly higher than expected (Table 18) and the highest WHPT O/E score of all Bourne sites (Figure 43). BRWC was the only site to maintain O/E values >1 for all surveys (Figure 43). The habitat surveying necessary to generate expected WHPT scores (WHPT-E) was not possible for BREC in autumn surveys when the channel was infilled with emergent macrophytes. However, observed autumn WHPT values for BREC were the lowest of all sites on the Bourne (Table 18).

On the Crane, where the watercress farm discharges small quantities of salad wash effluent intermittently, the WHPT-O scores were lower below the discharge at CRDS1 than above it at CRUS (Table 19). This was also true for mean O/E scores (Figure 44). However, WHPT-O scores for both CRUS and CRDS1 were marginally higher than expected. Further downstream at CRDS2 and CRDS3, the WHPT O/E ratio rose, with both sites having significantly higher observed WHPT scores than expected (Table 19).

On the Frome, all sites had significantly higher WHPT-O than WHPT-E (Table 20). No salad wash effluent is discharged into FRDS1, only watercress bed irrigation discharges, and here the mean WHPT O/E is higher in the site immediately downstream than above it (Figure 45).

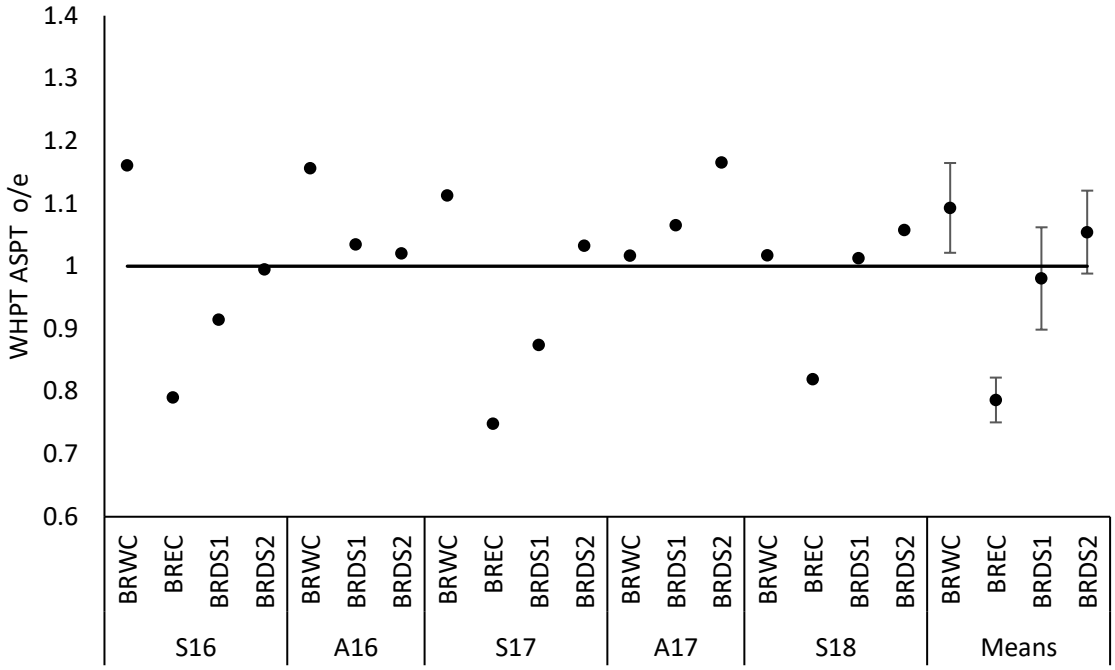

Figure 43 The Bourne Rivulet Observed/Expected Walley, Hawkes, Paisley Trigg Average Score Per Taxon (WHPT ASPT O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 5 for all sites except BREC where *n* = 3). Data points which fall below the horizontal line at a WHPT ASPT O/E of one indicate observed WHPT ASPT scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed WHPT ASPT scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected WHPT ASPT values, mean values and statistical comparison between observed and expected values are presented in Table 18

Table 18 The Bourne Rivulet multivariate biotic index scores for Walley, Hawkes, Paisley, Trigg average score per taxon observed (WHPT-O) and expected (WHPT-E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys (*n* = 5 for all sites except BREC where *n* = 3). The River Invertebrate Classification Tool (RICT) was used to generate WHPT-E and PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected WHPT and PSI scores were explored using two-way paired student's t-tests (α = 0.05)

				Date						
Site	*Measure*	*Mean*	*se*	*S16*	*A16*	*S17*	*A17*	*S18*	*T-value*	*P*
BRWC	WHPT obs.	6.097	0.155	6.436	6.300	6.272	5.608	5.867		
	WHPT pred.	5.581	0.055	5.542	5.448	5.634	5.514	5.766	2.93	**0.043**
	WHPT O/E	1.093	0.032	1.161	1.156	1.113	1.017	1.017		
BREC	WHPT obs.	4.440	0.158	4.436	3.931	4.682	4.305	4.843		
	WHPT pred.	5.924	0.186	5.611	n/a	6.253	n/a	5.907	-8.25	**0.014**
	WHPT O/E	0.786	0.021	0.791	n/a	0.749	n/a	0.820		
BRDS1	WHPT obs.	5.552	0.150	5.057	5.660	5.378	5.883	5.785		
	WHPT pred.	5.676	0.125	5.527	5.470	6.150	5.522	5.712	-2.60	0.060
	WHPT O/E	0.980	0.037	0.915	1.035	0.875	1.065	1.013		
BRDS2	WHPT obs.	5.939	0.189	5.505	5.552	5.924	6.467	6.248		
	WHPT pred.	5.632	0.084	5.531	5.440	5.736	5.547	5.906	1.87	0.135
	WHPT O/E	1.054	0.030	0.995	1.021	1.033	1.166	1.058		

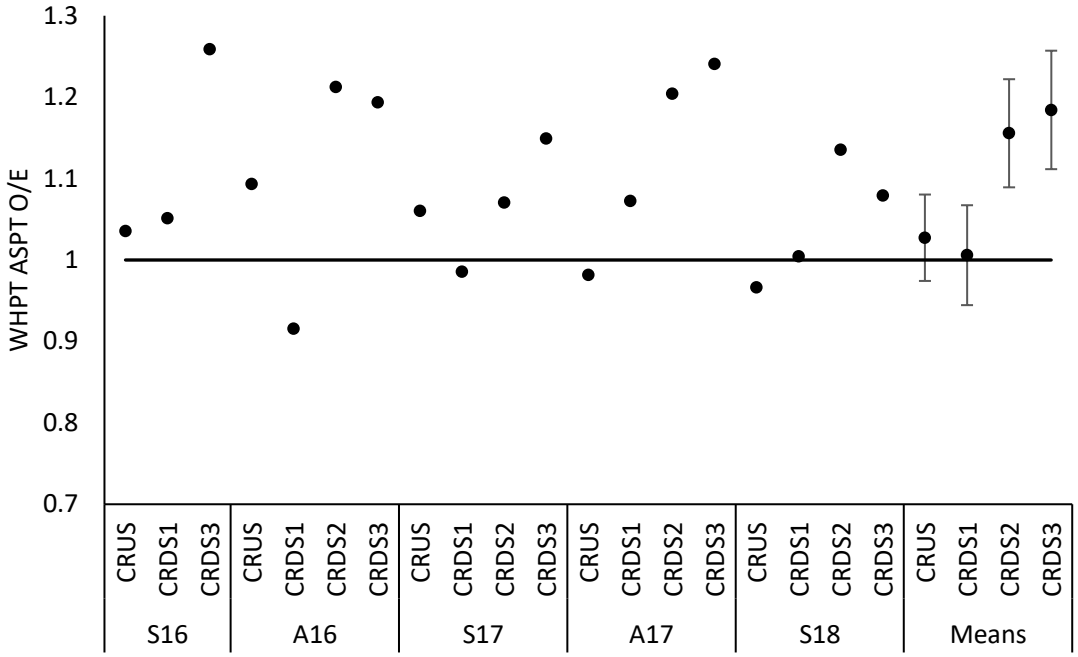

Figure 44 The River Crane Observed/Expected Walley, Hawkes, Paisley Trigg Average Score Per Taxon (WHPT ASPT O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 5 for all sites except CRDS2 where *n* = 4). Data points which fall below the horizontal line at a WHPT ASPT O/E of one indicate observed WHPT ASPT scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed WHPT ASPT scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected WHPT ASPT values, mean values and statistical comparison between observed and expected values are presented in Table 19

Table 19 The River Crane multivariate biotic index scores for Walley, Hawkes, Paisley, Trigg average score per taxon observed (WHPT-O) and expected (WHPT-E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys. The River Invertebrate Classification Tool (RICT) was used to generate WHPT-E and PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected WHPT and PSI scores were explored using two-way paired student's t-tests ($\alpha = 0.05$)

				Date						
Site	*Measure*	*Mean*	*se*	*S16*	*A16*	*S17*	*A17*	*S18*	*T-value*	*P*
CRUS	WHPT obs.	5.782	0.141	5.795	5.915	6.196	5.344	5.662		
	WHPT pred.	5.631	0.095	5.597	5.411	5.845	5.444	5.859	1.14	0.317
	WHPT O/E	1.027	0.024	1.035	1.093	1.060	0.982	0.966		
CRDS1	WHPT obs.	5.632	0.162	5.840	4.997	5.744	5.704	5.877		
	WHPT pred.	5.602	0.104	5.555	5.457	5.828	5.318	5.851	0.20	0.848
	WHPT O/E	1.006	0.027	1.051	0.916	0.986	1.073	1.004		
CRDS2	WHPT obs.	6.325	0.067	n/a	6.354	6.160	6.305	6.482		
	WHPT pred.	5.485	0.143	n/a	5.241	5.754	5.236	5.709	5.15	**0.014**
	WHPT O/E	1.156	0.033	n/a	1.212	1.071	1.204	1.135		
CRDS3	WHPT obs.	6.587	0.131	7.000	6.489	6.605	6.652	6.188		
	WHPT pred.	5.568	0.077	5.560	5.437	5.748	5.361	5.734	5.89	**0.004**
	WHPT O/E	1.184	0.033	1.259	1.193	1.149	1.241	1.079		

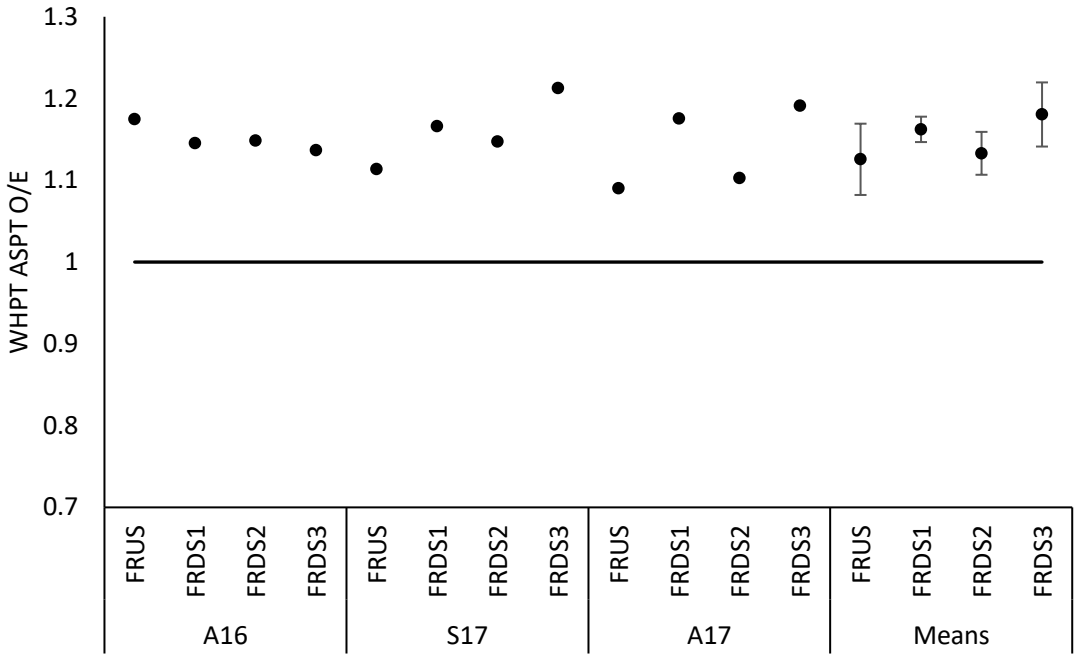

Figure 45 The River Frome Observed/Expected Walley, Hawkes, Paisley Trigg Average Score Per Taxon (WHPT ASPT O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 3). Data points which fall below the horizontal line at a WHPT ASPT O/E of one indicate observed WHPT ASPT scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed WHPT ASPT scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected WHPT ASPT values, mean values and statistical comparison between observed and expected values are presented in Table 20

Table 20 The River Frome multivariate biotic index scores for Walley, Hawkes, Paisley, Trigg average score per taxon observed (WHPT-O) and expected (WHPT-E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys (*n* = 3). The River Invertebrate Classification Tool (RICT) was used to generate WHPT-E and PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected WHPT and PSI scores were explored using two-way paired student's t-tests (α = 0.05)

				Date				
Site	*Measure*	*Mean*	*se*	*A16*	*S17*	*A17*	*T-value*	*P*
FRUS	WHPT obs.	6.394	0.054	6.481	6.493	6.208		
	WHPT pred.	5.680	0.052	5.517	5.829	5.694	5.390	**0.033**
	WHPT O/E	1.126	0.015	1.175	1.114	1.090		
FRDS1	WHPT obs.	6.306	0.047	6.221	6.470	6.228		
	WHPT pred.	5.426	0.042	5.432	5.548	5.297	19.290	**0.003**
	WHPT O/E	1.162	0.005	1.145	1.166	1.176		
FRDS2	WHPT obs.	6.218	0.065	6.246	6.397	6.011		
	WHPT pred.	5.488	0.025	5.438	5.575	5.452	8.550	**0.013**
	WHPT O/E	1.133	0.009	1.149	1.147	1.103		
FRDS3	WHPT obs.	6.363	0.094	6.078	6.641	6.369		
	WHPT pred.	5.390	0.025	5.347	5.475	5.347	7.610	**0.017**
	WHPT O/E	1.180	0.013	1.137	1.213	1.191		

3.4.9.10 Proportion of sediment-sensitive invertebrates (PSI)

The trends observed for proportion of sediment sensitive invertebrate (PSI) are similar to those reported in the WHPT analysis in section 3.4.9.9. Sites that received discharged salad wash effluent saw the greatest perturbations. For PSI, this took the form of a lowered percentage of sediment sensitive macroinvertebrates in samples, indicating increased sedimentation.

On the Bourne, the east channel had an observed mean PSI (PSI-O) that was significantly lower than the expected value (PSI-E), while both BRWC and BRDS2 both were significantly higher than expected (Table 21). The mean PSI O/E was >1 on BRDS1 (Figure 46), but the difference was not significant at this site. Observed mean PSI-O and PSI O/E scores suggest lowest siltation at BRWC, highest at BREC, with sediment decreasing with distance from the discharge (Table 21 and Figure 46). Similar trends between sites in PSI O/E occur across surveys in the study period, with spring surveys in particular showing relatively consistent between site trends (Table 21). With BREC only surveyed in spring, there was very little variation between its PSI O/E scores.

On the Crane, where salad wash effluent is occasionally discharged, all sites had higher mean PSI-O scores than PSI-E, significantly so for sites other than the immediate downstream site CRDS1 (Table 22). The PSI O/E values for all sites and surveys were >1 with the exception of CRDS1 in autumn 2016 (Figure 47). The mean PSI-O was lowest at CRDS1 and was higher upstream at CRUS and increased with increasing distance from discharge (Table 22). As on the Bourne, this suggests that fine sediment load decreased with increasing distance from the discharge

All Frome sites had mean PSI-O scores higher than mean PSI-E, significantly so for all but FRUS (Table 23). The PSI-O value was higher than the PSI-E for all sites and seasons on the Frome, resulting in PSI O/E ratios >1 for every site and survey (Figure 48). Observed mean PSI scores were highest on FRUS and lowest on FRDS1 and increased downstream to FRDS2 and FRDS3 (Table 23). Once again, this would suggest that fine sediment load was decreasing with distance from the source of discharge, the the case of the Frome, of watercress bed irrigation water only.

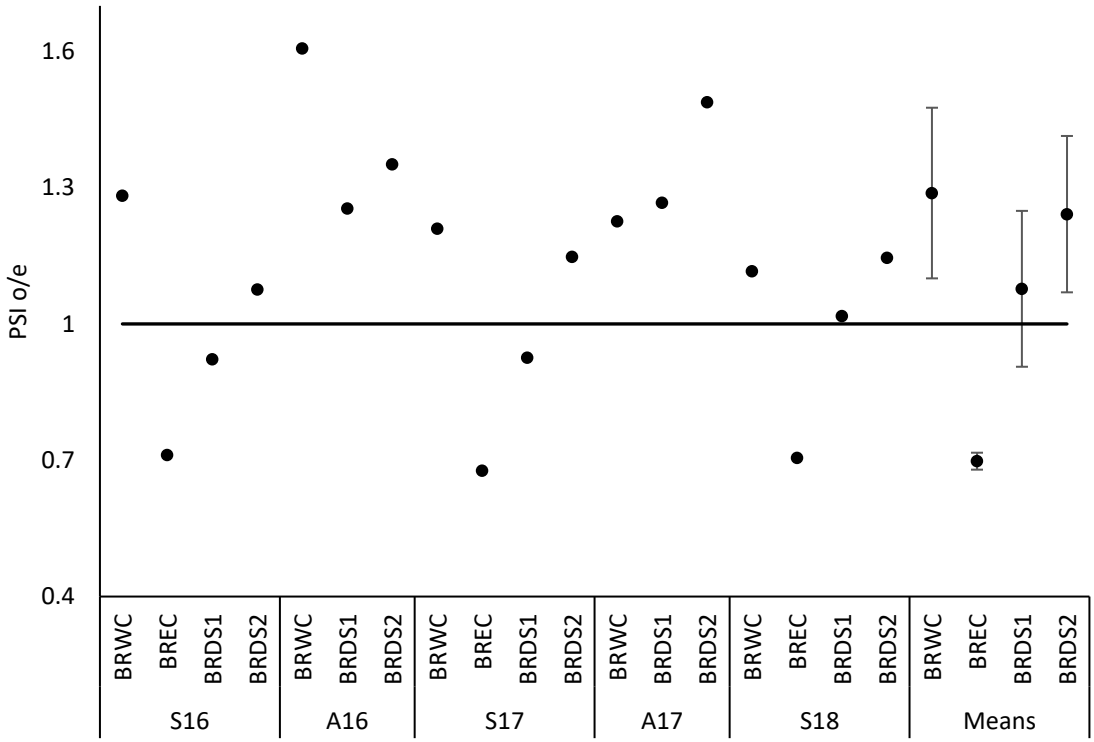

Figure 46 The Bourne Rivulet Observed/Expected Proportion of Sediment-sensitive Invertebrates (PSI O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 5 for all sites except BREC where *n* = 3). Data points which fall below the horizontal line at a PSI O/E of one indicate observed PSI scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed PSI scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected PSI values, mean values and statistical comparison between observed and expected values are presented in Table 21

Table 21 The Bourne Rivulet multivariate biotic index scores for the Proportion of Sediment-sensitive Invertebrates (PSI), both observed (PSI-O) and expected (PSI-E) and the O/E ratio (PSI O/E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys. The River Invertebrate Classification Tool (RICT) was used to generate PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected PSI scores were explored using two-way paired student's t-tests (α = 0.05)

				Date						
Site	*Measure*	*Mean*	*se*	*S16*	*A16*	*S17*	*A17*	*S18*	*T-value*	*P*
BRWC	PSI obs.	64.800	2.926	63.158	75.510	65.957	60.000	59.375		
	PSI pred.	50.586	1.409	49.248	47.003	54.531	48.949	53.200	3.75	**0.020**
	PSI O/E	1.288	0.084	1.282	1.606	1.210	1.226	1.116		
BREC	PSI obs.	36.093	1.763	36.170	29.730	40.426	36.364	37.778		
	PSI pred.	54.708	2.637	50.828	n/a	59.741	n/a	53.554	-11.81	**0.007**
	PSI O/E	0.698	0.011	0.712	n/a	0.677	n/a	0.705		
BRDS1	PSI obs.	54.972	2.991	45.098	60.000	54.348	62.222	53.191		
	PSI pred.	51.369	1.976	48.905	47.843	58.699	49.125	52.272	0.22	0.833
	PSI O/E	1.077	0.077	0.922	1.254	0.926	1.267	1.018		
BRDS2	PSI obs.	62.763	3.265	53.061	64.000	63.043	73.333	60.377		
	PSI pred.	50.724	1.360	49.327	47.367	54.939	49.277	52.708	3.28	**0.030**
	PSI O/E	1.242	0.077	1.076	1.351	1.148	1.488	1.146		

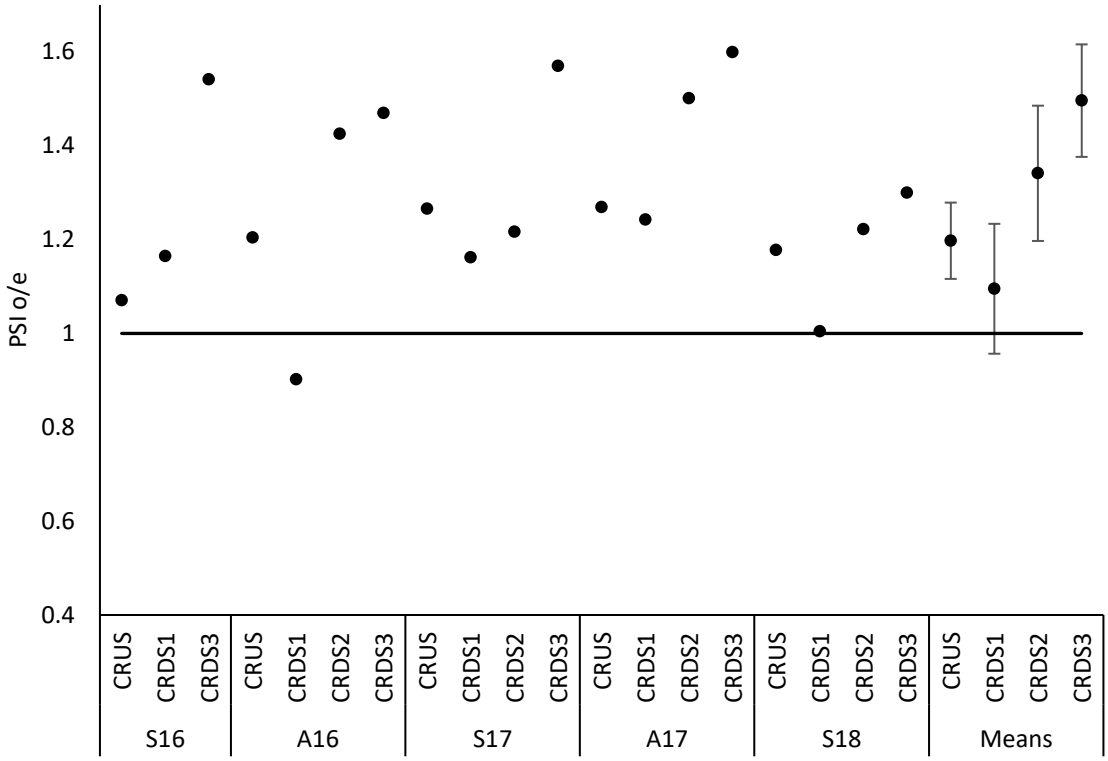

Figure 47 The River Crane Observed/Expected Proportion of Sediment-sensitive Invertebrates (PSI O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 5 for all sites except CRDS2 where *n* = 4). Data points which fall below the horizontal line at a PSI O/E of one indicate observed PSI scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed PSI scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected PSI values, mean values and statistical comparison between observed and expected values are presented in Table 22

Table 22 The River Crane multivariate biotic index scores for the Proportion of Sediment-sensitive Invertebrates (PSI), both observed (PSI-O) and expected (PSI-E) and the O/E ratio (PSI O/E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys (*n* = 5 for all sites except CRDS2 where *n* = 4). The River Invertebrate Classification Tool (RICT) was used to generate PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected PSI scores were explored using two-way paired student's t-tests (α = 0.05)

				Date						
Site	*Measure*	*Mean*	*se*	*S16*	*A16*	*S17*	*A17*	*S18*	*T-value*	*P*
CRUS	PSI obs.	59.465	2.476	53.846	55.319	67.308	58.140	62.712		
	PSI pred.	49.693	1.650	50.303	45.934	53.176	45.800	53.251	5.44	**0.006**
	PSI O/E	1.198	0.036	1.070	1.204	1.266	1.269	1.178		
CRDS1	PSI obs.	54.101	3.493	56.000	41.379	62.000	58.000	53.125		
	PSI pred.	49.376	1.573	48.068	45.876	53.359	46.678	52.899	1.60	0.185
	PSI O/E	1.095	0.062	1.165	0.902	1.162	1.243	1.004		
CRDS2	PSI obs.	61.595	0.775	n/a	61.364	60.870	63.830	60.317		
	PSI pred.	46.244	2.002	n/a	43.053	50.040	42.526	49.355	5.81	**0.010**
	PSI O/E	1.341	0.072	n/a	1.425	1.216	1.501	1.222		
CRDS3	PSI obs.	73.296	2.683	77.143	68.085	81.395	72.549	67.308		
	PSI pred.	49.071	1.367	50.056	46.325	51.836	45.351	51.785	9.60	**0.001**
	PSI O/E	1.496	0.054	1.541	1.470	1.570	1.600	1.300		

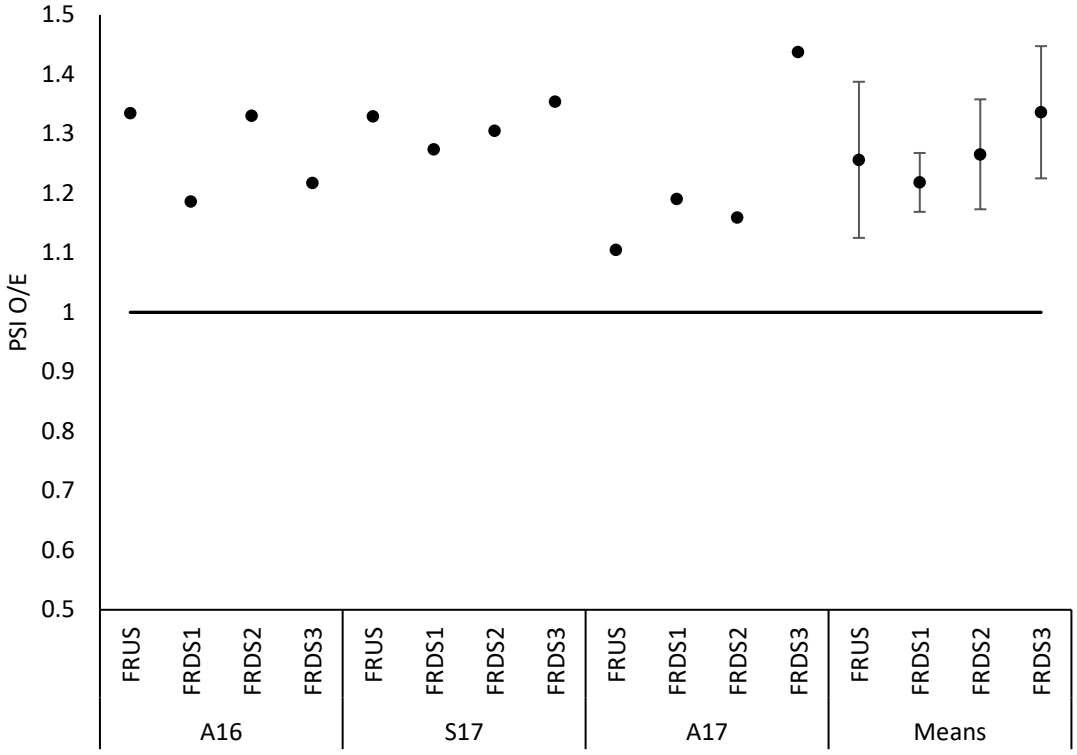

Figure 48 The River Frome Observed/Expected Proportion of Sediment-sensitive Invertebrates (PSI O/E) scores for macroinvertebrate samples for each survey (S = spring, A = autumn + year) and the mean (±SD) for all surveys (*n* = 3). Data points which fall below the horizontal line at a PSI O/E of one indicate observed PSI scores below expected scores for undisturbed habitats generated using the River Invertebrate Classification Tool. Conversely, data points on or above the horizontal line indicate the observed PSI scores were on parity or exceeded the expected scores, indicating undisturbed habitat. Observed and expected PSI values, mean values and statistical comparison between observed and expected values are presented in Table 23

Table 23 The River Frome multivariate biotic index scores for the Proportion of Sediment-sensitive Invertebrates (PSI), both observed (PSI-O) and expected (PSI-E) and the O/E ratio (PSI O/E) for each site and survey (S = spring, A = autumn + year) and the mean (±SE) for all surveys (*n* = 3). The River Invertebrate Classification Tool (RICT) was used to generate PSI-E based on prevailing habitat variables at each site/survey. Differences between observed and expected PSI scores were explored using two-way paired student's t-tests (α = 0.05)

				Date				
Site	*Measure*	*Mean*	*se*	*A16*	*S17*	*A17*	*T-value*	*P*
FRUS	PSI obs.	65.560	2.542	66.071	72.917	57.692		
	PSI pred.	52.183	0.893	49.490	54.849	52.209	3.370	0.078
	PSI O/E	1.256	0.044	1.335	1.329	1.105		
FRDS1	PSI obs.	56.999	1.667	55.769	62.500	52.727		
	PSI pred.	46.782	0.797	47.007	49.054	44.286	6.320	**0.024**
	PSI O/E	1.217	0.016	1.186	1.274	1.191		
FRDS2	PSI obs.	60.966	1.745	62.745	65.079	55.072		
	PSI pred.	48.172	0.492	47.151	49.865	47.499	4.900	**0.039**
	PSI O/E	1.265	0.031	1.331	1.305	1.159		
FRDS3	PSI obs.	61.412	1.701	55.556	63.768	64.912		
	PSI pred.	45.956	0.335	45.631	47.083	45.153	5.320	**0.034**
	PSI O/E	1.336	0.037	1.217	1.354	1.438		

3.4.9.11 WHPT, PSI and sediment correlations

On each river system, the PSI gradually increased downstream of the watercress farm discharge point while observations of percentage sediment cover did not follow this trend (3.4.5). To investigate this further, Pearson correlations were carried out between PSI-O and fine sediment cover and found to be non-significant for the Bourne (r = -0.299, P = 0.227), the Crane (r = -0.207, P = 0.396) or the Frome (r = -0.270, P = 0.395). In addition, the observed/expected PSI ratios (PSI O/E) did not correlate with fine sediment on either the Bourne (r = -0.240, P = 0.335), the Crane (r = -0.007, P = 0.978) or the Frome (r = 0.250, P = 0.434). A global correlation analysis of PSI O/E and percentage of fine sediment incorporating all rivers is presented in Figure 49, which highlights the poor correlation between observed sediment and the PSI O/E metric (r = 0.141, P = 0.333).

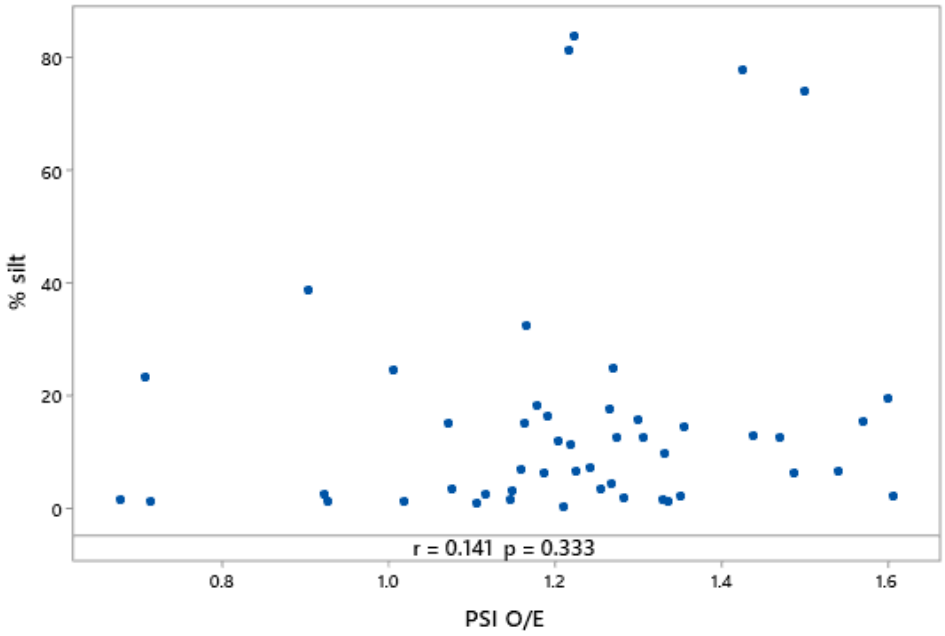

Figure 49 Pearson correlation between percentage of fine sediment (% silt) and Proportion of Sediment-sensitive Invertebrates observed/expected score (PSI O/E) for all sites and surveys on the Bourne Rivulet, the River Crane and the River Frome

To assess if PSI O/E scores may be reflecting general macroinvertebrate responses to organic pollution, Pearson correlations were carried out between PSI O/E and WHPT O/E. Correlations were positive and significant for the Bourne (r = 0.927, P = <0.001), the Crane (r = 0.875, P = <0.001) and the Frome (r = 0.664, P = 0.019). A global correlation analysis of PSI O/E and WHPT O/E incorporating all rivers is presented in Figure 50, showing the significant positive correlation between the two biotic indices (r = 0.880, P = <0.001).

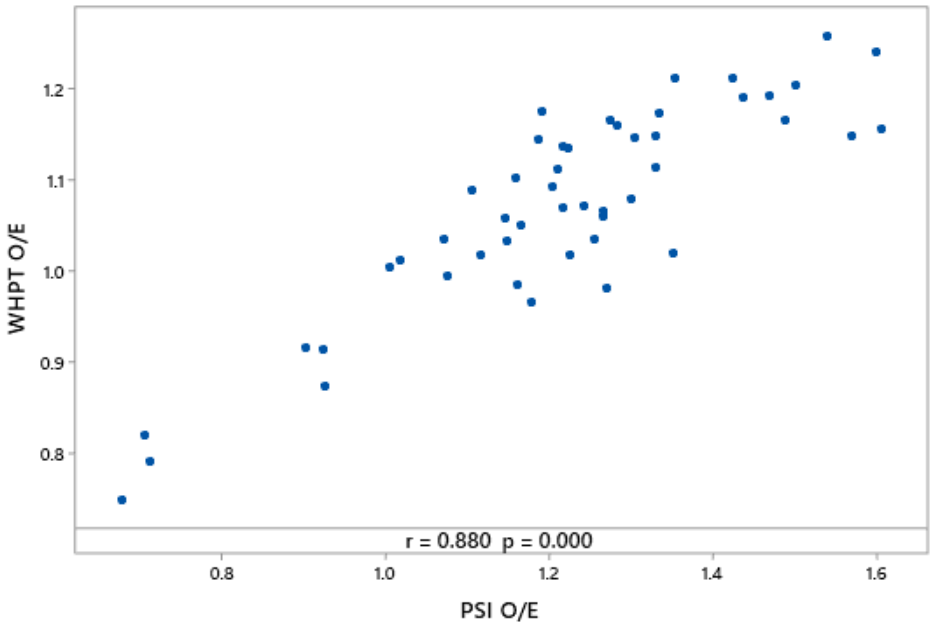

Figure 50 Pearson correlation between Walley Hawkes Paisley Trigg observed/expected score (WHPT O/E) and the Proportion of Sediment-sensitive Invertebrates observed/expected score (PSI O/E) for all sites and surveys on the Bourne Rivulet, the River Crane and the River Frome

3.4.10 Summary of key results

Table 24 presents a summary of the key findings of the chapter. It shows impacts were in a negative direction the greater the quantity of salad wash present in the discharge. Suspended solids and fine sediment deposits were slightly increased downstream of all three studied watercress farms. Abundances of pollution-sensitive taxa were reduced, and abundances of pollution-tolerant taxa increased under salad wash effluent discharges on both the Bourne and Crane, while the reverse was true for the Frome where no salad wash effluent is discharged. Total macroinvertebrate abundances were increased on the Bourne and reduced on the Crane and Frome. Macroinvertebrate diversity in terms of family richness, EPT richness and percentage of EPT in samples was reduced on the Bourne only and increased on the Crane and Frome. The biotic index score Walley Hawkes Paisley Trigg (WHPT) and the Proportion of Sediment Sensitive Invertebrates (PSI) was significantly lower on the Bourne, relatively lower on the Crane and increased on the Frome.

Table 24 Summary of discharge components and impacts for the Bourne Rivulet (comparing the east channel (BREC) and downstream one (BRDS1) to the west channel (BRWC) and BRDS2, the River Crane (comparing downstream one CRDS1 to upstream CRUS and sites further downstream) and the River Frome (comparing downstream FRDS1 to upstream FRUS and sites further downstream). Higher values downstream of discharge, ↑; lower values downstream of discharge, ↓. Showing macroinvertebrate family richness (NTAXA, number of taxa), Ephemeroptera, Plecoptera and Trichoptera (EPT), the biotic indices Walley, Hawkes, Paisley, Trigg (WHPT) and Proportion of Sediment-sensitive Invertebrates (PSI).

	Bourne Rivulet	*River Crane*	*River Frome*
Watercress bed irrigation	✓	✓	✓
Salad wash effluent	Large scale component	Intermittent small scale	×
Suspended solids	↑	↑	↑
Fine sediment	↑	↑	↑
Pollution-sensitive taxa	↓	↓	↑
Pollution-tolerant taxa	↑	↑	↓
Macroinvertebrate abundance	↑	↓	↓
Family richness (NTAXA)	↓	↑	↑
EPT (% EPT and EPT NTAXA)	↓	↑	↑
Shannon's index	↓	↑	↑
WHPT	↓	↓	↑
PSI	↓	↓	↑

3.5 Discussion

The aim of this investigation was to evaluate the effects of watercress farm discharges, both from watercress bed irrigation and salad wash effluent, on physicochemistry, habitat and macroinvertebrate assemblages. Overall, the findings showed that discharges from salad wash effluent had marked effects on macroinvertebrate composition, with an increase in pollution-tolerant taxa and decrease in pollution-sensitive taxa, which was evidenced by biotic index scores. Where watercress bed irrigation water only is discharged, there appeared to be no negative impact on macroinvertebrates, suggesting that salad wash effluent discharges are acting as a stressor to sensitive macroinvertebrate taxa.

3.5.1 Physicochemistry

Chalk streams have a relatively stable hydrology and physicochemistry due to being largely if not entirely groundwater fed from chalk aquifers (Cox 2009). Particularly in the upper reaches, the chalk geology exerts a strong buffering effect on pH which remains stable across seasons (Casey 1969; Bowes et al. 2005). Mean pH increased slightly with increasing distance from discharge at all sites, although there was no consistent trend in pH values between immediate up and downstream sites across rivers. The pH values were consistent with regular pH measurements taken by the

Environment Agency (2017). There was a notable peak in pH in spring 2017 across all sites. Higher pH values have been observed in chalk streams in the summer months when CO_2 concentrations are at a minimum as a result of higher rates of photosynthesis (Bowes et al. 2011). However, pH in spring 2017 stood out as being higher than other spring surveys. Spring 2017 was marked by low flows (see 2.7), so it is possible that shallower channels and decreased velocity during this survey facilitated increased rates of photosynthesis leading to the pH spike. Despite the spike in spring 2017, pH values at all times and at all sites remained within the acceptable levels for chalk streams stipulated by the Environment Agency of between pH 6 and 9 (Cox 2009).

Dissolved oxygen (DO) concentrations did not appear to be impacted by watercress farm discharges as there were no consistent differences across rivers between upstream and downstream sites. More pertinently, they remained high at all sites and the small differences are likely attributable to differences in hydrology and channel morphology. This is important as oxygen depletion in rivers often signals excessive anthropogenic and natural loading of organic matter, nutrients and pollution events (Yang et al. 2010; Dutton et al. 2018) which can have profound negative impacts on diversity and ecosystem functioning in rivers (Isbell et al. 2013). Low oxygen concentrations can cause shifts in macroinvertebrate communities (Calapez et al. 2018) and negatively impact on fish behaviour and metabolism (Franklin 2014).

Concentrations of suspended solids (SS) in discharge waters on all three rivers were slightly raised compared to upstream sites and were slightly higher than the ideal concentrations for unimpacted chalk streams for their distance from source (Mainstone 1999). As the high levels of SS in CRDS1 during watercress bed cleaning in August 2017 showed, watercress farming activities can create pulses of elevated SS which would likely be missed with infrequent surveys. Data collected by the Environment Agency (see section 1.1) show concentrations of suspended solids in watercress farm outfalls in the three study sites to exceed the EA maximum permitted release of 20 mg/L (Cox 2009) on occasion over the last ten years, so the infrequency of surveys in the present study may have missed high pulsed releases. Despite recent efforts by watercress producers to limit sediment discharges by employing settlement lagoons and traps, pulsed releases of suspended solids are clearly still an ongoing issue.

3.5.2 Substrate

Concentrations of SS are commonly used to assess fine sediment inputs (Collins and Anthony 2008), but the deposition of material is to a large degree a consequence of in-stream conditions such as water velocity (Hutchens et al. 2009). While spot water samples may miss pulsed releases of SS (Bilotta and Brazier 2008), these solids can fall out of suspension creating greater areas of silt which may persist, particularly in chalk streams which are typically not subjected to spates which would

otherwise clear the gravel beds (Acornley and Sear 1999). The increased areas of siltation found in all sites below watercress farm discharges in the present study are likely to be as a result of repeated pulses of SS release and/or chronic low-level releases. However, some sites situated further downstream had higher levels of fine sediments than the immediate downstream sites. Notably CRDS2, resulting from its low velocity, and the Frome downstream two and three due poaching by cattle (Wood and Armitage 1997; Walling and Amos 1999; Sutherland et al. 2002).

Increased fine sediments can lead to diminished macrophyte growth and increased algal growth (Heywood and Walling 2007; Neif et al. 2017). The classic habitat-forming macrophyte of chalk streams, *Ranunculus*, has a strong preference for silt-free gravels as low oxygen availability to roots in silted substrates tend to produce smaller root masses rendering the plant more vulnerable to wash-out (Mainstone 1999). The present study found lower macrophyte cover in downstream sites on the Bourne and Crane relative to upstream sites. However, it would not be possible to attribute such patterns solely to sedimentation, as on the Bourne the macrophytes at all sites are annually cut. The practice of 'weedcutting' is often performed in managed fisheries where a paucity of shading necessitates artificial management to keep the channel from being 'clogged' to maintain water conveyance and provide optimum salmonid habitat (Dawson et al. 1991; Old et al. 2014). On the Crane, there is no weedcutting activity, and here extensive areas of riparian shading over many reach areas effectively limits growth of macrophytes such as *Ranunculus* (Dawson and Kern-Hansen 1979; Davis et al. 2018) The upstream site CRUS had more extensive shading and more macrophyte coverage than CRDS1, so it could be speculated that sedimentation may be a limiting factor. However, *Ranunculus* growth was still strong with large areas of coverage under open canopy directly below the discharge weir, so it is arguable that siltation resulting from discharges from the watercress farm had not appreciably hindered its retention.

3.5.3 Biotic indices

The physicochemical determinands measured in the present study and also by the Environment Agency presented in chapter two, showed little differentiation between sites. However, the range of potential stressors assessed were limited and did not include the key stressor of interest in this research, phenethyl isothiocyanate (PEITC), due there currently existing no practical methodology for field measurements. Unlike physicochemical measurements, in this study biotic indices and macroinvertebrate assemblages clearly differed between sites, and by extension the discharges, particularly between those receiving discharges of salad wash effluent containing higher concentrations of PEITC (Worgan and Tyrell 2005; Dixon and Shaw 2011; Cotter 2012) and watercress bed irrigation discharge.

To take account of differing environmental variables at each site, the macroinvertebrate biotic indices used to assess organic pollution (WHPT) and sedimentation (PSI) were compared to expected scores generated by RIVPACS using RICT. As RICT analysis takes into consideration the substrate composition and other abiotic variables when predicting biotic index scores, it provided a tool to disentangle physicochemical and habitat variables when examining observed macroinvertebrate assemblages under potential pollution stress (Clarke and Davy-Bowker 2014). The PSI metric was found mirror WHPT scores and was not an effective indicator of sediment levels, as discussed later in detail section 3.5.8. The discussion on biotic indices in relation discharges that follows will therefore focus on solely WHPT.

WHPT was designed to assess organic pollution, but previous studies using observed WHPT scores have been shown to be sensitive to a range of stressors. For example, on the River Medlock in north east England, WHPT scores have been shown to be lower beneath sewer overflows which intermittently discharge untreated waste than at upstream sites (Medupin 2019). Sites below dam impoundments have been shown to produce lower WHPT values relative to upstream and reference sites (Krajenbrink et al. 2019; Aspin et al. 2020), and patches of invasive riparian vegetation have shown a moderate decrease in WHPT scores relative to controls (Seeney et al. 2019). However, the present study is as far as the author is aware, the first to use WHPT scoring to describe changes in macroinvertebrate communities in response to watercress farm discharges. Moreover, by comparing observed WHPT scores to expected scores using RICT, site-specific differences in key habitat variables were accounted for.

Downstream of salad wash effluent on the Bourne, the observed WHPT score was significantly lower than predicted in the east channel and lower than predicted at downstream one. The indicator species analyses add further support, with sites downstream of salad wash effluent harbouring greater abundances of pollution-tolerant taxa and fewer pollution-sensitive taxa. The situation was more complicated on the Crane, where there was a split apparent between the immediate up and downstream sites CRUS and CRDS1 and the two furthest downstream sites CRDS2 and CRDS3. This was highlighted in the NMDS and ANOSIM analysis, showing that macroinvertebrate assemblages in CRUS and CRDS1 were relatively similar to each other and CRDS2 and CRDS3 being relatively similar each other, while there was a greater dissimilarity between the two pairs of sites. This was reflected in WHPT scores, which were lower in CRUS and CRDS1 than CRDS2 and CRDS3. A likely explanation may be the presence of a WWTP, at an upstream distance of 1.26 km from the upstream site CRUS. Discharges from WWTPs can increase nutrient loads, release micropollutants such as pesticides, pharmaceuticals and personal care products which may adversely impact sensitive macroinvertebrates (Schwarzenbach et al. 2006; Eggen et al. 2014). Although CRDS1 had the lowest mean observed WHPT score, it was still marginally above the mean

expected score, which suggests that the reach was in relatively good ecological health. However, both CRUS and CRDS1 appear to have low WHPT scores for the river. Environment Agency surveys conducted between 2007 and 2009 upstream of the WWTP produced scores on par with those seen in CRDS2 and CRDS3, which led them to give the river the highest possible rating for biology, indicating a river unimpacted by pollution (Environment Agency 2020). This suggests that both CRUS and CRDS1 had unusually low WHPT scores for reach of river, potentially as a result of the WWTP discharge upstream. In this respect, the CRUS site was not an optimal upstream reference due to possible contamination from the WWTP which may have negatively affected sensitive macroinvertebrates in both CRUS and CRDS1. However, in the absence of the watercress farm discharge, the WHPT observed/expected ratio may have been expected to be higher in CRDS1 than CRUS due to its further downstream distance from the WWTP. Therefore, the observed drop in the WHPT scores from CRUS to CRDS1 is likely to have arisen due to discharges from the watercress farm.

The sites that received watercress bed irrigation only, BRWC and FRDS1, both had WHPT scores significantly above expected, which suggests that strong impacts seen in the Bourne downstream of the east channel discharge and to a lesser extent downstream of the Crane may be driven by a component of salad wash such as PEITC. This is reinforced by the high abundances of indicative pollution-sensitive taxa and low abundances of pollution-sensitive taxa downstream of the sites that discharged only watercress bed irrigation. That salad wash effluent exerts an impact on macroinvertebrates rather than watercress bed irrigation discharge is corroborated by research by Cotter (2012) on six watercress farms in Southern England which included the Bourne site used in the present study along with five other watercress farms. In Cotter's (2012) study, only the Bourne site saw negative impacts on leaf litter processing rates and lower abundances of Gammarids while such effects were not seen on the five farms that did not discharge salad wash effluent.

Biotic indices such as WHPT provide good evidence of degradation but they have limited ability to indicate which stressor is causative (Berger et al. 2018). Macroinvertebrate assemblages are rarely affected by single stressors, with typically a range of interacting pressures affecting resident communities (Friberg 2010; Schäfer et al. 2016). It is therefore difficult to definitively isolate a single stressor which may have led to the results of the present study. The application of fertilizer and pesticides to growing watercress crops is minimal at most and the results would appear to rule out their influence. The sites that receive discharge from cress bed irrigation only, such as the western channel of the Bourne and downstream sites on the Frome would be subject to both stressors, yet the results showed these sites to be some of the least degraded of all under study. Previous experiments have shown higher Gammarid mortality rates in salad wash effluent, which has been linked to PEITC release (Dixon 2010), while laboratory studies have identified the toxicity of PEITC

to macroinvertebrates (Newman et al. 1990; Worgan and Tyrell 2005; Dixon and Shaw 2011). With sites that receive only watercress bed irrigation discharge displaying no signs of diminishment of sensitive macroinvertebrate abundances, and sites that receive salad wash effluent having macroinvertebrate assemblages reflecting degradation, this suggests that the presence of PEITC may be the prime stressor acting on macroinvertebrates downstream of salad wash effluent discharges

3.5.4 Macroinvertebrate abundance and diversity

Mean total macroinvertebrate abundances were lowest on sites upstream of watercress farm discharge and highest in downstream sites on the Bourne, suggesting that on the Bourne the discharge from salad wash increases prey availability to higher trophic levels such as fish. Similar increases in total macroinvertebrate abundances have been linked to nutrient enrichment below WWTP discharges (Gücker et al. 2006; Askey et al. 2007; Álvarez-Cabria et al. 2011) and fish farms (Guilpart et al. 2012). Some watercress growers add phosphate fertilizers to watercress beds during the growing season as P is a limiting nutrient for growth (Casey and Smith 1994). N is typically found at lower concentrations in discharge water than in the aquifers from which it was first abstracted as the nutrient is assimilated during watercress growth (Casey and Smith 1994; Cox 2009). Abstracted water typically has P levels of 0.01 mg/L, while watercress bed outflow concentrations are on average raised to 0.06-0.08 mg/L (Cox 2009) which is low when compared to releases from WWTPs (Cox 2009). Due to the limited number of site visits, N and P levels were not recorded in the present study as a small number of spot samples may inflate or underestimate long-term loadings due to the wide diel and seasonal fluctuations (Cox 2009). However, none of the sites displayed the symptoms of chronic eutrophication, including the growth of epiphytes on instream macrophytes such as *Ranunculus* (Wilby et al. 1998) and filamentous algae on substrates (Carr and Goulder 1990). Such observations, while not quantitative, are likely to be more instructive than temporally distant measurements which may miss or catch nutrient pulses.

Relative to immediate upstream sites, sites downstream of the discharges showed decreased macroinvertebrate richness and diversity on the Bourne yet increased on the Crane and Frome. The lack of a consistent response between sites is reflected in inconsistencies in other studies which investigated discharges. For example, Pinder (1987) quantified macroinvertebrate diversity indices on sites on the Frome, Dorset, where there was visible evidence of organic pollution from sewerage, fish farm effluent and farm waste. At the sites investigated, diversity indices were found to be positively correlated with DOC. In contrast, Ortiz and Puig (2007) investigated macroinvertebrate assemblages upstream and downstream of WWTP effluent in rivers in NE Spain and found that at downstream sites Shannon index was mostly unchanged while taxa richness was 20% higher.

Camargo (1992) used a similar surveying methodology as the present study with four sites, one upstream and three downstream of a discharge from a trout farm on a limestone stream in Central Spain. His study found diversity measures were ineffective at quantifying differences between sites, while biotic indexes clearly differentiated them. Other studies investigating macroinvertebrate responses to pollutants in streams have similarly reported higher sensitivity in compositional metrics than richness and diversity metrics, which do not take into account taxon identity (Smith and Tran 2010; Turunen et al. 2016). In agreement with these studies, the present study found the composition of macroinvertebrate assemblages and biotic indices to be more informative in distinguishing sites than diversity and richness metrics.

3.5.5 Gammarids

Gammarids were identified using SIMPER as the macroinvertebrate taxon which contributed the greatest degree of dissimilarity between the Crane and Frome sites and the second greatest dissimilarity between Bourne sites. Across all sites and rivers, they accounted for 26.3% of all dissimilarity. To place into context the extent to which gammarids contributed towards differences in composition, the taxa responsible for the second greatest contribution to differences was Ephemerellidae at 16.5%. A three minute kick sample of a healthy chalk stream headwater would be expected to reveal several hundred individuals, and over one thousand individuals would not be uncommon (Medgett and Court 2008). The Environment Agency in conjunction with Salmon and Trout Conservation charity agreed a target of >500 gammarids per kick sample to indicate a healthy population in their Riverfly Census chalk stream monitoring project (S&TC 2019). Smith (1992) surveyed macroinvertebrate populations at upstream and downstream locations at 15 watercress farm discharges on chalk streams in southern England and found the crustacean to be absent or at much lower abundances below discharges than in sites above or at reference sites. This led to several studies on gammarid populations on the watercress farm on the Bourne Rivulet surveyed in the present study. Due to concerns over PEITC discharges from salad wash processes negatively impacting gammarid abundances, in November 2007 work was completed to reroute salad wash effluent from its original path directly into BREC. The salad wash effluent was diverted to recirculate through watercress beds prior to discharge into the east channel in order to dilute and provide time for the breakdown of volatile PEITC. Dixon (2010) carried out in situ acute 7-day caged tests of *Gammarus pulex* which found mortality was significantly higher in salad wash water before entering the watercress bed than after leaving it, so the operation was deemed successful. She surmised that UV breakdown and/or adsorption into sediment in the watercress beds effectively lowered PEITC concentrations. Prior to the action to re-route salad wash water, gammarid abundances had historically been very low in the east channel, with numbers increasing from being virtually absent between 1989-2006 to 700 in in a kick sample in 2007 following the reroute of salad wash effluent.

Meanwhile, abundances in the west channel over that time period ranged from 500 – 4500 individuals (Medgett and Court 2008). The present study found the lowest abundance of gammarids in east channel, and indeed, the site had the lowest mean abundance of all sites studied, with a mean abundance of 228, while the west channel mean abundances just met the S&TC target of 500. However, mean abundances were highest on The Bourne Rivulet just after the east/west confluence at the BRDS1, suggesting that any potential impact on gammarids does not extend far downstream. The watercress farm on The Crane was also found to have a depauperate gammarid population immediately downstream of the discharge In 1991, with fewer than 100 individuals found in CRDS1 during a survey by Smith (1992). The present study found a mean abundance of close to 700 at the same site, indicating an appreciable improvement. However, despite recent improvements, gammarids were still more abundant above the watercress farm than immediately below. For the Frome, gammarids were found to be absent in FRDS1 in a survey in spring 1991 (Smith 1992). At the time, a fish farm immediately upstream from the cress farm was considered by Smith (1992) as the prime driver for the absence at this site. Aerial photographs on Google Maps indicate that the fish farm was closed between 2006 and 2008. Moreover, the use of pesticides and zinc has been discontinued since the 1991 surveys. Gammarid abundances are up from 0 in 1991 to a mean of 850 at FRDS1 in the present study, a substantial improvement. Abundances were still twice as high upstream in FRUS compared to FRDS1. The habitat in FRUS, however, with its higher velocity and greater coverage of cobbles is a more favourable habitat for gammarids (Dahl and Greenberg 1996) so is likely to have been a key driver for the differences in abundance between these sites.

3.5.6 Ephemeroptera, Plecoptera and Trichoptera (EPT)

EPT are sensitive to organic pollution, and their absence or relative abundance has often been used to identify impacted habitats (Herman and Nejadhashemi 2015). They are considered useful in describing changes in stream quality following spot organic pollution sources (Lydy et al. 2000; Ortiz and Puig 2007), and inorganic pollution such as heavy metals from mining activities have been shown to reduce the EPT taxon richness in rivers (Wright and Ryan 2016). Of the three rivers studied, only the Bourne showed a negative impact on EPT taxa richness and percentage of EPT from the discharge. Both metrics were substantially lower in the east channel that received salad wash effluent than the west, and both gradually increased in a downstream trend although by BRDS2 some 1.1km from the discharge had still not returned to levels observed at BRWC. These findings conform with Loch et al. (1996) who found EPT richness lower for at least 1.5k downstream of discharges from three commercial trout farms in North America relative to upstream controls. It should be stressed that BREC is unusual in that it is not connected upstream to the main river channel, so in that respect it may be better considered as a discharge channel. In contrast to other

sites it would receive less macroinvertebrate drift from upstream to replace drift lost downstream. It is also plausible that the annual infilling of the channel with emergent macrophytes may render it unsuitable habitat for egg depositing for some flying insects, which may in part explain the very low EPT richness and abundance. Moreover, the annual clearance of emergent macrophytes in BREC in the early spring may remove with them large numbers of attached macroinvertebrates. Dawson et al. (1991) found as many as 30 individuals per g dry weight were removed with cut weed on the Frome in Dorset, equivalent to 4900 individuals per square metre of riverbed. Weedcutting occurs on all river sites on the Bourne, but at other sites the practice involves thinning rather than clearance of the whole channel at BREC, so this site would be most impacted. Such loss of macroinvertebrates at the east channel would be compounded by a limited potential for repopulation from upstream. However, BRDS1, the site below the confluence of the east and west channels is not subject to infilling with emergent macrophytes and channel clearing and would receive drift from both the eastern and western channels. At this site EPT richness and %EPT was still lower than western channel and further downstream at BRDS2. This slow recovery downstream of EPT downstream of the east channel was most marked in Plecoptera and Trichoptera abundances, with the pollution-tolerant and numerous Ephemeropteran Baetidae making up a large proportion of EPT abundance downstream of salad wash effluent. In contrast, on the Crane and Frome, EPT richness and EPT percentage both increased downstream relative to upstream. This may be linked to quantity of organic pollution in the discharge as EPT response may be influenced by the magnitude of the stressor. For example, Quinn and Hickey (1993) examined the response of macroinvertebrates to WWPT discharges in eight New Zealand streams and found that low organic solid loadings increased densities of EPT by up to 50%, while higher loadings reduced densities of sensitive EPT taxa by more than 50%.

The Bourne was marked by its high abundance of Ephemeroptera larvae in comparison to the Crane and Frome, which may be due to the higher abundance of submerged macrophyte cover, typically a favoured habitat for the order (Wright et al. 1994; Wright et al. 2003). However, the most sensitive Ephemeroptera family, Heptageniidae, is typically found clinging to substrates rather than in macrophytes (Wright et al. 1998) so its absence from the east channel, which had low macrophyte abundance may be attributable to other factors. This family was absent from both east channel and downstream at BRDS1, while the least sensitive Ephemeropteran, Baetidae, was the most abundant at these sites. A similar pattern was true on the Crane, where Baetidae represented the most abundant Ephemeroptera in the immediate downstream site, CRDS1.

Plecoptera are considered the most pollution-sensitive of all benthic macroinvertebrates (Clarke and Davy-Bowker 2014). This group were absent in east channel on the Bourne that receives salad wash effluent, but present in the west channel that receives watercress bed irrigation discharges.

Similarly, they were at their lowest abundance at the immediate downstream reach on Crane while there was no apparent negative impact from the watercress farm on Plecopterans on the Frome, where abundances were higher downstream than upstream.

The distribution of Trichoptera on The Bourne Rivulet in the present study concur with a previous study by Medgett and Court (2008), which looked at abundances between 2004-2007. They found abundances and diversity to be highest in west channel and very low in the east channel, while no Trichoptera individuals at all were found in the east channel prior to 2006 before salad wash water was rerouted through watercress beds. In both Medgett and Court's (2008) study and the present study, the recovery of Trichoptera downstream was gradual and progressive. At the lowest downstream survey point at 1.8km from the watercress farm, Medgett and Court's (2008) study found Trichoptera abundances to have failed to return to densities in the western channel. They too failed to return to levels In the western channel at the furthest downstream sampling point in the present study at 1.1 km from the farm (BRDS2). Similarly, the site immediately downstream of the watercress farm discharge on the Crane had the lowest abundance of Trichoptera, but the reduction relative to upstream was not as marked, and on this river, abundances increased to levels above CRUS further downstream. As was observed for Ephemeroptera and Plecoptera, once again the Frome had the greatest abundance and diversity of this group, which may be a function of the longitudinal location of the sites as middle reaches, which are known to have greater diversity of arthropods than upper reaches (Dunn et al. 2006). in contrast to the Crane and Frome, the abundances increased downstream of the watercress farm discharge.

3.5.7 Pollution-tolerant macroinvertebrates

On all the rivers, the increase in pollution-tolerant taxa in downstream sites scaled with the magnitude of salad wash discharge, with the Bourne most affected, followed by the Crane, while the Frome not showing any consistent pattern and having comparatively low abundances at all sites. Particularly on the Bourne, there was an increase in some of the most pollution-tolerant taxa such as Oligochaeta, Tricladida, Hirudinea, and Asellidae (Armitage et al. 1983; Clarke and Davy-Bowker 2014). Pollution-tolerant taxa generally have an affinity for silt substrates (Descloux et al. 2013; Descloux at al. 2014; Mathers and Wood 2016), so a plausible explanation for increased abundances of pollution-tolerant taxa might be the increase in observed fine sediment cover in these sites. However, the percentage of silt cover was approximately three times higher in the low-velocity intermediate site on the Crane, CRDS2, compared to CRDS1. If depositing substrate was the primary driver for high abundances of Oligochaeta, Hirudinea, and Asellidae, it would be expected that these families would be more abundant in CRDS2 than CRDS1. However, this was not the case; these taxa were approximately twice as abundant in CRDS1 relative to CRDS2. Camargo

(1992) found similar increases in Oligochaeta, Hirudinea and Chironomidae downstream of discharges from a fish farm in Central Spain which he attributed to much lower dissolved oxygen levels compared to an upstream control reach. In contrast to his study, the present study found high DO levels downstream of watercress farm discharges. This suggests that a stressor other than fine sediment and low DO resulted in the observed patterns.

3.5.8 WHPT, PSI and fine sediment correlations

For all rivers, the mean observed PSI (PSI-O) and the observed and expected PSI ratio (PSI O/E) showed a pattern of increasing with distance from watercress farm discharges, suggesting that the quantity of fine sediment was highest immediately downstream and decreased with distance. However, the visual surveys of fine sediment deposited on the streambed did not support this assumption. While at all rivers the upstream sites had less fine sediment than immediate downstream sites, in a number of instances fine sediment deposition was greater further downstream while the PSI-O continued to increase. This discrepancy was highlighted by weak and non-significant correlations between both PSI-O and PSI O/E and the percentage of fine sediment recorded at all rivers. A notable example was the site CRDS2 whose substrate was approximately 80% fine sediment, over fourfold higher than the other Crane sites. Consequently, it had the lowest PSI-E score, but the observed PSI-O score was higher than all sites except CRDS3. This may be explained by a large contribution of sediment-sensitive taxa from the largely gravel/cobbled upper 20% of the reach. The 3-minute kick sample was applied proportionate to the substrate, but it may be that the deep fine sediments of the lower reach were relatively depauperate in macroinvertebrates, while the upper eroding substratum was rich, leading to greater densities of sediment-sensitive macroinvertebrate than predicted. However, this is speculative. Only by using a different sampling methodology whereby substrate types were sampled independently could this hypothesis be investigated. It seems more likely that the PSI-O was reflecting the pollution-sensitivity of the macroinvertebrate assemblage more strongly than its sediment-sensitivity, bolstered by the strong and significant positive correlations on all rivers between WHPT O/E - which accounts for substrate composition. Indeed, it is understood that taxa that have an affinity for fine sediments tend to be those that are also broadly pollution-tolerant (Gammon 1970; Nutall and Bielby 1973; Wright and Symes 1999; Jones et al. 2012). Moreover Berger et al. (2018) call into question the ability of stressor-specific indices to detect responses to a range of different chemical stressors, though their study did not incorporate fine sediments.

A thorough search in the literature for studies that analysed correlations between WHPT and PSI to ascertain if the strong correlations seen in the present study were unusual or typical proved unfruitful. However, Clarke and Davey-Bowker (2014) utilised the full RIVPACS dataset of 685

reference sites and found BMWP to be highly correlated with WHPT. As such, BMWP may cautiously be used as a proxy for WHPT. Extence et al. (2013) generally found poor correlations between PSI and BMWP at the three river sites used to test their then newly developed PSI index. Two of the rivers were in the North of England, one of which was in a clay catchment (Eye Brook) and one in chalk (Laceby Beck) and the River Chess in Buckinghamshire was a southern chalk stream. Only at the latter were significant positive correlations between BMWP and PSI discovered. This suggests that the strong positive correlations between WHPT and PSI seen in the present study may not be atypical for southern chalk streams habitats, while surface water fed rivers may show less correlation and the PSI metric may be more useful at pinpointing fine sedimentation of substrates.

Extence et al. (2017) note a tendency for RICT to produce PSI-E values lower than expected from parallel analysis using the channel substrate index (CSI) when assessing natural gravel dominated sites such as chalk streams. The CSI metric is similar to the HABSCORE methodology employed in the present study in that it visually estimates substrate type. In the present study, mean PSI-O values were higher than mean PSI-E for all sites except the Bourne Rivulet east channel. In most cases, they were significantly higher. As chalk streams are gravel dominated and largely natural, the high PSI O/E are likely to have resulted from low PSI-E scores due to bias in RICT predictions. As such, it would be unwise to assume from PSI O/E scores that the majority of sites in the present study had unexpectedly high proportions of sediment-sensitive macroinvertebrates. Moreover, the lack of significant and positive correlation between PSI and observed fine sediments and the strong positive correlation between WHPT and PSI suggest that PSI may not be well-suited to assess fine sediments in chalk streams.

3.6 Conclusions

This study highlights the disturbance to pollution-sensitive macroinvertebrates from discharges of salad wash effluent into chalk streams. Notably, the magnitude of salad-wash effluent discharge scales with the level of perturbation. Such disturbances were shown to reduce abundances and species richness of sensitive EPT insect larvae, whilst promoting higher abundances of pollution-tolerant taxa. In contrast, where discharges consisted of watercress bed irrigation effluent only, biotic indices indicated higher than predicted quality habitat, which was reinforced by high abundances of pollution-sensitive taxa.

None of the discharges resulted in measured water quality variables that were uncharacteristic of chalk stream habitats. While siltation is known to impact macroinvertebrate composition, the results of the study suggest a factor in salad wash effluent not tested in the suite of physicochemical and environmental variables was exerting a stressor on sensitive taxa. Previous studies have shown that PEITC content in salad wash can exert a toxic effect on macroinvertebrates and so it is

speculated that the discharge of PEITC may be impacting sensitive macroinvertebrates in sites downstream of salad wash discharge.

Using the same survey sites in the present chapter, the next chapter will investigate fish populations to determine if discharges from watercress farms, particularly changes in macroinvertebrate abundances, biotic indices and habitat predict any observed changes in fish population structures.

3.7 Further research and limitations

Future studies could use reference sites on reaches unconnected to sites downstream of watercress farm discharges to explore the potential for more statistical comparison, which was constrained in the present study due to pseudoreplicaton. However, as NMDS ordination in the present study indicates, different rivers have different macroinvertebrate assemblages which may not provide robust comparisons. Another potential experimental redesign might reduce sites to a single upstream and single downstream site on each farm. It proved difficult to find rivers where multiple downstream sites could be accessed and/or were not affected by tributaries, which limited the number of potential watercress farms to survey. Now that the downstream extent of perturbations has largely been established in this study, future studies focussing on single upstream and downstream sites could increase the number of rivers. Doing so would allow data from all farms to be analysed concurrently with the use of mixed effect models with site identity held as a random factor and so avoiding pseudoreplicaton in assessing upstream and downstream effects.

The field data collection for substrate type using HABSCORE could be improved in future studies. Though more time-demanding, estimating percentage cover for each substrate category rather than recording the ASCFD categorical data required by HABSCORE would bring enhanced accuracy. In the present study, assumptions had to be made to infer percentage values from the collected ASCFD field data. Recorded percentages could later be converted into ASCFD categories for running the HABSCORE model.

Temperature was omitted from analysis when it became apparent that differences in temperature between sites were masked by temporal differences, especially on warm days, when temperatures recorded in the afternoon surveys were higher than those recorded first thing in the morning. Future studies should aim to obtain temperature readings as close to simultaneously as possible. Ideally, data loggers could be used to not only record temperature and other variables simultaneously, but to generate a time series data set to better explore patterns.

Environmental concentration of PEITC in downstream sites needs to be quantified to assess environmental concentrations. Ecotoxicology experiments could be performed on sensitive taxa using environmentally relevant PEITC concentrations. This may isolate the role of PEITC exposure on the observed reduced abundances of sensitive taxa.

THE IMPACT OF WATERCRESS FARM DISCHARGES ON FISH POPULATIONS IN CHALK STREAMS

4.1 Introduction

Freshwaters account for approximately 40% of global fish diversity and freshwater fish are considered among the most threatened group of vertebrates worldwide (Dudgeon et al. 2006). Freshwater fishes are subjected to wide range of anthropogenic stressors, such as habitat degradation (eutrophication, acidification and sedimentation), altered hydrology (dams, flow regulation and abstraction) and the introduction of non-native species and transfer of pathogens (Cowx 2002; Soto et al. 2006; Cowx and Portocarrero Aya 2011). The susceptibility of freshwater fish to pathogens is likely to increase with forecasted increases in river temperatures due to climate change (Marcos-López et al. 2010). Freshwater ecosystems are often relatively small, with species that have both restricted distributions and ability to disperse (Suski and Cooke 2007). Consequently, the threat of chemical contamination to fish populations is most acute in freshwater habitats (Hamilton et al. 2016). In the UK, contamination of inland waters from anthropogenic toxicants began with Roman mines in Wales, and later expanded in range with industrial discharges (Meybeck and Helmer 1989). Today, a wide range of anthropogenic toxicants pose hazards to fish populations in surface waters, such as persistent organic pollutants from industry and agriculture, surfactants, flame retardants, polycyclic aromatic carbons, pesticides, discharges from aquaculture and an increasing range of pharmaceuticals from STW effluents (Sacher et al. 2001; Moldovan 2006; Barnes et al. 2008; Loos et al. 2009; Fenlon et al. 2010; Brozinski et al. 2012). Over 60,000 anthropogenic chemicals are in regular use and find their way into industrial and domestic wastewater discharges (Hamilton et al. 2016). Most of these compounds are present in low concentrations, but many are still of ecotoxicological concern, particularly when present as a suite of complex mixtures (Loos et al. 2009; Hamilton et al. 2016). To address the issues associated with aquatic pollution, the European Water Framework Directive (WFD) produces environmental objectives to achieve “good water status”. The European Commission defines Environmental Quality Standards (EQS) for a list of priority substances that are to be monitored by member states (European Commission 2017). However, it has been speculated that many organic compounds and their metabolites that contaminate aquatic ecosystems have neither been identified, nor their impact on aquatic organisms determined (Van der Oost et al. 2003; Hamilton et al. 2016)

Organic pollution can directly impact on fish communities through a variety of pathways (Karr 2004; Peterson et al. 2011). Most organic pollution in rivers arises from domestic, industrial or agricultural sources and in exceptional cases can lead to mortalities of all fish species (Hendry et al. 2003) or

selective mortality of sensitive species (Maitland 1995). The input of chronic low level pollution may lead to altered population structures of fish in an impacted site, as conditions which may be advantageous for one fish species may disadvantage another (Johnson and Sumpter 2014). For example, chemical stressors have been shown to reduce abundances of intolerant, lithophilic and insectivorous fish species, while increasing abundances of generalist omnivorous species (Schmutz et al. 2007). Such changes may lead to decreased abundance of native stream species while increasing abundances of invasive species (Tsai 1970). In the more economically developed world, impacts to fish from poorly treated sewerage and toxic chemicals peaked for many rivers between 1950 and 1975, however, while improvements have been made in recent decades, the range of low-level stressors riverine fish may be subject to has increased (Johnson and Sumpter 2014). Chronic sublethal concentrations of a wide range of pollutants can bring about adverse effects on the reproductive potential of a broad range of fish species (Kime 1995) and can lead to changes in sex-ratios of populations by mimicking natural hormones (Murl Rolland 2000). In addition, they can increase susceptibility of fish to ubiquitous pathogens which in turn increases the probability of disease-related mortalities (Dunier and Siwicki 1993; Arkoosh et al. 1998; Hinck et al. 2008). Sensitivity to toxicants typically changes over life history stages, with concentrations which may be harmless to adult fish proving fatal or inducting teratogenic effects to early life stages (Murl Rolland 2000).

4.1.1 Surveying fish populations in rivers

A wide range of methods are available to survey fish populations in rivers. These can be broadly divided into active and passive methods or gear. Passive gear relies on fish to swim into it; examples being hoop nets, fyke nets and trap nets. This type of gear tends to be used to survey a small area over a long period of time (Portt et al. 2006). In contrast, active gear is used to survey a wider area over a shorter period of time and relies on the gear being taken to the fish (Teixeira-De Mello et al. 2014). Most commonly, active methods are employed to survey stream fish communities, while passive methods to survey lentic habitats (Portt et al. 2006). The efficiency of gear to catch fish varies among habitats, among species and among size classes of each species. Gears that capture a narrow range of species and size classes are termed selective while gears that capture a wide range of species and sizes are termed non-selective gears. As passive gears rely on fish moving to the gear, most are highly selective, while active gears tend to be non-selective (Teixeira-De Mello et al. 2014).

The most commonly used sampling method for estimating fish populations in lotic waters is electric fishing; an active and relatively non-selective method (Vehanen et al. 2013; Matson et al. 2018). It is considered to be the least biased and least destructive method (Teixeira-De Mello et al. 2014), and has higher capture rates and is less selective than seine, gill and hoopnetting (Wiley and Tsai

1983; Growns et al. 1996; Pugh and Schramm 1998). It is particularly well-suited for surveying small and wadable streams with low turbidity (Paller 1995; Portt et al. 2006) making it an ideal technique to survey the fish populations in chalk streams.

While it is relatively straightforward to quantify and estimate salmonid densities, direct comparison between sites when seeking evidence of potential impacts of degraded water quality can only be satisfactorily achieved when habitat variables are corrected for (Armstrong et al. 2003). HABSCORE is an empirical habitat-fish model developed to aid the interpretation of salmonid fishery data (Milner et al. 1998), which is frequently used for impact assessments (Cowx et al. 2009). The system is based on a series of empirical statistical models relating populations of salmon and trout to observed habitat variables. The models were developed using salmonid population estimates obtained by electric fishing of 602 pristine sites in England and Wales. The modelling involved stepwise multiple regression analysis of population sizes of three size/age classes of *S. trutta* and salmon (0+; >0+; <20 cm) against habitat variables. HABSCORE software is able to predict expected densities of each size class at a site from location and habitat variables. Comparisons with expected densities and observed densities can then be used to flag up sites that may be underpopulated due to stressors such as organic pollution.

4.1.2 Mark-recapture

Spatial and temporal tracking of individual animals enables the gathering of ecological data which can reveal insights of life-history, population size, mortality rates (Pine et al. 2011), growth, movement and reproduction of wild populations (Lucas and Baras 2000). An important tool in fisheries stock assessments are mark-recapture studies, whereby individual animals are captured and given a physical mark or tag so that it is identifiable as the same individual at a later point in time and/or space. In fishery research, individual fish most are commonly given an external mark on the body, or a physical tag is attached, either externally or internally. External marks such as panjetting (dyeing), tattooing, freeze branding and fin-clipping have been widely used, but have various drawbacks, such as confusion with natural injury, occlusion by developing pigmentation, as well as risk for the operator during freeze branding and to the fish during tattooing (Lucas and Baras 2000). Popular internal tags for salmonids include the use of small wire coded tags, which some workers have found to significantly reduce return rates of migrating salmon smolts (Crozier and Kennedy 2002). More recently, passive integrated transponder (PIT) tags that can be read with remote detection antennae are used (Lucas and Baras 2000), but these can have negative effects on growth (Adams et al. 1998). All such markings are *invasive*, in that physical damage or alteration to the body of the fish occurs, and all involve specialist equipment which can be costly (Lucas and

Baras 2000). Such tags can lead to altered behaviours, raise ethical issues and may lead to biases in the estimation of the parameters of interest (Moya et al. 2015).

Using photographs of natural body markings to identify individual animals has been used successfully (Van Tienhoven et al. 2007), and circumvents the problems associated with physically marking individuals. In addition, with the use of digital cameras and free-to-use spot matching software such as I^3S (see section 4.3.2) it can be a low-cost solution. An additional cost incurred by researchers wishing to physically tag fish is the cost of a Home Office licence under the Animals (Scientific Procedures) Act 1986 to carry out tagging, which is not necessary for photographic methods. Spot recognition software has been successfully employed to monitor whale shark (*Rhincodon typus*) (Speed et al. 2007) and sand tiger shark (*Carcharias taurus*) (Van Tienhoven et al. 2007) populations, but has as yet not been used on salmonids. The present study was the first to trial spot recognition software as an accessible, low cost and non-invasive alternative to physical tags and markings.

4.1.3 Watercress farms and fish populations in chalk streams

To date there have been no published peer-reviewed studies assessing fish population structures downstream of watercress farm discharges. A privately commissioned survey of *S. trutta* abundances on The Bourne Rivulet by Gent (2005) surveyed *S. trutta* populations on six occasions between 1985 and 2004. The surveys found sites downstream of discharges to hold lower densities of *S. trutta* than reference sites in most years. However, the research was limited to quantifying densities of fish and did not assess changes in habitat which may have precipitated the changes observed. A study of fish population structures on The Bourne Rivulet was undertaken by the Environment Agency (Longley 2006) used HABSCORE modelling and so accounted for habitat variables. The study found that young-of-year *S. trutta* were absent in the site directly below the confluence of the east channel and the west channel (see chapter two). However in contrast to Gent (2005), the study found a healthy population in the east effluent channel. In addition, Longley (2006) found *C. gobio* densities to be very low in the east channel relative to other surveyed sites on the river. These two unpublished reports examine fish populations on The Bourne Rivulet only, did not use comparable survey methodologies and found contradictory results. There is therefore a need to examine more widely how chalk stream fish population structures may be impacted by watercress farm discharges, by incorporating more than a single watercress farm and using comparable methods which incorporate habitat variables. Moreover, there is a need to examine potential stressors which may drive any changes to fish populations observed.

The chalk stream headwaters where watercress farms are typically sited are important spawning and nursery grounds for salmonids and other fish species (Mainstone et al. 1997). As early life

stages of fish are particularly sensitive to pollutants and toxicants (McKim 1977; Weiss 1989; Kristensen 1994; Belanger et al. 2010), the organic pollution downstream of salad wash suggested by the macroinvertebrate survey results in chapter three may have implications for fish recruitment if exposure to PEITC occurs. Moreover, as variations in prey availability can have implications for growth, condition, survival and recruitment success for fish (Armstrong et al. 2003; Nunn et al. 2012), the altered macroinvertebrate assemblages below salad wash discharges observed in chapter three may have indirect effects on fish population structure and the condition of individuals if suitable food resources are limited (Nunn et al. 2012).

4.2 Objectives

This study is the first of its kind to utilise comparable approaches to investigate fish populations around three watercress farms operated by three different growers. In chapter three, macroinvertebrate biotic index scores indicated that the two farms that discharged salad wash effluent had macroinvertebrate communities reflecting organic pollution, with the farm with the most intensive salad wash regime showing greatest impact. Conversely, the farm which did not wash salad had a biotic index score which did not suggest negative impacts from organic pollution. With reference to the habitat, macroinvertebrate and physicochemical data obtained in chapter three, this study will investigate if watercress farm discharges are affecting fish populations in terms of the following:

I. Species composition, condition, density and diversity of fish populations at each site allowing comparison between sites upstream of watercress farm discharges and those receiving them.
II. The observed densities of three size classes of salmonids compared to expected densities predicted by HABSCORE modelling to assess if watercress farm discharges are impacting on specific cohorts
III. Site fidelity and comparative rates of growth of *S. trutta* using spot-recognition techniques to investigate if sites receiving discharges from watercress farms differ from upstream reference sites. In addition, population estimates using spot recognition as a form of mark-recapture will be trialled to assess if the technique may be suitable for the estimation of *S. trutta* populations

4.3 Methods

Surveys were conducted on sites linked to the discharges of three watercress farms on the Bourne Rivulet, the River Crane and River Frome. These same sites were surveyed for habitat and macroinvertebrates in chapter three and are described fully in chapter two. Specific survey dates for each site and season can be found in chapter two. For ease of interpretation of the dataset, survey dates are referred to as spring (S) or autumn (A) followed by the last two digits of the year; i.e. S16 for the first surveys in spring 2016 and S18 for the final surveys in spring 2018.

Electric fishing was reviewed and approved by the University of Brighton's Animal Welfare & Ethics Review Board (AWERB). The procedures involved were exempt from licence by the UK Government Home Office under The Animals (Scientific Procedures) Act 1986 working with animals taken from the wild.

4.3.1 Electric fishing

Prior to electric fishing, water quality parameters were measured to obtain temperature and conductivity values to inform electrical current settings for electric fishing equipment. All stream reaches were fished using multiple pass electric fishing, a technique considered to obtain the most reliable estimations of abundace and richness (Hedger et al. 2013; Teixeira-De Mello et al. 2014). Multiple pass electric fishing was conducted in closed stream sections using stop nets at both ends of the reach to prevent emigration/migration (Peterson et al. 2005). Multiple pass methods sample the same reach on more than one occasion during each survey, thereby multiply the fishing effort (Bohlin et al. 1989) and allowing for estimations of original size of the population (Hedger et al. 2013) as later discussed in section 4.4.3.

Each site was netted off at the top and bottom of the reach using stop nets with a 6.5 mm mesh (seine net 210/12 (Collins Nets Ltd, Dorset, UK)). Electric fishing was carried out using an Easyfisher EFU1 electric fishing unit and Easyfisher anodes (East Anglian Electrical Services Ltd, Suffolk, UK), powered by a Honda inverter EU20i (Honda, Japan). To avoid driving fish upstream, fishing was carried out in the discontinuous method, whereby the current is temporarily cut as the gear is moved upstream. The current settings were tailored to the prevailing conductivity and temperature of the water, but in all cases used pulsed DC on 25% duty cycle at 50hz. Starting voltage depended on conductivity and was typically set at 150v which was increased if capture efficiency was low and conversely decreased if caught fish showed slow recovery. This equipment was operated from the hull of a Seahopper Nifty 50 boat (Seahopper, Exeter, UK), which was pushed upstream with the fishing team or towed by a rope around the anode operator's waist. The team was configured depending on the stream width and number of volunteers. Typically, on a wide stream (>5m) two

anodes operators would be employed, with a net operator allocated to each anode, while a single anode operator and two net operators would be used for narrower streams as per Kennedy and Strange (1981).

Netted fish were transferred into a 121.1L round white plastic Brute bin with water aerated using a battery-powered aerator (tecTACKLE, Jarvis Walker). The team member pushing the boat was also responsible for monitoring fish recovery in the bucket, and to regularly extract and replenish water to maintain its temperature and aid gas diffusion. Following each run, the fish were returned to the riverbank and identified to species level. *S. trutta* were measured as fork length (FL), and all other species as total length (TL) to the nearest mm. Smaller fish were weighed to the nearest 0.1g on an EPS precision compact balance (County Scales Ltd, Nottingham, UK). Fish that were too large to fit on the balance (> c.275 mm) were instead weighed in a sling using digital angling scales (Korum, Telford, UK) attached to a tripod to the nearest 10g. Measuring the length of live eels proved impossible using the measuring board due to their high activity levels and resistance to adopt a straight body posture. Instead, they were placed in a cylindrical bucket where their body would naturally rest against the inner side. The length of the eel was then marked on the inner circumference of the bucket which was subsequently measured using a tape measure. In addition, the weight of small individual eels was recorded inside an empty bucket that had been tared and placed on the electronic balance, while larger eels were weighed in the sling.

Following measurements and in the case of *S. trutta* photography (see section 4.4.4), the fish were transferred to an aerated recovery bucket. The recovery bucket was a 121.1 L plastic bucket which was set up half-submerged in the river in a shaded spot maintain the temperature. The fish in the recovery bucket were closely monitored, and partial water changes were frequently performed. Following the final depletion run, the recovery bucket was taken to the middle of the reach and the fish released.

4.3.2 Spot recognition

4.3.2.1 Photography

Photographs were taken in the field following length measurements, with the fish lying flat on a measuring board. Several different models of digital camera were used throughout the study to take high resolution .jpg files. As far as practicable, photographs were taken with the camera perpendicular to fishes' flank. However, the i^3S software algorithms allow for spot-matching of images of animals taken from a range of angles. As pigmentation marks will differ on either flank, the left-hand side was chosen. However, a small number of mistakes were made in autumn 2016 on the Frome when images of right-hand flank were taken in error. These were for two individuals

from FRUS, eight individuals from FRDS1 and ten individuals from FRDS3. These individuals were excluded from analysis.

4.3.2.2 Image preparation.

The images were cropped and straightened in the Windows 10 photo application, so that the fish image filled the frame and was horizontal. To reduce image size for more rapid comparisons in i^{3}S, all the images were resized to 1000x700 pixels in IrfanView as recommended by the i^{3}S developers. The images were batch renamed with the standard DSCN prefix replaced by the site name, and the image number suffixed with the date of capture. For example, DSCN4392 became FRUS4392_april17.

4.3.2.3 I^{3}S: Interactive Individual Identification System

The Interactive Individual Identification System (I^{3}S) software used is freely available for download at http://www.reijns.com/i3s/. The I^{3}S software was developed by Van Tienhoven et al. (2007) and enables large numbers of image files of individual animals to be compared and rapidly matched against a library of images. The software consists of three processing steps. The first step is pre-processing, where a common region of interest on the animal is identified and selected using three reference markers. Within the region of interest, markers are manually placed centrally on spots, forming a two-dimensional map for each individual. The second stage is an automated comparison between a sample image and the library of images, which presents the user with a list of possible matches arranged in order of best fit, based on scores calculated by a distance metric. The last stage is for the user to match candidates visually from the list of best possible matches, thereby reducing the number of images to be manually inspected (Moya et al. 2015).

4.3.2.4 Method development: area of interest and spot placement

Opercular melanophore patterns have been used in previous studies of young salmon (Donaghy et al. 2005). Stien et al. (2017) found that melanophores get larger as fish grow, so that largest melanophores are oldest and smallest most recently formed. These two studies did not use automatic spot recognition software, but rather a manual visual analysis, which would be impractical to repeat with the large number of fish analysed in the present study. Many young *S. trutta* were found to lack opercular melanophores entirely, and all but the very largest specimens had more than the minimum of 12 required for the spot recognition software. As that was the case, the most appropriate area for spot recognition was the flank which typically had the minimum of 12 melanophores and usually considerably more. It was apparent from looking at images of *S. trutta* of various sizes, that larger individuals had more melanophores than smaller ones, so like salmon opercular spots in the studies by Donaghy et al. (2005) and Stien et al. (2017), the number and size

of flank melanophores increased with age, with the largest melanophores being the oldest. The software allows for the selection of up to a maximum of 40 reference spots, so with *S. trutta* that possess considerably more than 40 distinct melanophores, it was important to develop a methodology that consistently picked out, as closely as possible, the same reference spots each time. Several trial runs comparing known matched individuals were undertaken to construct a methodology that produced replicable results and obtained the lowest match scores. The process is described in five stages below:

1. The triangular area of interest is selected from the anterior bases of the dorsal fin, anal fin and pectoral fin (Figure 51)
2. Initially, only melanophores below or on the lateral line were considered, maximising the base of the triangle reference area and minimising the number of spot options on highly spotted individuals
3. Working anterior to posterior, the most ventral melanophores were selected. Secondly, working back posterior to anterior, melanophores that were dorsal to the previously marked melanophores, but did not overlap the ventral melanophores when viewing a line from dorsal to ventral were selected
4. Where there were fewer than 12 melanophores available in the reference area, melanophores on or below the lateral line outside of the reference area were selected. The I^3S software accepts spots located outside of the reference area, though ideally the selection of spots outside of the reference area should be minimised
5. If there are still fewer than 12 melanophores selected after stage 4, then the most ventral melanophores above the lateral line were selected until 12 were allocated.

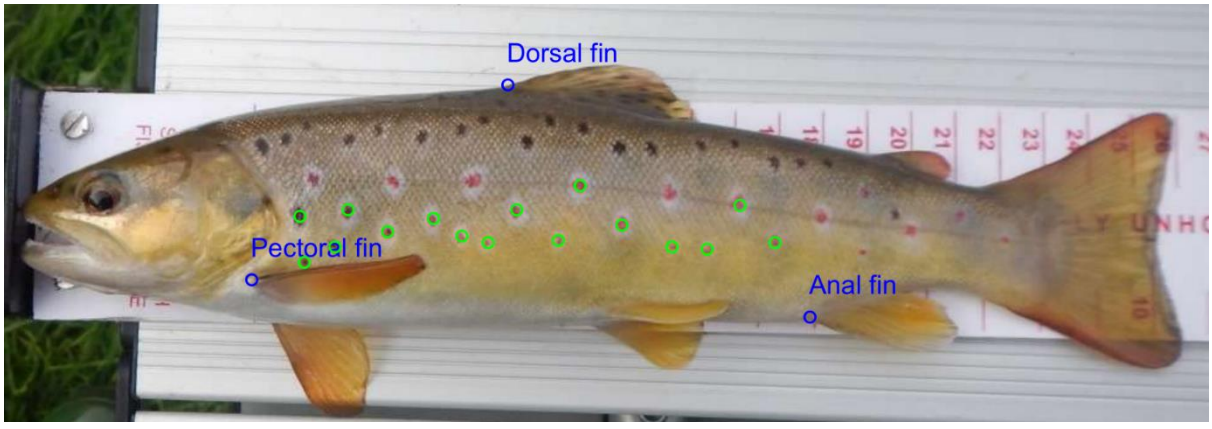

Figure 51 Example of reference area (blue) and melanophores selected (green) for spot analysis in i^3s. The methodology developed produces a line of the most ventral melanophores within the reference area for analysis

4.3.3 Analysis

The analytical methods applied to the fishery data are summarised in Table 25. The table presents an overview of each test in terms of the its characteristics, metrics used, and provides the chapter number in which each test described in more detail. The table includes only primary analytic techniques and tests, with the statistical analyses described in section 4.3.3.5

Table 25 Summary of the tests used to analyse fishery data

Test	*Analysis type*	*Specific characteristics*	*Metrics*	*Section*
Carle and Strub (Carle and Strub 1978)	Population estimate	Estimates fish populations following multiple pass sampling, based on the principal that in a closed system, the number of fish captured decreased on each sample	Fish abundance data from multiple pass electric fishing	4.3.3.1
Relative Weight (Wege and Anderson 1978)	Condition factor	Provides a value for the condition of fish to indicate its health based on body weight. A fish with expected weight for its length will score 100, an underweight specimen <100 and one heavier than typical >100	Length and weight of fish, and species-specific standard weight W_s equation	4.3.3.2
Standard growth	Growth equation	Provides a value for growth between two captures for individual fish	Weight at first and second capture and time between captures	4.3.3.3.2
Jolly-Seber (Jolly 1965; Seber 1965)	Population estimate	Estimates the size of an open population from mark recapture data	Encounter history matrix	4.3.3.3.3
HABSCORE (Milner et al. 1998)	Habitat evaluation method	Predicts densities of three age classes of brown trout and salmon based on environmental features. Outputs metrics based on observed populations of each size class to indicate if observed densities are significantly higher or lower than expected for the habitat	Date of sampling, site location (NGR), altitude, distance from source, distance from tidal limit, site gradient, catchment gradient, link number, downstream link number, migratory access for brown trout and salmon, riparian shading (% deciduous trees and shrubs, coniferous trees and herbaceous vegetation), substrate embeddedness (low, medium or high), discharge category, mean conductivity, mean channel width and depth, substrate composition (% bedrock/artificial substrate, boulders, cobbles, gravel/ coarse sand, fine sand, silt and compacted clay), flow type (% deep and shallow of cascade, turbulent, glide and slack), sources of cover for >100 mm trout (% of submerged vegetation, boulder and cobbles, tree root systems, branches and logs, undercut banks, other submerged cover, undercut banks and areas of deep water)	4.3.3.4
Similarity Percentage (SIMPER) (Clarke 1993)	Method	Identifies the percentage contribution of each species to overall average dissimilarity observed between samples	Bray-Curtis dissimilarity matrix of fish abundance data	3.3.6.6

4.3.3.1 Fish populations

Estimations of abundance following multiple pass (M-P) fishing can be made based on the principal that in a closed, finite population the number of fish present will decrease if captured fish are removed on each pass. This will lead to a reduction in catch-per-unit-effort (CPUE), the decline of which can be used to estimate the original size of the population (Hedger et al. 2013). The method developed by Carle and Strub (1978), known either as the Carle and Strub method, or Maximum Weighted Likelihood (MWL), is commonly used in fisheries research and considered one of the most robust and statistically reliable methods (Cowx 1983; Hedger et al. 2013). The Carle and Strub method assume an exponential decline in fish numbers with each successive pass, and is calculated as follows:

The parameter M is calculated using:

$$M = \sum_{i=1}^{k} (k - i)\, u_i$$

Where k is the number of samples taken and u_i the number of fish caught in the ith sample.

The population size, *N* is estimated as the smallest integer greater than the total catch, *T*, that satisfies the inequality

$$\left(\frac{N+1}{N-T+1}\right)\left(\frac{kN-M-T+0.5k}{kN-M+1+0.5k}\right)^{k} \leq 1.$$

The above inequality is an approximation of a summation and can fail for samples when k=2.

The Carle and Strub (Carle and Strub 1978) method was used to estimate fish abundances using Population Estimation by Removal Sampling v1.23 (Pisces Conservation Ltd, Lymington). Estimates for *S. trutta* were generated using the maximum likelihood method (ML). The method relies on the assumption that the number of animals captured will decline in successive M-P sampling, which occurred in all but two instances with *S. trutta*. For other less common and benthic species, there was considerable variation in the proportion of the population taken on each pass, and the ML model failed. In addition, where abundances of a species are low, M-P does not provide a robust estimate of abundance (Hedger et al. 2013). For example, Riley and Fausch (1992) set a minimum abundance of 30 individuals before estimating abundance from M-P catch. For species other than

S. trutta, and on the two occasions when *S. trutta* catch data did not fit the assumptions of ML, the numbers of individuals caught on all passes were totalled and used in place of population estimates.

As each site was not of uniform dimensions, to allow site-to-site comparison of fish abundances between sites, the density of fish (*n* per 100m^2) was calculated for each site and survey. The surface area of each site on each survey date was generated from channel measurements taken during the HABSCORE surveying and forms part of the output from the HABSCORE for Windows software. The density of *S. trutta* of three size categories is automatically generated by the software. For all other species, the population density (*n* per 100m^2) at each site at each surveying date was generated by dividing the population size (*n*) by the surface area and multiplying by 100.

Species diversity was investigated in terms of the number of unique fish species at each site (species richness), and Shannon's Index (see 3.3.6.1).

4.3.3.2 Condition

The collection of length and weight data is a standard practice in fishery surveys, and these data are commonly used to perform condition assessments (Blackwell et al. 2000). As a fish increases in length, it will increase in weight, and the ratio of these two measurements have been used in a number of ways as non-invasive measures of condition. The objective of condition assessment is to measure the 'plumpness' of fish, which can be used to indicate general health, as well as inform of environmental characteristics such as habitat quality, water quality and prey availability (Liao et al. 2004). The traditional condition assessment has been the use of Fulton's condition factor (K), calculated as

$$K = W/L^3$$

where *W* is weight and *L* is length. Fulton's K assumes isometric growth - that fish shape does not change with growth. In practice isometric growth is rarely the case as fish increase in length, they become more rotund, resulting in increasing K with fish length (Bolger and Connolly 1989). This condition factor is both length and species dependent, so that it isn't possible to compare fish of different species or fish of the same species of disparate lengths (Murphy et al. 2004). Relative weight (W_r) was introduced by Wege and Anderson (1978) and is now widely used in fisheries research and management (Murphy et al. 2004). It is generated from a species-specific standard weight W_s equation derived from length and weight regressions of fish from a variety of populations in good health. W_s equations have been developed for a wide range of species (Blackwell et al. 2000; Liao et al. 2004).

Relative weight is calculated

$$W_r = \frac{W}{W_s} \times 100$$

where W is the measured wet weight (g) in the field and W_s is the standard weight predicted by a weight-length regression of the whole surveyed population.

W_r has several advantages over Fulton's K and other condition assessments: (1) standard weights compensate for inherent changes and species specific body form, (2) W_r does not change with different measurement units, (3) variation in W_r may be primarily due to existing ecological factors, and (4) W_r can be compared between fish of different lengths and from different populations (Wege and Anderson 1978). To compare the condition of species between sites, it was important that that a) they were represented at all sites, and b) that they were in sufficient number in location and time to make comparisons. Two species fit these conditions; *S. trutta* and *C. gobio*. *A. anguilla* were represented at most sites, but were absent on many occasions, and when present were in low number. In addition, their length could only be measured approximately when alive, which would not give sufficiently accurate measures of condition, so the condition of this species was not explored.

The sites and seasons of fishery data used for W_r is presented in Table 26. There were found to be significant differences in W_r between seasons on the Bourne and Crane (see 4.4.2), so it was important to have representative data sets for both seasons when examining site-specific W_r. The W_r analysis was therefore run for each site using equal numbers of spring and autumn surveys to avoid seasonal bias. It was necessary to exclude some data from W_r analysis. The electronic balance used to weigh fish malfunctioned during the first spring survey in 2016 on the Bourne and during Crane surveys in spring 2017. However, the omission of these data still allowed two seasons of spring and autumn data to be analysed (Table 26). The site BREC was omitted from W_r analysis for as no autumn data exists for it with the channel is blocked by emergent macrophytes in the autumn and winter and so impassable to fish. As such, any fish surveyed in spring would have migrated into the site following weedcutting having spent an unknown duration elsewhere on the river. Instead, the focus of fish condition on the Bourne is on BRWC, BRDS1 and BRDS2 only. The location of BRDS1, just after the confluence of BRWC and BREC, allows it to be considered the downstream site of watercress farm effluent.

Table 26 Electric fishing site and survey seasons (S = spring, A = autumn + year) used to produce relative weight values from length and weight data of fish. A tick denotes data from site and season used, nd; no data available, nu; data available but not used in order to have equitable seasonal representation

River	*Site*	*Season*				
		S16	*A16*	*S17*	*A17*	*S18*
The Bourne Rivulet	BRWC	nd	✓	✓	✓	✓
	BREC	nd	nd	nu	nd	nu
	BRDS1	nd	✓	✓	✓	✓
	BRDS2	nd	✓	✓	✓	✓
The River Crane	CRUS	✓	✓	nd	✓	✓
	CRDS1	✓	✓	nd	✓	✓
	CRDS2	✓	✓	nd	✓	✓
	CRDS3	✓	✓	nd	✓	✓
The River Frome	FRUS	nd	nu	✓	✓	nd
	FRDS1	nd	nu	✓	✓	nd
	FRDS2	nd	nd	✓	✓	nd
	FRDS3	nd	nu	✓	✓	nd

W_s equations have been developed for a wide range of species (Liao et al. 2004), and a search for suitable W_s equations was undertaken for the two target species in this study. No W_s equations could be found for *C. gobio* or related *Cottus sp*. in any habitat. W_s equations for *S. trutta* were found for North American populations in both lentic and lotic habitats, but none could be found for UK chalk streams. As UK chalk streams are known for their high productivity of *S. trutta* (Westlake et al. 1972), the published W_s from North American rivers were not considered a good benchmark for W_s in chalk streams. For these reasons, W_s equations were developed using the length-weight data gathered for both *C. gobio* and *S. trutta* during the surveys in the present study. While these data may include 'impacted' sites, it would still allow comparison of relative weight between sites to highlight which sites, if any, had populations at higher or lower than average condition.

In calulating W_s equations and applying W_r analysis, Murphy et al. (2004) state that it is neccesarry to set a minimum length threshold for two principal reasons. Firstly, there exists an increased potential for inaccurate weight measurements in the field when measuring small live and wet specimens. Secondly, fish go through large ontogenetic changes in body morphology form juveniles to adult form. For stream-dwelling *S. trutta*, Milewski and Brown (1994) found in developing standard weight W_s and W_r equations, that there was a decrease in variance/mean ratios in *S. trutta* below 140mm, and so this length was set as the minimum when developing W_s equations and subsequent condition analysis.

Length in FL (fork length) and weight data for trout, and total length (TL) and weight data for *C. gobio* were log10 transformed to provide linear weight length relationships for each species. Linear regression analysis was then performed for each species.

As no standard weight W_s values could be found for chalk stream *S. trutta* in the UK, W_s was generated from all surveyed fish ≥ 140 mm FL (*n* = 732 from a total *S. trutta* catch of 2136). This provided the regression formula:

$$\log 10\ W_s\ (\mathrm{g}) = -1.95007 + 3.030787\ \log 10\ \mathrm{FL}\ (\mathrm{cm})$$

No standard weight equation could be found for *C. gobio*, so as with *S. trutta*, a W_s was generated from all surveyed fish ≥ 50 mm TL (*n* = 315 from a total *C. gobio* catch of 351) to circumvent issues with accuracy of weighing of small fish in the field. The regression formula was for *C. gobio* was calculated:

$$\log 10\ W_s\ (\mathrm{g}) = -1.84403 + 3.035559\ \log 10\ \mathrm{TL}\ (\mathrm{cm})$$

There is a danger that W_r values can be biased by fish length, so to investigate biases in the W_s equation, the W_r values were regressed against length. If the regression is significant, i.e. the slope is significantly different from zero at α 0.05, then size specific W_r estimates are needed. If the regression is not significant, then the W_r could be used to represent all sizes classes.

A W_r of 100 would apply to a fish which had an average length weight relationship, while sub 100 W_r score would apply to a fish with a lower than expected body weight, and a >100 W_r for a fish with a higher body weight for its length. The individual W_r values were used to compare variation in fish condition between sites using one-way ANOVA.

4.3.3.3 Spot recognition

Spot-recognition was used to identify individuals to examine site fidelity, site-specific growth rates and to generate population estimates. A total of 1098 images were used, with 883 young-of-year *S. trutta* were excluded from the analysis as salmonid melanophore constellations have been shown to be unstable in juvenile fish (Merz et al. 2012) and no successful matches were made for any young-of-year fish. In addition, 17 *S. trutta* from the Frome were excluded from analysis as they lacked the requisite minimum of 12 melanophores required for spot analysis (Figure 52), in addition to the 20 individuals excluded from the Frome due to the wrong flank being photographed as previously discussed.

Figure 52 *Salmo trutta* captured on the River Frome, one of 17 excluded from spot recognition analysis due to having fewer than the minimum of twelve melanophores required for I^3S spot recognition software

4.3.3.3.1 Site fidelity

Stream dwelling Individual *S. trutta* are typically territorial and remain within a reach, while a small percentage have been shown to roam more freely (Knouft and Spotila 2002; Giller and Greenberg 2015). *S. trutta* that were caught at one site, and later recaptured at another were considered non-resident individuals. The percentage of resident and non-resident *S. trutta* was calculated for each river system from the number of individuals recaptured at the same site and the number recaptured at alternative sites.

4.3.3.3.2 Growth

Growth rates were calculated from matched individuals of resident *S. trutta* matched to the database of weight and length data collected in the field. When positive matches were achieved for resident *S. trutta*, the standard growth (G_w) was calculated using the standard growth equation

$$G_w = \frac{ln\, W_t - ln\, W_0}{T\ x\ 100}$$

Where W_0 is the fresh mass of fish at first capture, and W_t is the fresh mass after T number of days

Due to seasonal variations in salmonid growth rates (Klemetsen et al. 2003), it would be inappropriate to compare growth rates between sites spanning different seasons. Instead, it would only be appropriate to compare growth rates between sites spanning a homologous timeframe.

While some specimens were caught repeatedly across several seasons, and at some particular sites there were large numbers of recaptured individuals, there were insufficient recaptures at each site during the same timeframe to allow statistical comparisons of growth rates between sites.

4.3.3.3.3 Population estimates

Population estimates were generated using a mark-recapture method principally to ascertain the effectiveness of spot-recognition to estimate *S. trutta* populations. The effectiveness was assessed by comparison of mark-recapture estimates with population estimates generated using the M-P depletion methods. As *S. trutta* are not enclosed at any site, population estimates for each site were generated using the Jolly-Seber (J-S) method for open populations (Jolly 1965; Seber 1965). This methodology allows for mortality and immigration and emigration. A further advantage of the model is that the time interval between samples need not be constant and series data extending over a number of years is permitted. The J-S model estimates population size at each sampling occasion (*i*) by the observed proportion of marked and unmarked individuals from the preceding and subsequent samples. As such, a minimum of three samples are required, and estimates are not generated for the first and last survey occasions. The J-S model has the following assumptions:

1. Every marked animal present in the population at sampling period *i* has the same probability of being captured or resighted
2. Every marked animal present in the population at sampling period *i* has the same probability of survival until sampling period *i*+1
3. Marks are neither lost nor overlooked and are recorded correctly
4. Sampling periods are instantaneous (in reality they are very short periods) and recaptured animals are released immediately

Population estimates using J-S were generated for each site, with fish that were known to have emigrated due to being recaptured at a different site excluded from the analysis. There was no photography of fish in the Bourne in spring 2016 which led to BREC only having spot match data for two surveys, which meant J-S could not be used at this site. Due to the low rates of recapture on the Frome, only one site, FRUS, contained enough recaptured individuals for population estimating using J-S, so populations were not estimated at all other Frome sites.

4.3.3.4 HABSCORE and salmonids

HABSCORE is able to predict densities of three salmonid size/age classes; 0+, 1+ and > 200 mm. These categories can also be described as juvenile, sub-adult and adult fish (Milner et al. 1998). Correct interpretation of 0+ and 1+ year classes is considered important for the accuracy of HABSCORE (Wyatt et al. 1995). Previous studies using scale analysis has shown that there is overlap

in year class length at most sites (Longley 2006). During the present study, it was not possible to obtain a Home Office licence to remove scales for accurate age analysis, and so fork length was used to distinguish 0+ and < 200 mm classes. Under the advice of the lead developer of HABSCORE, Nigel Milner (pers. comms., December 2016), the 0+ class was defined as any fish with a fork length < 99 mm and the < 200 mm class was set as any fish between 100 – 199 mm. The third class was any fish over >200 mm.

Physical site variables (see 3.3.3) and *S. trutta* population estimates using Carle and Strub (1978) were entered into HABSCORE for each site for each survey, which produces outputs for the following outputs for the three size/age categories (Wyatt et al. 1995):

- *Habitat Quality Score* (HQS): This is derived using habitat data from the habitat surveys and represents the potential population of the site given the habitat characteristics. The HQS is expressed as the estimated population density (N ± CL/100m^2) of each age class at a given site derived purely using physical habitat data
- *Habitat Utilisation Index* (HUI): This is generated by comparing the observed population data of a site with the expected (HQS) population size calculated from the habitat. A HUI of one indicates that the population is on par with the estimate (HQS). A HUI below one indicates that the population falls below the estimate, indicating a factor other than habitat quality (degradation in water quality or overfishing, for example) may be limiting population density of the particular size class. A HUI above one indicates that a site is sustaining a higher population density than expected, suggesting a good ecological condition.

HUI is the measure of habitat utilisation and is derived as the ratio (± 90% CL) between the observed densities recorded during fishing surveys, and the predicted population density (HQS) (Wyatt et al. 1995). When the observed density and the HQS are identical HUI = 1. When observed densities fall below the expected HQS, HUI < 1. When the upper CL limit of the HUI distribution is < 1, then the difference is considered significant. (Wyatt et al. 1995) Conversely, HUI > 1 indicates that the population density is greater than the predicted HQS. When the lower CL limit of the HUI distribution is > 1, then the observed density is significantly greater than the predicted HQS (Wyatt et al. 1995).

Some dates were not accepted by the HABSCORE software as they were out of the seasonal range used to generate the models. In such situations, in order to run the software, the closest accepted date was input. For example, to enable data from the River Frome survey which took place on 29th April 2017 to be entered, the inputted date was changed to 1st May 2017.

4.3.3.5 Statistical analysis

Similarity and dissimilarity in fish community structure across sites and subsequently between sites on each river were explored using NMDS and SIMPER. These analyses are described in sections 3.3.6.5 and 3.3.6.6 respectively.

Canonical correspondence analysis (CCA) was used to explore patterns of fish community composition in relation to the macroinvertebrate abundances and environmental and habitat variables obtained in chapter three. The technique directly relates community variation to environmental variation (ter Braak 1986). Autocorrelation of variables was explored using Spearman rank correlations, with correlations >0.5 and significant considered co-correlated. Channel width was removed due to its strong correlation with depth. The four substrate categories, Silt, sand, gravel and cobbles are percentage variables totalling 100%. To avoid co-correlation in the substrate category, gravel and cobbles were pooled as 'coarse substrate' and silt and sand were omitted. All percentage variables were arcsine square-root transformed. Multiple stepwise regression was used to narrow down the three variables most influential on the abundances of fish species on each river as per Langford et al. (2012). The mean densities of fish were then ordinated against site and the most influential variables using CCA, whereby fish density and site are plotted as points and environmental variables as vectors in two-dimensional space. Where there were fewer than three individuals of any species captured over the duration of the study on any river, these were omitted from the CCA analysis.

Relative condition (W_r) data for site and season was tested for normality of distribution and homogeneity of variance. It was found to be normally distributed, so significant differences W_r between site on each river system, and between seasons, were explored using one-way ANOVA (α 0.05). Linear relationship between W_r and non-transformed habitat variables, macroinvertebrate abundance, diversity and WHPT biotic index (see chapter three) were examined using Pearson product moment correlation to investigate factors that affect fish condition.

Multiple stepwise regression, Pearson correlation and ANOVA were performed using Minitab v19 (Minitab, Inc, USA). NMDS, SIMPER and CCA were performed using PAST v3.26 (Hammer et al. 2001). Jolly-Seber population estimates were calculated using the FSA package in R (Ogle 2016).

4.4 Results

4.4.1 Fish species composition

Twelve species of fish were caught across all sites over the course of the study (May 2016 to June 2018). The total number of individuals captured of each species from each site is presented in Table 27 along with the species richness and Shannon's Index. Shannon's Index showed that diversity was higher in the sites immediately downstream of watercress farm discharges than those immediate upstream on all three river systems. Species richness was less consistent, with the Bourne having a higher species richness below the discharge in BREC (4) than BRWC (2) and BRDS1 (3). Species richness was higher in the Crane upstream site CRUS (6) than the immediate downstream site CRDS1 (4), while the Frome had an equal species richness of seven for both the upstream FRUS and the immediate downstream site FRDS1.

Table 27 Total catch of fish from all surveys on the Bourne Rivulet (BRWC, BREC, BRDS1 and BRDS2), the River Crane (CRUS, CRDS1, CRDS2 and CRDS3) and the River Frome (FRUS, FRDS1, FRDS2 and FRDS3) between May 2016 and June 2018, the species richness and Shannon's diversity index

Site	*n*	**Brown trout** *Salmo trutta*	**Bullhead** *Cottus gobio*	**Eel** *Anguilla anguilla*	**Stickleback** *Gasterosteus aculeatus*	**Brook lamprey** *Lampetra planer*	**Minnow** *Phoxinus phoxinus*	**Stone loach** *Barbatula barbatula*	**Pike** *Esox lucius*	**Perch** *Perca fluviatilis*	**Grayling** *Thymallus thymallus*	**Roach** *Rutilus rutilus*	**Tench** *Tinca tinca*	*Species richness*	*Shannon's index*
BRWC	5	170	25	-	-	-	-	-	-	-	-	-	-	2	0.383
BREC	3	28	35	1	2	-	-	-	-	-	-	-	-	4	0.870
BRDS1	5	185	41	-	2	-	-	-	-	-	-	-	-	3	0.520
BRDS2	5	214	42	1	1	-	-	-	-	-	-	-	-	4	0.494
CRUS	5	338	15	3	3	4	1	-	-	-	-	-	-	6	0.345
CRDS1	5	215	42	6	2	-	-	-	-	-	-	-	-	4	0.584
CRDS2	4	143	14	5	2	-	-	-	-	-	-	-	-	4	0.490
CRDS3	5	403	57	7	-	-	-	-	-	-	-	49	3	5	0.750
FRUS	3	195	13	4	-	-	30	15	1	-	6	-	-	7	0.953
FRDS1	3	82	32	36	-	1	165	15	1	2	-	-	-	7	1.363
FRDS2	2	77	19	15	-	-	69	9	-	1	2	-	-	7	1.381
FRDS3	3	77	52	18	-	-	107	22	2	1	4	-	-	8	1.522

NMDS ordination of total catch data cumulative for all seasons against site shows little overlap in ordination space between sites on the three river systems, indicating that there are river-wide differences in fish population structures (Figure 53). The sites BREC and FRUS stand out as being distinct from other sites on their respective rivers. Sites on the Crane appear more similar in their fish communities, but of these, CRDS2 appears to be the most distant. SIMPER analysis revealed that cumulatively up to 95% of differences between all sites were contributed by the four most numerous species, brown trout (*Salmo trutta* L.) (62.02%), minnow (*Phoxinus phoxinus* L.) (13.92%), bullhead (*Cottus gobio* L.) (13.83%) and eel (*Anguilla anguilla* L.) (3.736%) (Table 28).

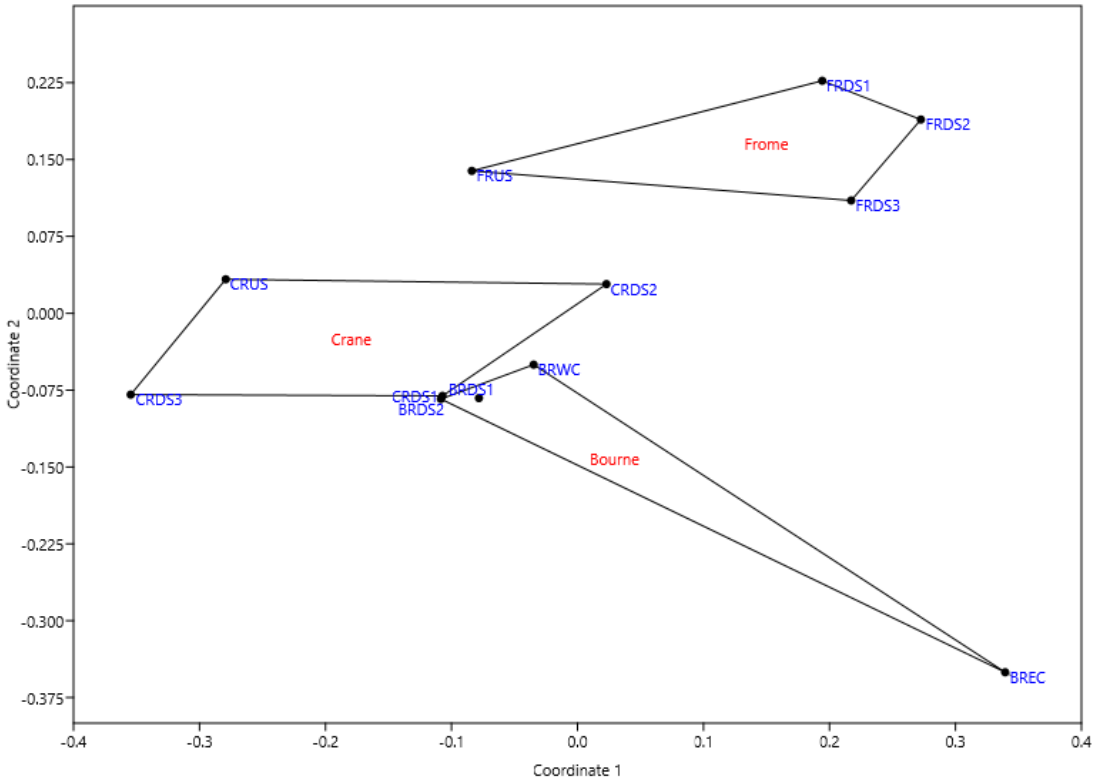

Figure 53 Non-metric multidimensional scaling plot of the total abundance of fish species captured by electric fishing between and site identity for all surveys on the Bourne Rivulet (BRWC, BREC, BRDS1 and BRDS2), The River Crane (CRUS, CRDS1, CRDS2 and CRDS3) and The River Frome (FRUS, FRDS1, FRDS2 and FRDS3) between May 2016 and June 2018 (stress 0.04065)

Table 28 SIMPER analysis of the percentage contribution to differences between rivers (Con. %), the cumulative percentage differences (Cum. %) between all rivers and the mean densities (*n*/100m^2 ± SD) of each species on the Bourne Rivulet, the River Crane and the River Frome

	Con. %	*Cum. %*	*Bourne Rivulet*	*River Crane*	*River Frome*
Salmo trutta	62.08	62.08	6.87 ± 3.08	21.03 ± 10.74	6.05 ± 2.16
Phoxinus phoxinus	13.92	76	-	0.01 ± 0.03	5.49 ± 3.33
Cottus gobio	13.83	89.83	2.68 ± 2.68	2.09 ± 1.59	1.69 ± 0.86
Anguilla anguilla	3.736	93.57	0.06 ± 0.09	0.38 ± 0.19	1.08 ± 0.80
Barbatula barbatula	2.365	95.93	-	-	0.83 ± 0.17
Rutilus rutilus	1.979	97.91	-	0.78 ± 1.57	-
Gasterosteus aculeatus	0.8236	98.74	0.12 ± 0.18	0.11 ± 0.09	-
Thymallus thymallus	0.4739	99.21	-	-	0.16 ± 0.11
Lampetra planer	0.3219	99.53	-	0.06 ± 0.11	0.01 ± 0.03
Perca fluviatilis	0.1829	99.71	-	-	0.06 ± 0.05
Esox lucius	0.1603	99.88	-	-	0.05 ± 0.04
Tinca tinca	0.1248	100	-	0.04 0.08	-

4.4.1.1.1 The Bourne Rivulet fish assemblage

Four fish species were captured on the Bourne during the duration of the study (Table 29). SIMPER analysis showed that differences in density between Bourne sites were greatest for *S. trutta* (68.52%) and in descending order; bullhead *C. gobio* (29.24%), stickleback *Gasterosteus aculeatus* (1.56%) and eel *A. anguilla* (0.68%). Densities of *S. trutta* were highest in BRWC and lowest in BREC which receives salad wash effluent and increased with increasing distance from BREC (Table 29). In contrast, the three non-salmonid species were found at the highest densities in BREC and declined in abundance with increasing distance from BREC. Both *G. aculeatus* and *A. anguilla* were absent from BRWC and in very low abundance at other sites, with <2 individuals of either species captured at any site over the entire duration of the study (Table 27).

Table 29 SIMPER analysis of the percentage contribution to differences between sites (Con. %) , the cumulative percentage differences (Cum. %) between sites and the mean density (*n*/100m^2 ± SD) of each species at each site on the Bourne Rivulet (BRWC, BREC, BRDS1 and BRDS2). Mean densities from electric fishing surveys between May 2016 and June 2018; *n* = three spring surveys, BREC; *n* = three spring and *n* = two autumn surveys; BRWC, BRDS1 and BRDS2

	Con. %	*Cum. %*	*BRWC*	*BREC*	*BRDS1*	*BRDS2*
Salmo trutta	68.52	68.52	10.25 ± 9.82	4.82 ± 3.02	5.95 ± 4.75	6.20 ± 2.78
Cottus gobio	29.24	97.76	1.33 ± 1.06	6.70 ± 1.06	1.43 ± 1.69	1.26 ±1.23
Gasterosteus aculeatus	1.558	99.32	-	0.38 ± 0.66	0.06 ± 0.08	0.03 ± 0.07
Anguilla anguilla	0.6784	100	-	0.19 ± 0.33	-	0.03 ± 0.07

Canonical correspondence analysis was carried out on the three size classes of *S. trutta* and *C. gobio*. *A. anguilla* and *G. aculeatus* were excluded from CCA as fewer than three individuals of each were captured over duration of study. Stepwise multiple regression showed that the three habitat variables that were the best predictor of fish abundance of *S. trutta* of three size classes and *C. gobio* were macrophyte cover, LWD and coarse substrate. CCA analysis showed that young-of-year and sub-adult *S. trutta* densities were higher in macrophyte cover, while adult *S. trutta* densities were associated LWD (Figure 54). *C. gobio* were mainly associated with high coverage of coarse substrates.

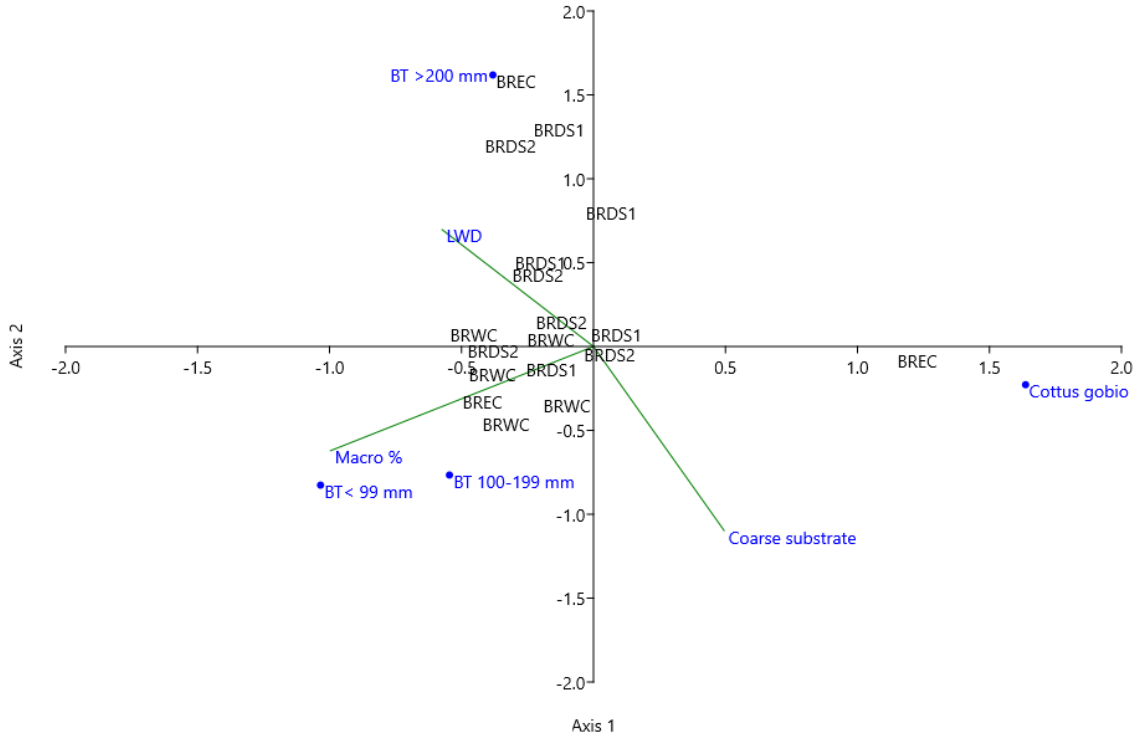

Figure 54 Canonical correspondence analysis triplot of species and densities of fish in relation to the three most influential habitat variables and sites on the Bourne Rivulet (BRWC, BREC, BRDS1 and BRDS2). BT < 99 mm, BT 100-199 mm, BT > 200 mm, size classes of *Salmo trutta,* brown trout; *Cottus gobio*, bullhead: LWD, large woody debris; Macro %, percentage of macrophyte cover; course substrate, gravel, cobbles and boulders

4.4.1.1.2 The River Crane fish assemblage

Eight fish species were captured on the Crane over the duration of the study (Table 30). 95% of the difference between sites in density were from three of the species, *S. trutta* (78.93%), *C. gobio* (10.87%) and roach *Rutilus rutilus* (5.75%). *S. trutta* densities were lowest downstream of the discharge in CRDS1 and increased with increasing distance from the discharge. Upstream of the

discharge at CRUS densities were higher than both CRDS1 and CRDS2, but greatest densities were found in CRDS3. *C. gobio* densities were higher in CRDS1 than CRUS and CRDS2 but were highest of all in CRDS3. *A. anguilla* were present at all sites, with densities increasing downstream for the length of the river studied. *R. rutilus* and tench *Tinca tinca* were present only in CRDS3, where a high abundance of roach (49 individuals) at this one site led to the relatively high SIMPER value. *L. planer* and *P. phoxinus* were captured in CRUS only and in very low abundances, with just four and one individuals captured over the duration of the study respectively.

Table 30 SIMPER analysis of the percentage contribution to differences between sites (Con. %) the cumulative percentage differences (Cum. %) between sites and the mean density (*n*/100m^2 ± SD) of each species at each site on the River Crane (CRUS, CRDS1, CRDS2 and CRDS3). Mean densities from electric fishing surveys between May 2016 and June 2018; *n* = two spring and *n* = two autumn surveys, CRDS2; *n* = three spring and *n* = two autumn surveys; CRUS, CRDS1 and CRDS3

	Con. %	*Cum. %*	*CRUS*	*CRDS1*	*CRDS2*	*CRDS3*
Salmo trutta	78.93	78.93	25.80 ± 20.2	10.11 ± 6.7	15.93 ± 12.3	35.19 ± 10.5
Cottus gobio	10.87	89.81	1.08 ± 1.0	2.13 ± 1.2	0.99 ± 0.9	4.38 ± 2.6
Rutilus rutilus	5.75	95.56	-	-	-	3.70 ± 3.3
Anguilla anguilla	2.218	97.77	0.24 ± 0.3	0.30 ± 0.3	0.47 ± 0.7	0.67 ± 0.5
Gasterosteus aculeatus	1.06	98.83	0.24 ± 0.5	0.10 ± 0.1	0.18 ± 0.4	-
Lampetra planer	0.6873	99.52	0.28 ± 0.3	-	-	-
Tinca tinca	0.3586	99.88	-	-	-	0.20 ± 0.4
Phoxinus phoxinus	0.1213	100	0.07 ± 0.1	-	-	-

Canonical correspondence analysis was carried out on *S. trutta* of three size classes, *C. gobio* and *A. anguilla*. *T. tinca* and *R. rutilus* were omitted from the analysis as they appeared in only CRDS3, the likely result of escapes via an overflow from a nearby fishing lake and so were not native to the river. *P. phoxinus*, *G. aculeatus* and brook lamprey were excluded due to their scarcity in samples.

CCA using the three most influential variables, selected by stepwise multiple regression, showed that the three habitat variables that were the best predictor of fish abundance were macrophyte cover, water velocity and quantity of overhanging vegetation. The analysis showed that *A. anguilla*, adult and sub-adult *S. trutta* had a positive relationship with overhanging vegetation, while young-of-year *S. trutta* were once again associated with higher macrophyte coverage (Figure 55). *C. gobio* were mainly associated with high water velocities.

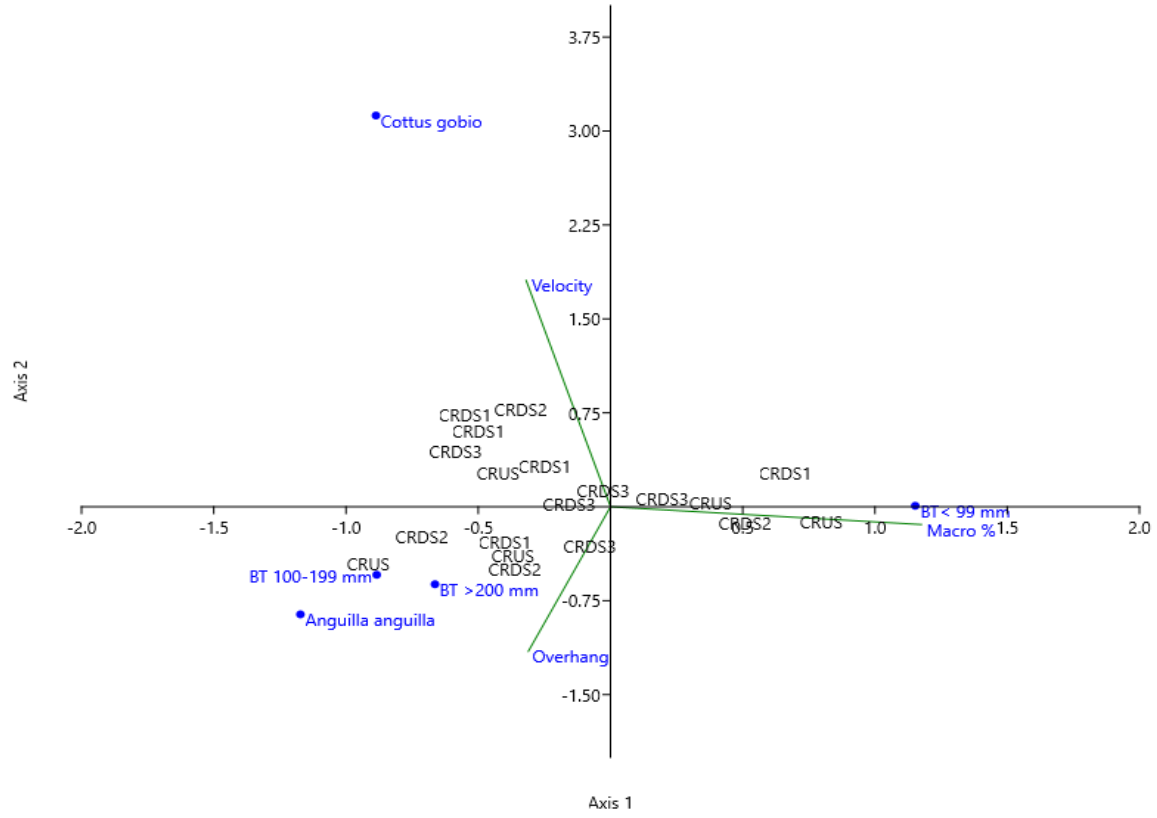

Figure 55 Canonical correspondence analysis triplot of species and densities of fish in relation to the three most influential habitat variables and sites on the River Crane (CRUS, CRDS1, CRDS2 and CRDS3). BT < 99 mm, BT 100-199 mm, BT > 200 mm, size classes of *Salmo trutta*, brown trout; *Cottus gobio*, bullhead; *Anguilla anguilla*, eel; Overhang, vegetation overhanging the channel by < 50cm; Macro %, percentage of macrophyte cover; Velocity, mean water flow

4.4.1.1.3 The River Frome fish assemblage

A total of nine fish species were captured on the River Frome. Just over 95% of the cumulative differences between sites were from five species, in descending order of percentage difference; *S. trutta* (38.44%), *P. phoxinus* (35.69%), *C. gobio* (10.81%), *A. anguilla* (9.11%) and stone loach *Barbatula barbatula* (3.55%) (Table 31).

S. trutta were in greatest densities in FRUS, dropped below the discharge at FRDS1 before rising again at FRDS2. The lowest densities were found in FRDS3. Both *P. phoxinus* and *A. anguilla* were at greatest densities below discharge in FRDS1 and at lowest densities upstream in CRUS, with densities downstream in FRDS2 and FRDS3 intermediate.

Benthic species followed a similar pattern of distribution across sites, with *C. gobio* and stone loach being at their lowest density in FRUS, second highest density in FRDS1 and at greatest densities in FRDS3. A single brook lamprey was captured at FRDS1 over the duration of the study.

Grayling *Thymallus thymallus* were captured in highest densities upstream at FRUS, were absent in FRDS1 and increased downstream. However, actual catches were low, with a total of six individuals captured in FRUS and two and four in CRDS2 and CRDS3 respectively (Table 27).

Pike *Esox lucius* and perch *Perca fluviatilis* were rarely captured, with fewer than two individuals captured at any one site over the duration of the study.

Table 31 SIMPER analysis of the percentage contribution to differences between sites (Con. %) the cumulative percentage differences (Cum. %) between sites and the mean density (*n*/100m^2 ± SD) of each species at each site on the River Frome (FRUS, FRDS1, FRDS2 and FRDS3). Mean densities from electric fishing surveys between October 2017 and October 2018; *n* = one spring and two autumn surveys for all sites

	Con. %	*Cum. %*	*FRUS*	*FRDS1*	*FRDS2*	*FRDS3*
Salmo trutta	38.44	38.44	8.64 ± 6.0	4.94 ± 5.9	6.89 ± 8.1	3.73 ± 2.9
Phoxinus phoxinus	35.69	74.13	1.30 ± 1.3	9.45 ± 6.6	5.76 ± 3.0	5.46 ± 4.5
Cottus gobio	10.81	84.94	0.56 ± 0.6	1.91 ± 1.4	1.66 ± 0.1	2.62 ± 1.9
Anguilla anguilla	9.11	94.05	0.17 ± 0.3	2.08 ± 2.0	1.23 ± 1.2	0.85 ± 1.3
Barbatula barbatula	3.545	97.59	0.64 ± 0.8	0.86 ± 0.1	0.76 ± 0.3	1.05 ± 0.4
Thymallus thymallus	1.055	98.65	0.27 ± 0.2	-	0.17 ± 0.1	0.18 ± 0.1
Perca fluviatilis	0.617	99.26	-	0.11 ± 0.1	0.08 ± 0.1	0.04 ± 0.1
Esox lucius	0.513	99.78	0.04 ± 0.1	0.05 ± 0.1	-	0.10 ± 0.1
Lampetra planer	0.2237	100	-	0.05 ± 0.1	-	-

Canonical correspondence analysis was performed on the fish species which accounted for up to 97.6% of cumulative difference between sites; *S. trutta*, *C. gobio*, *A. anguilla* and *B. barbatula*. The remaining species excluded due to their scarcity in samples. CCA using the three most influential variables, selected by stepwise multiple regression, showed that the three habitat variables that were the best predictor of fish abundance of *S. trutta* of three size classes and of *P. phoxinus*, *C. gobio*, *A. anguilla* and stone loach were channel depth, macrophyte coverage and overhanging vegetation. The analysis showed that *S. trutta* densities were mainly associated with high macrophyte cover and shallow water, particularly young-of-year and sub-adult (Figure 56). *C. gobio* were positioned close to the centroid, so appeared not to be strongly associated with any of the three habitat variables. Both *P. phoxinus* and *A. anguilla* were associated with deeper water.

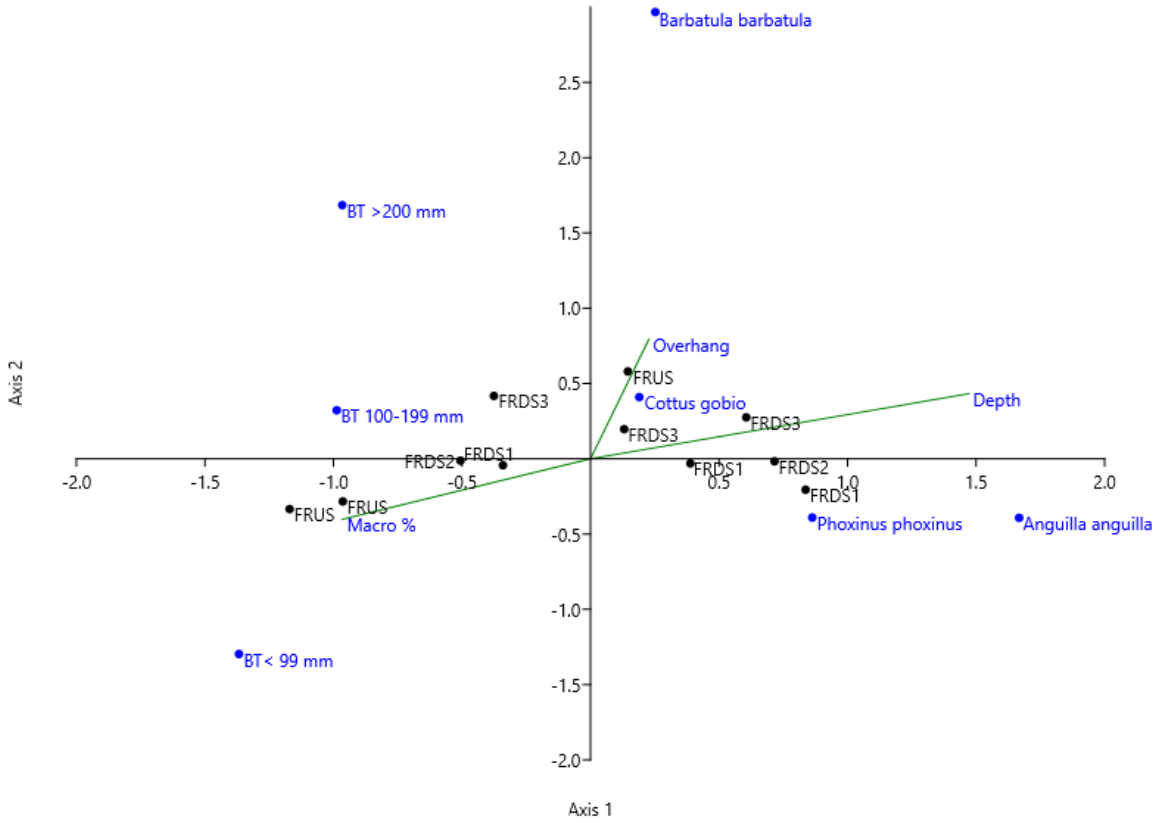

Figure 56 Canonical correspondence analysis triplot of species and densities of fish in relation to the three most influential habitat variables and sites on the River Frome (FRUS, FRDS1, FRDS2 and FRDS3). BT < 99 mm, BT 100-199 mm, BT > 200 mm, size classes of *Salmo trutta*, brown trout; *Cottus gobio*, bullhead; *Anguilla anguilla*, eel; *Phoxinus phoxinus*, minnow; *Barbatula barbatula, stone loach*: Overhang, vegetation overhanging the channel by < 50cm; Macro %, percentage of macrophyte cover; Depth, mean depth

4.4.2 Condition

4.4.2.1 Validating the W_r model

Relative weight was regressed against length to ascertain if there were biases for larger fish in the W_r equations. For *S. trutta*, the regression slope for fish of FL ≥ 140mm (n = 732) was very close to zero (W_r = 100.8 + 0.001578 FL (mm)) (Fig 62), which was not significant ($F_{1,729}$ = 0.04, P = 0.849), so the W_r equation was deemed suitable to describe condition for all *S. trutta* with a fork length greater than 140mm.

For *C. gobio*, the regression analysis of fish with total length >50 mm (n = 315) (W_r -73.18 + 2.785 TL (mm)) was significant ($F_{1,312}$ = 595.72 P >0.001), showing that there were biases for smaller and larger fish in the W_s equation (Figure 57). Further regressions were performed for ever narrower

total length bands, to see if restricting the length range analysed would result in a non-significant regression slope and therefore an applicable W_s equation for this species. However, even at 10 mm bands, i.e., 70-79 mm, there was still a significant (P <0.001) relationship between W_r and TL. By this point, n per site was too low to make robust comparisons between sites. It was decided that only the W_r model for *S. trutta* was suitable for exploration.

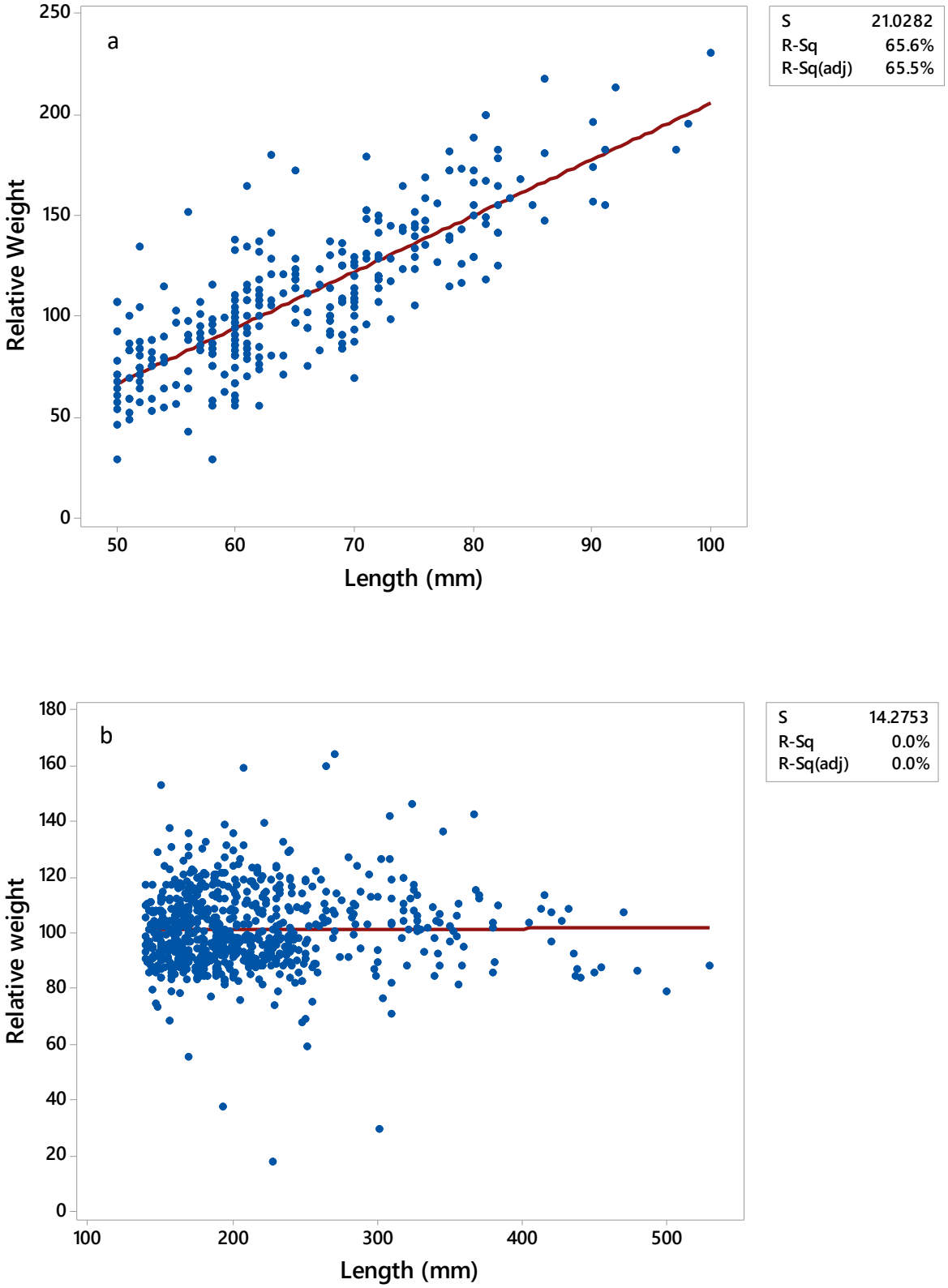

Figure 57 Fitted line plots of variation in relative weight (W_r) among individual *Cottus gobio* (n = 315) of total length ≥ 50mm (a) and *Salmo trutta* (n = 732) of fork length ≥ 140mm (b) from all sites on the Bourne Rivulet, the River Crane and River Frome. Data points represent the relative weight by length of fish, which would be 100 for a perfect fit to the standard weight equation. The slope of the fitted line for *Cottus gobio* (a) shows that for increases in length the subsequent increase in relative weight exceeds the model. For *S. trutta* (b), the slope is extremely close to zero (0.001), indicating that increasing fish length does not significantly affect the W_r equation

4.4.2.2 Relative weight (W_r) condition of *S. trutta* by season

With data from all rivers combined, W_r was significantly higher in spring (108.62 ± 13.07) than in autumn (94.85 ± 11.31) ($F_{1,718}$ = 229.23, P >0.001). This was true on both the Bourne which had significantly higher W_r in spring (117.38 ± 10.55) than autumn (102.12 ± 10.55) ($F_{1,208}$ = 98.18, P < 0.001), and also the Crane which has a spring W_r of 105.51 ± 11.46 compared to autumn W_r of 91.81 ± 9.22 ($F_{1,399}$ = 173.23, P < 0.001). This seasonal pattern was not apparent on the Frome, however, where there were no significant differences between seasons ($F_{1,107}$ = 0.92, P = 0.339). In contrast to the Bourne and Crane, on the Frome the autumn W_r (93.08 ± 11.31) was marginally higher than the spring (90.07 ± 11.02).

4.4.2.3 Relative weight (W_r) condition of *S. trutta* by site

The relative weight was calculated using data from all seasons and years combined. There were no significant differences in W_r on the sites on the Bourne ($F_{2,207}$ = 1.31, P = 0.271) (Figure 58)). However, W_r was notably consistent between BRWC (108.49 ± 13.04) and BRDS2 (108.28 ± 14.51) and was higher at the downstream site BRDS1 (111.4 ± 12.83). There were significant differences in W_r on the Crane ($F_{3,397}$ = 16.45, P = >0.000) with Tukey's post-hoc analysis showing that CRUS and CRDS1 both had significantly higher W_r than CRDS2 and CRDS3. There were no significant differences on the Frome ($F_{3,105}$ = 1.65, P = 0.182), where FRUS2 had the highest W_r followed by FRUS, FRDS3 and finally the immediate downstream site FRDS1.

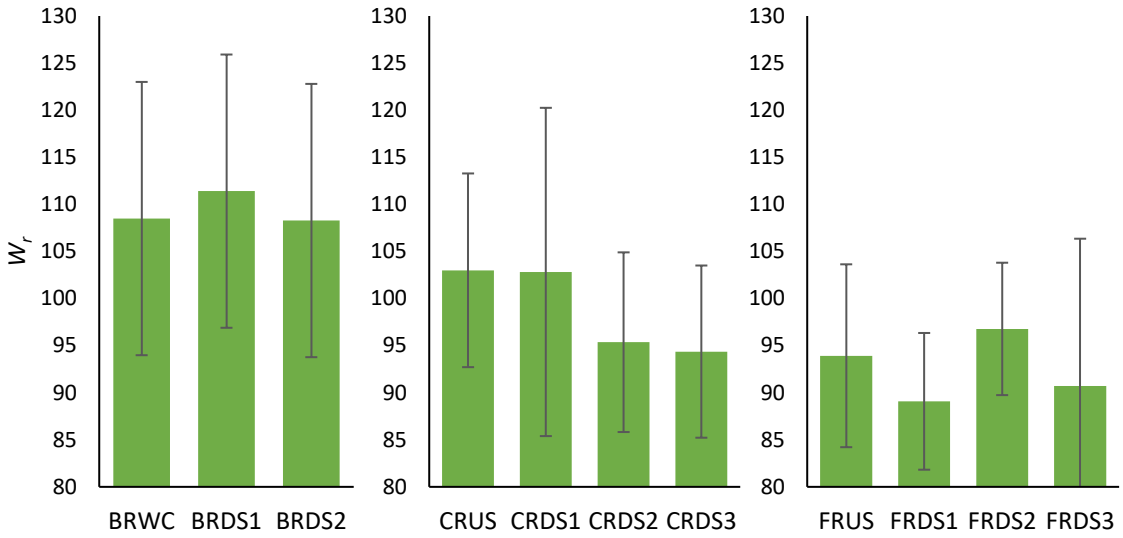

Figure 58 Mean (±SD) relative weight (W_r) of *Salmo trutta* on the Bourne Rivulet (BRWC, BRDS1 and BRDS2), the River Crane (CRUS, CRDS1, CRDS2 and CRDS3) and the River Frome (FRUS, FRDS1, FRDS2 and FRDS3). Means were generated from fish of fork length >140 mm during spring and autumn surveys between May 2016 to June 2018. A W_r of 100 indicates a body condition on par with the standard weight for all surveyed populations, while a value below 100 indicates a lower condition, and a value above 100 indicates superior condition. One-way ANOVA was performed for each river system individually, with mean values that do not share the same group letter being significantly different (Tukey's post-hoc α 0.05)

4.4.2.4 Relationships between W_r and environmental variables

Pairwise Pearson correlation analysis between W_r and all habitat variables found significant correlations between W_r and four variables (Figure 59). Significant positive correlations occurred between W_r and macrophyte cover (r = 0.538, P = <0.001) and macroinvertebrate abundance (r = 0.455, P = 0.004). Significant negative correlations occurred between the WHPT biotic index (r = -0.357, P = 0.026) and LWD (r = -0.346, P = 0.033). No other variables were significantly correlated with W_r.

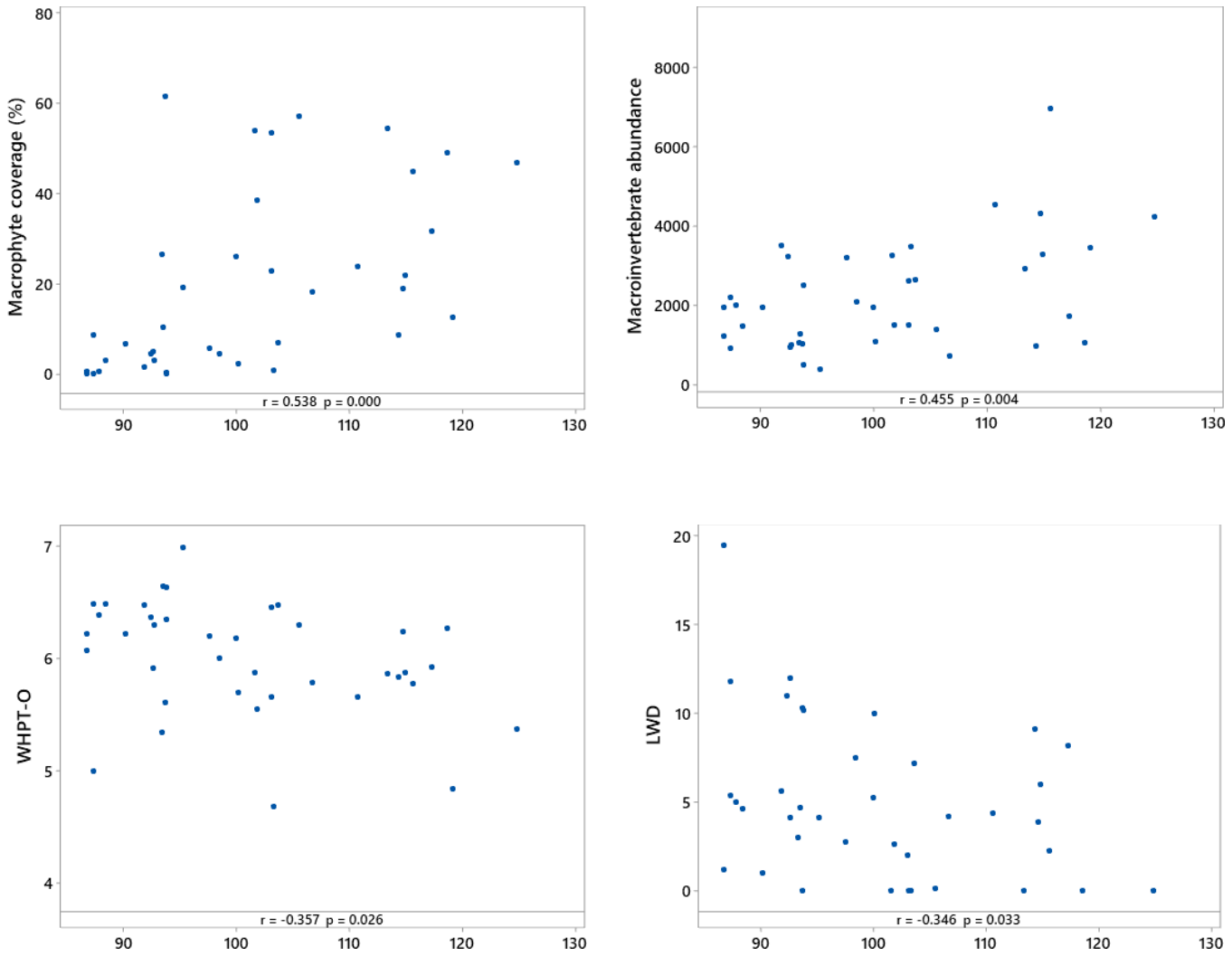

Figure 59 Matrix plots of four variables with linear correlations ($P < 0.05$) with relative weight of *Salmo trutta* (W_r) displayed on the *x* axis; macrophyte coverage; macroinvertebrate abundance, numbers of individuals in standard kick and sweep sample; WHPT-O, the observed Walley Hawkes Paisley Trigg biotic index score of macroinvertebrate assemblage; LWD, mean quantity of large woody debris. Data points from $n = 39$ surveys between May 2016 and June 2018 on the Bourne Rivulet, the River Crane and the River Frome

4.4.3 The Bourne Rivulet HABSCORE

Juvenile *S. trutta* (>99 mm fork length) were found in densities below predicted by HABSCORE in both the east channel (BREC) and BRDS1, with the Habitat Utilisation Index (HUI) being <1 for both sites (0.06 and 0.51 respectively) (Table 32). On BREC, the difference was considered significant with the upper HUI confidence limit being <1. While HQS indicated that BREC should support the highest abundances of juvenile trout, there were none found in BREC at any time (Figure 60). Significantly low abundances of juvenile *S. trutta* occurred in the spring of 2017, when none found on BRDS1 or BRDS2 (Figure 60). The west channel (BRWC) consistently held the highest densities relative to other sites surveyed and both BRWC and BRDS2 had mean HUI >1.

Sub-adult *S. trutta* (FL < 100-199 mm) were at higher mean densities than predicted for all sites except BREC, where the mean HUI was <1 (Table 32). However, no autumn surveying was possible on BREC when densities of sub-adults were greater than spring at the other three sites. Autumn 2016 saw significantly higher observed densities on BRWC, BRDS1 and BRDS2 (Figure 60). The spring of 2017 held significantly lower observed densities of sub-adults in BRDS1.

Adult *S. trutta* (FL > 200 mm) were at mean densities below expected in BREC and BRWC and above expected in BRDS1 and BRDS2 (Table 32). The only occasion when observed densities significantly differed from expected was the lower than predicted density in the BREC in spring 2016

Table 32 Bourne Rivulet HABSCORE summary of mean observed densities, Habitat Quality Score (HQS), Habitat Utilisation Index (HUI) and the lower and upper confidence limits (CL) for HUI for three size classes of brown trout *Salmo trutta* (Fork length <99, 100-199 and > 200 mm). Significant differences highlighted in bold font are considered to occur when the upper HUI CL is <1 (habitat utilisation significantly lower than predicted) and when HUI CL is >1 (habitat utilisation significantly higher than predicted)

Size class (FL) mm	*Site*	*n*	*Mean obs. density (N/100m²)*	*Mean HQS (N/100m²)*	*HUI*	*HUI lower CL*	*HUI upper CL*	*Significance*
< 99	BRWC	5	3.25	2.02	1.61	0.23	11.28	NS
	BREC	3	0.00	7.70	**0.06**	0.01	**0.43**	**Lower**
	BRDS1	5	1.21	1.95	0.51	0.08	3.57	NS
	BRDS2	5	1.05	1.44	1.08	0.15	7.62	NS
100 - 199	BRWC	5	4.62	1.20	3.81	0.61	23.58	NS
	BREC	3	2.41	3.33	0.70	0.11	4.51	NS
	BRDS1	5	3.13	1.04	3.92	0.65	23.75	NS
	BRDS2	5	3.04	0.89	4.14	0.68	25.16	NS
> 200	BRWC	5	1.75	1.84	0.91	0.29	2.81	NS
	BREC	3	1.84	3.54	0.50	0.16	1.55	NS
	BRDS1	5	2.28	1.73	1.33	0.43	4.05	NS
	BRDS2	5	2.65	1.52	1.74	0.57	5.28	NS

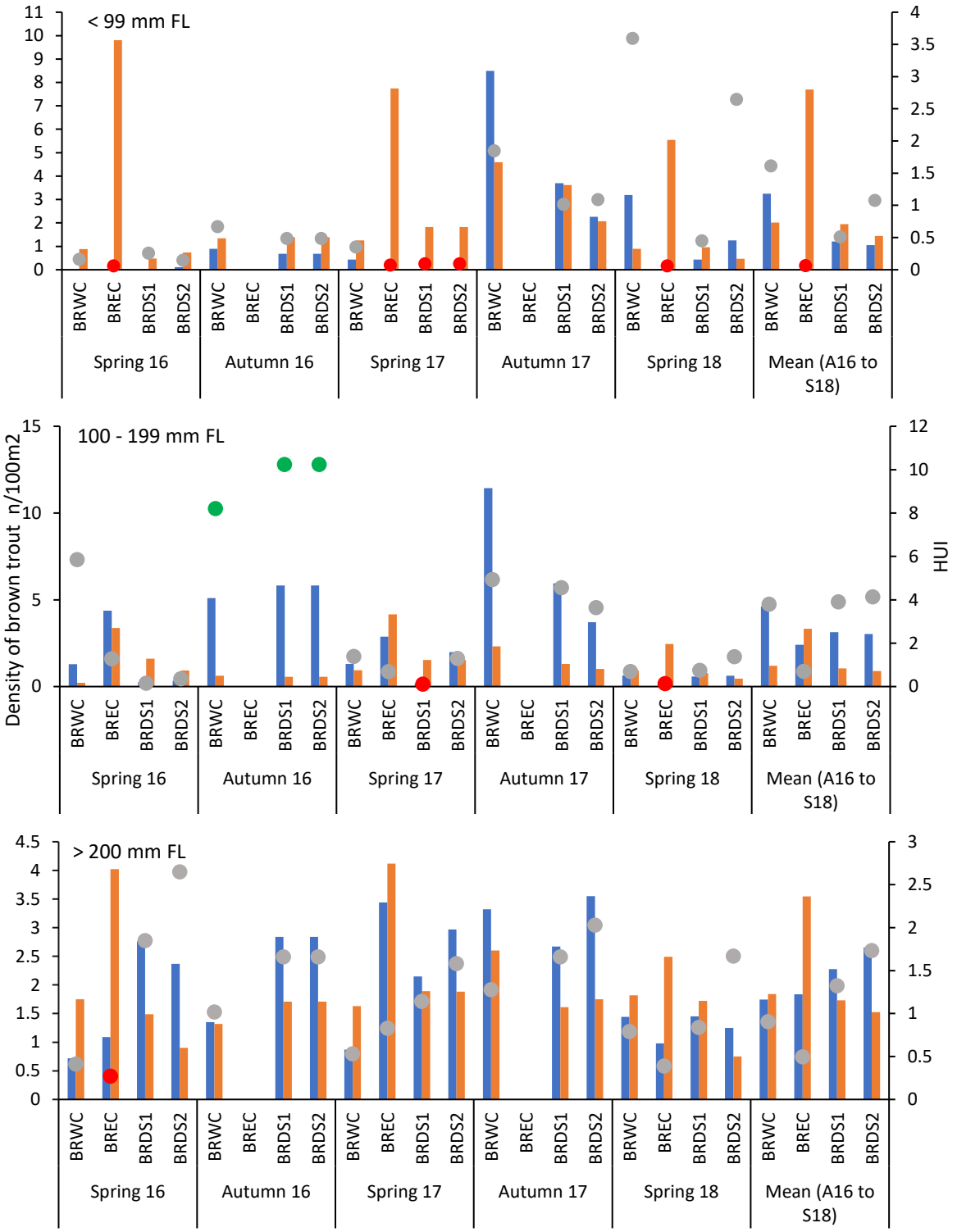

Figure 60 Observed density (blue bars) and predicted density/habitat quality score (HQS) (orange bars) of *S. trutta* (n/100 m2) of three different size classes (Fork Length (FL) <99, 100-199 and >200mm) caught by electric fishing in the Bourne Rivulet. Habitat utilisation index (HUI) is represented by dots and describes the relationship between observed density and predicted density. A HUI of one indicates parity between observed and predicted densities. A HUI below one indicates observed densities are below expected, and a red dot indicates it is significantly so. A HUI above one indicates observed densities are above predicted densities, and a green dot indicates significantly so. Note no data were available for BREC in October 2016 and 2017 due to the channel being blocked with emergent macrophytes. The mean data for BREC excludes autumn surveys, while the mean data for all other sites is the mean of surveys from autumn 2016 through to spring 2018. Note differences in density axis for each size class

4.4.4 The River Crane HABSCORE

Juvenile *S. trutta* (>99 mm fork length) were found at a lower than predicted mean density at the immediate downstream site CRDS1 and above predicted densities at all other Crane sites (Table 33). The site CRDS2 in spring 2018 was the only occurrence of juvenile *S. trutta* having an observed density significantly higher than predicted (Figure 61). There were two occurrences of juvenile *S. trutta* having observed densities significantly below predicted, both of which occurred in spring 2017 at both CRUS and CRDS1 (Figure 61).

Sub-adult (FL < 200 mm) *S. trutta* were found at mean densities exceeding expected densities at all sites, with CRDS2 holding significantly higher mean density than predicted (Table 33). The site CRDS3 was notable in having high observed densities of sub-adults, over double that found in the next most densely populated site CRUS. In both autumn surveys, both CRDS2 and CRDS3 held significantly more sub-adult *S. trutta* than predicted (Figure 61).

Adult *S. trutta* (FL > 200 mm) mean densities were below predicted at both CRUS and CRDS1 and above predicted at the two farthest downstream sites CRDS2 and CRDS3 (Table 33). Adult *S. trutta* observed densities were significantly lower than predicted in spring and autumn 2017 in both CRUS and CRDS1 (Figure 61). The only significantly higher observed density was in autumn 2016 in CRDS2 (Figure 61).

Table 33 River Crane HABSCORE summary of mean observed densities, Habitat Quality Score (HQS), Habitat Utilisation Index (HUI) and the lower and upper confidence limits (CL) for HUI for three size classes of brown trout *Salmo trutta* (Fork length <99, 100-199 and > 200 mm). Significant differences highlighted in bold font are considered to occur when the upper HUI CL is <1 (habitat utilisation significantly lower than predicted) and when HUI CL is >1 (habitat utilisation significantly higher than predicted)

Size class (FL) mm	Site	n	*Mean obs. density (N/100m²)*	*Mean HQS (N/100m²)*	*HUI*	*HUI lower CI*	*HUI upper CI*	*Significance*
< 99	CRUS	5	15.92	12.38	1.29	0.16	7.29	NS
	CRDS1	5	6.10	7.85	0.78	0.25	11.19	NS
	CRDS2	4	6.52	1.88	3.47	0.42	19.46	NS
	CRDS3	5	12.83	5.92	2.17	0.31	14.13	NS
100 - 199	CRUS	5	8.86	5.69	1.56	0.31	11.11	NS
	CRDS1	5	4.35	3.73	1.17	0.25	9.11	NS
	CRDS2	4	6.86	1.05	**6.52**	**1.03**	39.04	**Higher**
	CRDS3	5	18.41	3.25	5.66	0.97	34.33	NS
> 200	CRUS	5	0.93	1.56	0.60	0.26	2.72	NS
	CRDS1	5	0.74	1.16	0.63	0.28	2.61	NS
	CRDS2	4	3.15	1.37	2.31	0.68	6.69	NS
	CRDS3	5	2.50	2.31	1.08	0.35	3.32	NS

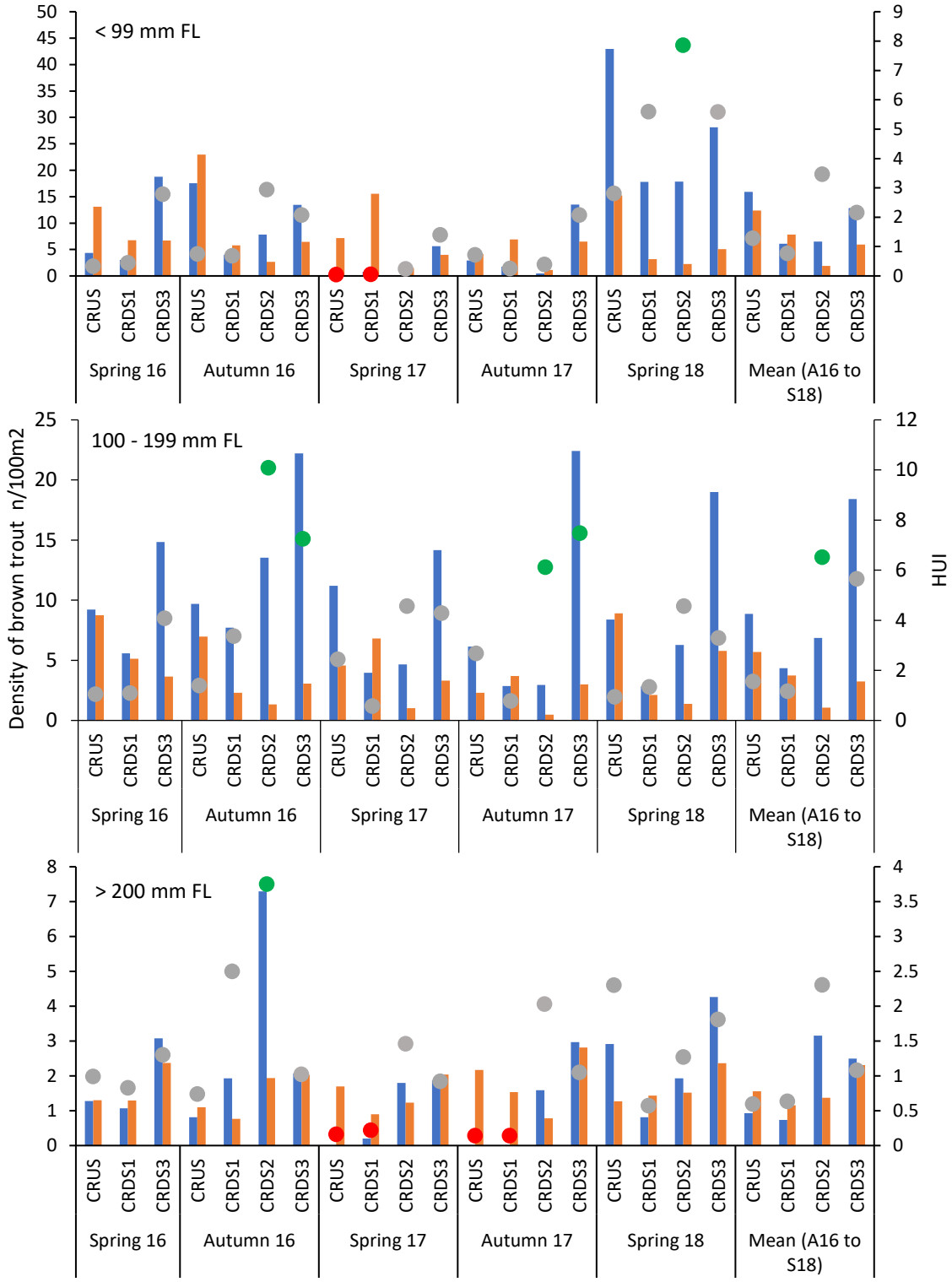

Figure 61 Observed density (blue bars) and predicted density/habitat quality score (HQS) (orange bars) of *S. trutta* (*n*/100 m^2) of three different size classes (Fork Length (FL) <99, 100-199 and >200mm) caught by electric fishing in the River Crane. Habitat utilisation index (HUI) is represented by dots and describes the relationship between observed density and predicted density. A HUI of one indicates parity between observed and predicted densities. A HUI below one indicates observed densities are below expected, and a red dot indicates it is significantly so. A HUI above one indicates observed densities are above predicted densities, and a green dot indicates significantly so. The mean data is the average of seasons autumn 2016 through to spring 2018

4.4.5 The River Frome HABSCORE

Juvenile *S. trutta* (>99 mm fork length) occurred at mean densities above predicted at all sites except FRDS3 (Table 34). Of the three remaining sites, FRUS had the highest mean habitat utilisation by juvenile *S. trutta*, and in autumn 2017 was the only site and date to have a significantly higher than expected density (Figure 62). The same site was the only site to have a significantly lower than expected density, which occurred in spring 2017 (Figure 62).

Sub-adult (FL < 200 mm) *S. trutta* occurred at densities above predicted at all sites and dates (Table 34). The upstream FRUS site consistently held significantly higher observed densities than predicted at all survey occasions (Figure 62), which led to the mean observed density being significantly higher (Table 34). Observed densities at all sites were highest in autumn 2017, and here the only other significantly higher abundance was found, which occurred at CRDS2.

Adult *S. trutta* (FL > 200 mm) had observed mean densities above predicted in FRUS only, with all other sites having lower observed densities than predicted (Table 34). Spring 2017 had significantly lower densities than predicted in both FRDS1 and FRDS2, and in autumn 2016, FRDS1 had a significantly lower density than predicted (Figure 62).

Table 34 River Frome HABSCORE summary of mean observed densities, Habitat Quality Score (HQS), Habitat Utilisation Index (HUI) and the lower and upper confidence limits (CL) for HUI for three size classes of brown trout *Salmo trutta* (Fork length <99, 100-199 and > 200 mm). Significant differences highlighted in bold font are considered to occur when the upper HUI CL is <1 (habitat utilisation significantly lower than predicted) and when HUI CL is >1 (habitat utilisation significantly higher than predicted)

Size class (FL) mm	*Site*	*n*	*Mean obs. density (N/100sq.m)*	*Mean HQS (N/100sq.m)*	*HUI*	*HUI lower CI*	*HUI upper CI*	*Significance*
< 99	FRUS	3	4.00	0.93	4.89	0.72	33.19	NS
	FRDS1	3	2.70	1.42	1.79	0.27	12.01	NS
	FRDS2	2	2.91	1.14	2.57	0.38	17.34	NS
	FRDS3	3	0.41	1.09	0.39	0.06	2.61	NS
100 - 199	FRUS	3	4.22	0.35	**11.19**	**1.84**	68.04	**Higher**
	FRDS1	3	2.61	1.37	1.84	0.31	11.15	NS
	FRDS2	2	4.55	0.74	5.88	0.98	35.52	NS
	FRDS3	3	2.24	0.76	2.57	0.42	15.72	NS
> 200	FRUS	3	0.83	0.74	1.30	0.41	4.08	NS
	FRDS1	3	0.75	1.89	0.44	0.14	1.32	NS
	FRDS2	2	1.29	1.55	0.84	0.28	2.55	NS
	FRDS3	3	1.28	1.43	0.91	0.30	2.80	NS

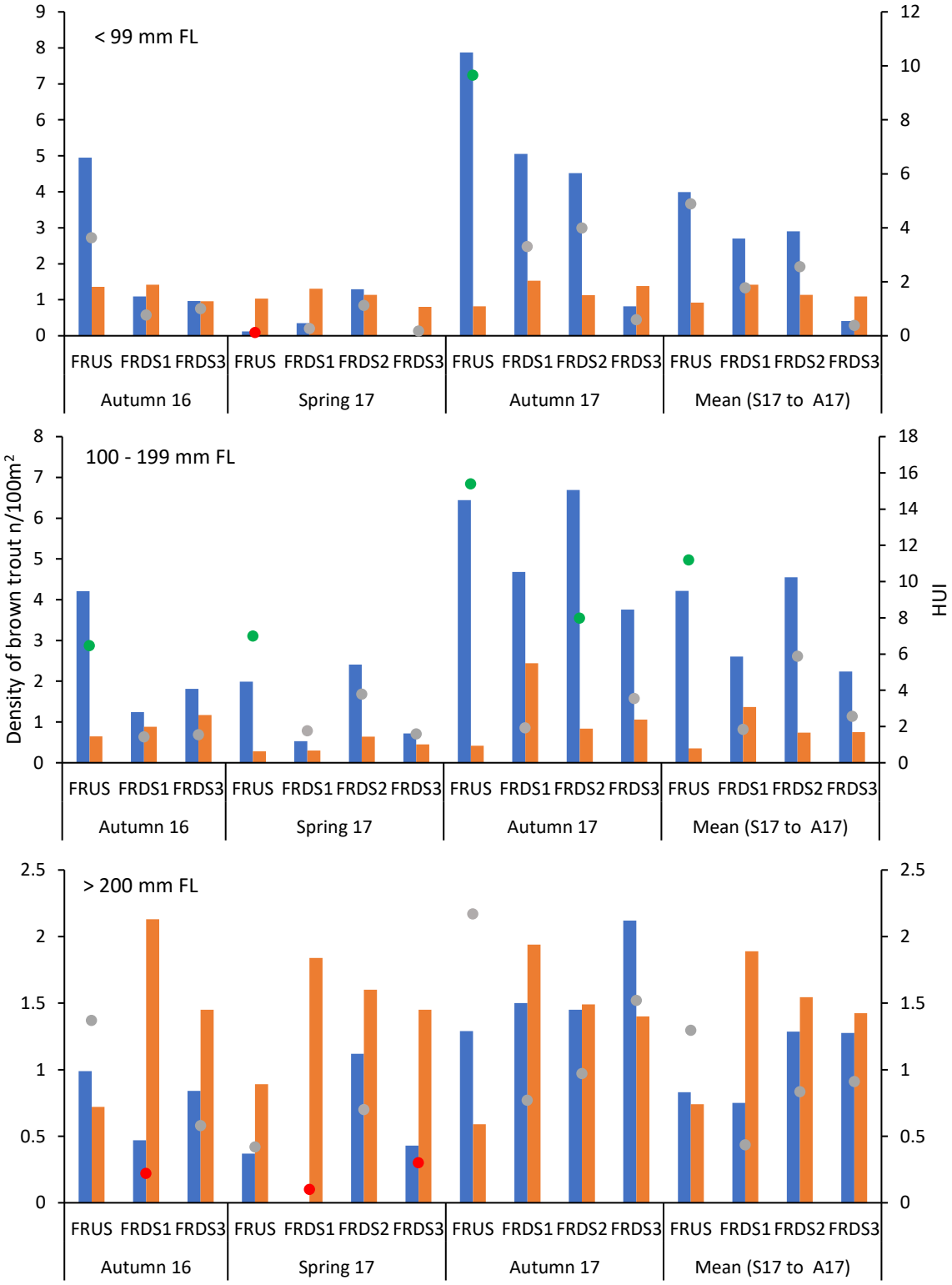

Figure 62 Observed density (blue bars) and predicted density/habitat quality score (HQS) (orange bars) of *S. trutta* (*n*/100 m^2) of three different size classes (Fork Length (FL) <99, 100-199 and >200mm) caught by electric fishing in the River Frome. Habitat utilisation index (HUI) is represented by dots and describes the relationship between observed density and predicted density. A HUI of one indicates parity between observed and predicted densities. A HUI below one indicates observed densities are below expected, and a red dot indicates it is significantly so. A HUI above one indicates observed densities are above predicted densities, and a green dot indicates significantly so. Note no data exists for FRDS2 in October 2016 when access to site was unavailable. The mean data is generated from both surveys in 2017

4.4.6 Spot recognition

Spot-recognition was used to identify recaptured *S. trutta* to assess site fidelity, growth rates and to generate population estimates to assess the efficacy of spot recognition for population estimation. Overall, 12.4% of the 1098 photos taken were matched (Table 35). Examples of an individual photo matched large adult *S. trutta* from BRDS2 is presented in Figure 63 and a sub-adult from CRDS3 is presented in Figure 64. Both examples highlight the technique used by the user to verify matches made by the spot matching algorithm. There was considerable variation in the number of matched individuals between sites, ranging from 0% in FRDS1 and FRDS2, to the highest percentage of matching in FRUS (35.1%) and CRDS3 (26.6%). The number of photos analysed is lower than the number of *S. trutta* caught, as 0+ *S. trutta* had to be excluded as their spot patterns had not developed and not all images were useable, as discussed in 4.3.3.

Table 35 Recapture rates of *S. trutta* sampled on sites on The Bourne Rivulet (BRWC, BREC, BRDS1 and BRDS2), The River Crane (CRUS, CRDS1, CRDS2 and CRDS3) and The River Frome (FRUS, FRDS1, FRDS2 and FRDS3) between May 2016 and June 2018

	n photos	*Individuals matched*	*% individuals matched*	
BRWC	83	9	10.843	
BREC	13	2	15.385	
BRDS1	116	9	7.759	
BRDS2	138	17	12.319	
			Bourne Rivulet mean (%)	*11.576*
CRUS	157	17	10.828	
CRDS1	110	11	10	
CRDS2	82	14	17.073	
CRDS3	263	70	26.616	
			River Crane mean (%)	*16.129*
FRUS	17	6	35.294	
FRDS1	48	0	0	
FRDS2	32	0	0	
FRDS3	39	1	2.564	
			River Frome mean (%)	*9.465*
			Total mean matched (%)	*12.390*

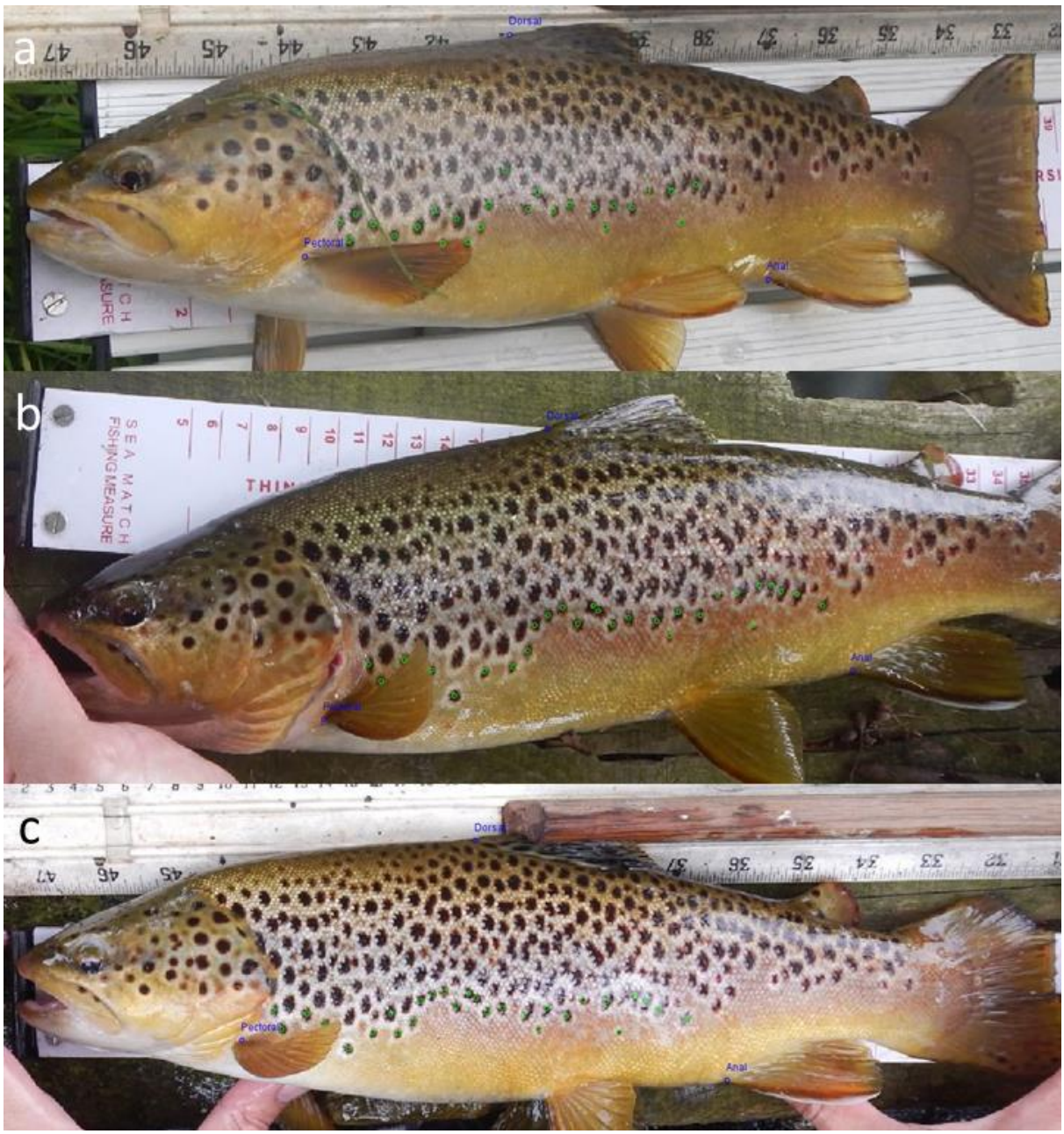

Figure 63 An individual *S. trutta* from BRDS2 first captured in October 2016 (a) and recaptured in June 2017 (b) and October 2017 (c). Green spots mark out the melanophores selected for spot-recognition using i3s software. Images rated by i3s from database as best match based on spot locations were confirmed by the user. In the case of this individual, two distinctive melanophores on the upper mandible aided confirmation.

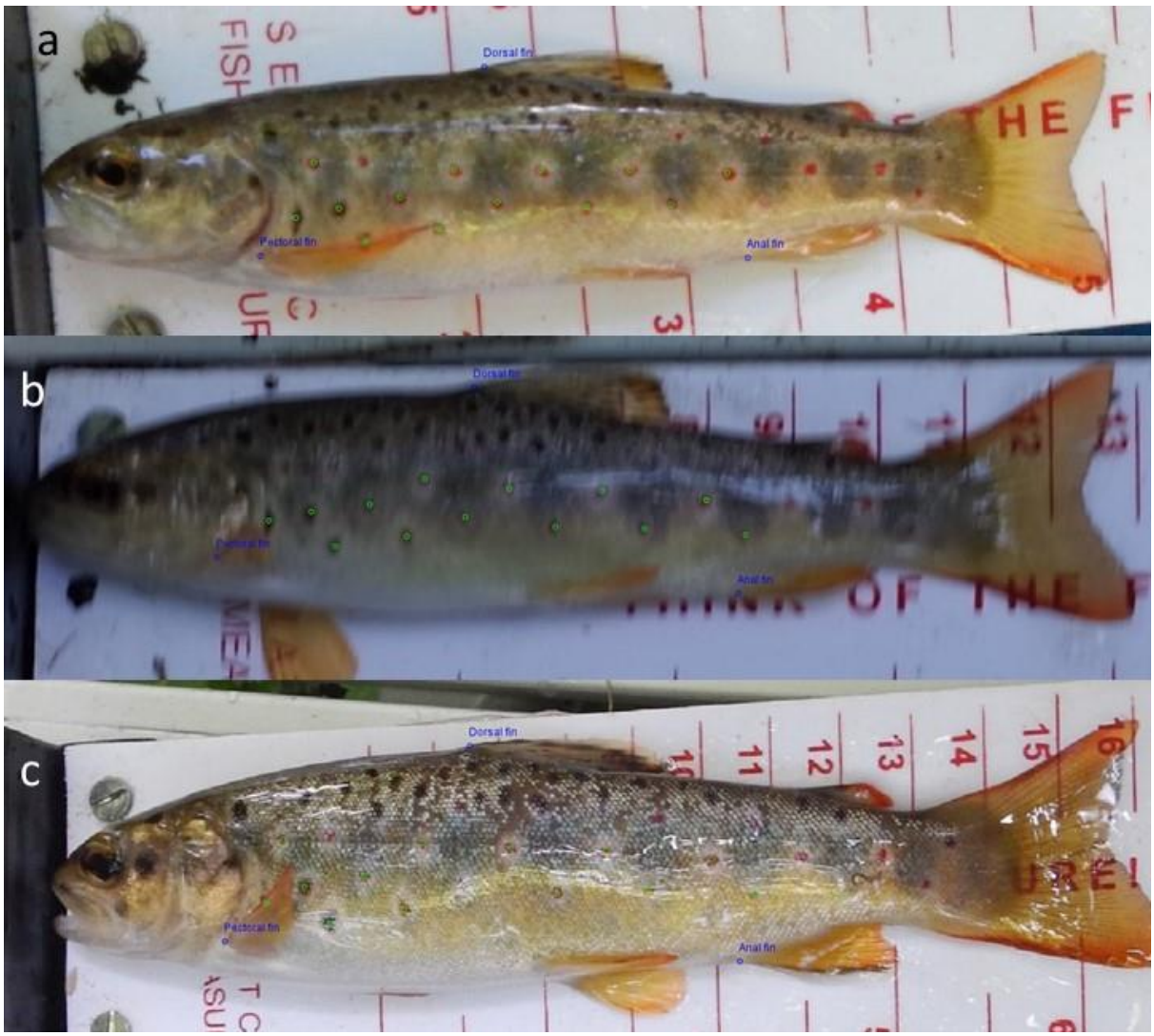

Figure 64 An individual *S. trutta* from CRDS3 captured first on June 2016 (a), recaptured in October 2016 (b) and again in June 2017 (c). Green spots mark out the melanophores selected for spot-recognition using i3s software, which are difficult to make out in image c. Images rated by i3s from database as best match based on spot locations were confirmed by the user. This individual was confirmed by the user by a rectangular pattern of large dark melanophores from anterior of the eye to the operculum and above. These images show that spot matching is still achievable even with relatively poor-quality images such as image b

4.4.6.1 Site fidelity

Site fidelity of *S. trutta* > 99 mm were investigated in terms of the proportion of fish that were recaptured in different sites compared to those that were recaptured at the same site on two or more occasions (Table 36)**.** The percentage of *S. trutta* that were not found to move between sites was broadly similar for the Bourne (94.59%) and the Crane (95.69%). The number of recaptured *S. trutta* on the Frome was very low (*n*=7), and of these *n*=6 occurred in FRUS. Due to the low *n* of recaptured fish, the 100% site fidelity found on the Frome should be treated with caution.

Table 36 Site fidelity of *S. trutta* on the River Bourne, the River Crane and the River Frome

River	*no. individuals matched*	*no. moved*	*% moved*	*% site fidelity*
Bourne Rivulet	37	2	5.41	94.59
River Crane	116	5	4.31	95.69
River Frome	7	0	0	100

4.4.6.2 Growth

The mean standard growth of resident *S. trutta* populations is presented in Table 37. On the Bourne *S. trutta* growth appeared lowest at BREC where G_w was 0.06. However, this was value was for a single individual recaptured between S17 and S18. At the same sampling season, the single individual recaptured on BRWC had a lower G_w of 0.02. Mean G_w was higher at BRDS1 than BRWC and BRDS2. On the Crane, mean G_w was highest at CRDS2 and lowest at CRUS. G_w was higher downstream at CRDS1 than the immediate upstream site, while growth at CRDS3 was intermediate. On the Frome, the four individuals recaptured between S17 and A17 showed high growth as did the one individual recapture in the same timeframe at CRDS3. Due to low and inconsistent recapture rates between sites at each season-to-season time period, there were no time spans when $n > 3$ at all sites on any river, where the differences in growth between upstream and downstream sites could be statistically examined. The use of mean G_w for all time spans at each site encompassed a range of seasons where growth rates will differ, so should be interpreted with caution.

Table 37 Mean standard growth (G_w) (±SD) of resident < 99mm *S. trutta* between years (2016, 2017 and 2018) and season (S; spring A; Autumn) at each site. The total mean G_w of all between year and season surveys is summarised as mean G_w. Caution should be exercised in comparing the mean G_w between sites which have varying sample *n* between seasons and years, as somatic growth is greater during summer than winter.

Dates	*n*	*BRWC*	*sd*	*n*	*BREC*	*sd*	*n*	*BRDS1*	*sd*	*n*	*BRDS2*	*sd*
S17-A17	6	0.57	1.01	0	-	-	2	0.11	0.60	12	0.45	0.29
A16-S17	0	-	-	0	-	-	0	-	-	2	0.05	0.04
S17-S18	1	0.02	-	1	0.06	-	1	0.23	-	3	0.13	0.33
A17-S18	2	0.14	0.08	0	-	-	2	0.44	0.01	1	0.20	-
Mean G_w	9	0.24	0.29	1	0.06	-	5	0.26	0.17	18	0.21	0.17

Dates	*n*	*CRUS*	*sd*	*n*	*CRDS1*	*sd*	*n*	*CRDS2*	*sd*	*n*	*CRDS3*	*sd*
S16-A16	7	0.16	0	5	-0.22	0.4	0	-	-	19	0.03	0.1
S16-A17	1	0.02	-	0	-	-	0	-	-	19	0.13	0.1
S16 -S18	0	-	-	0	-	-	0	-	-	4	0.09	0.1
A16-A17	0	-	-	0	-	-	6	0.10	0	19	0.19	0.1
A17-S18	0	-	-	1	0.43	-	4	0.27	0.1	19	0.15	0.1
Mean G_w	8	0.09	0.1	6	0.11	0.4	10	0.18	0.1	80	0.12	0.1

Dates	*n*	*FRUS*	*sd*	*n*	*FRDS1*	*sd*	*n*	*FRDS2*	*sd*	*n*	*FRDS3*	*sd*
S17 - A17	4	0.79	0.1	0	-	-	0	-	-	1	0.88	-
Mean G_w	4	0.79	0.1	0	-	-	0	-	-	1	0.88	-

4.4.6.3 Population estimation using Jolly-Seber

S. trutta density estimates were generated using the Jolly-Seber (J-S) methodology for open populations to compare values to those produced using multiple pass depletion methods and Carle and Strubb estimates (C&S) (Table 38). The J-S methodology generally produced density estimates that were higher than C&S estimates, leading to a composite J-S – C&S difference of 12.87 *n*/100m^2 and a mean difference of 15%. BREC and all Frome sites except FRUS have been omitted as the low number of surveys and captures did not allow J-S to produce estimate values.

Table 38 Jolly-Seber estimates (±SE) of *S. trutta* abundance at survey occasion *i*. J-S estimates are not computable for the first and last surveys for any site. Carle and Strubb (C&S) abundance estimates (±SE) are presented in addition the difference between J-S and C&S abundance estimates for comparison between the two methodologies.

Site	*i*	*J-S*	*se*	*C&S*	*se*	*J-S - C&S*
BRWC	*i*2	7.45	n/a	2.17	1.02	5.28
	*i*3	48.52	69.20	14.76	0.25	33.76
BRDS1	*i*2	3.31	n/a	3.64	0.35	-0.33
	*i*3	28.79	28.97	7.26	0.49	21.53
BRDS2	*i*2	4.79	n/a	4.95	0.23	-0.16
	*i*3	31.82	25.16	7.26	0.20	24.56
CRUS	*i*2	16.09	7.50	10.51	3.31	5.58
	*i*3	33.57	29.70	11.19	0.55	22.38
	*i*4	1.76	0.00	6.15	0.87	-4.39
CRDS1	*i*2	7.07	n/a	9.65	1.11	-2.58
	*i*3	3.19	n/a	4.17	0.33	-0.98
	*i*4	2.43	n/a	2.87	1.47	-0.44
CRDS2	*i*2	78.39	93.15	6.47	0.42	71.92
	*i*3	5.65	1.95	4.54	0.59	1.11
CRDS3	*i*2	43.94	7.88	24.29	0.62	19.65
	*i*3	38.18	7.78	16.04	0.70	22.14
	*i*4	25.21	1.78	25.38	0.62	-0.17
FRUS	*i*2	2.11	n/a	2.36	0.47	-0.25
Mean diff between J-S and C&S estimated ab.						12.87
Mean % diff between J-S and C&S estimated ab.						15.23

4.5 Discussion

This study was conducted to ascertain if discharges from watercress farms were altering fish population structures in light of the changes in macroinvertebrate assemblage and biotic indices presented in chapter three. Overall, sites downstream of discharges were associated with higher densities of non-salmonid species and lower densities of salmonids. In particular, young-of-year *S. trutta* densities were lower downstream of salad wash effluent discharges. The discharges appeared to have a positive effect on *S. trutta* condition, potentially resulting from increased food availability associated with the higher macroinvertebrate abundances at these sites.

4.5.1 Species diversity

It has long been established that in riverine systems fish diversity and richness increases, mainly by species addition, in a gradient from headwater to mouth (Hynes and Hynes 1970; Horwitz 1978;

Vannote et al. 1980; Casatti 2005). Taken on the river scale, the species richness (SR) on each river reflects the relative longitudinal positions of the sites, whereby the Bourne and Crane sites, being headwaters less than 10 km for the source had a more limited range of species than the Frome sites which were c.33km from the source. The Frome sites contained a greater range of cyprinids, while the Bourne and Crane sites are dominated by salmonids, which is consistent with their relative longitudinal locations (Sear et al. 1999). There existed no consistent trend in SR between sites upstream of watercress farm discharges and sites immediately downstream of them. Upstream SR was identical on the Frome, lower on the Bourne, and higher on the Crane. In contrast, Shannon index was consistently lower at upstream sites than the respective immediate downstream sites. While it is expected that species richness and diversity may increase downstream, the upstream and immediate downstream sites were almost contiguous, separated only by between 2-10 m of river. Therefore, the increase in species diversity in downstream sites is unlikely to have resulted from longitudinal effects. Rather, it was a result of the numerical dominance of *S. trutta* in the fish communities in upstream sites relative to the immediate downstream sites.

4.5.2 *Salmo trutta* densities

S. trutta are the most dominant salmonid in chalk stream headwaters (Elliott 1989; Mann et al. 1989), and this species was ubiquitous across all sites under study. *S. trutta* are a key species of interest in the present research as they spawn in chalk stream headwaters where watercress farming occurs and are of high ecological and commercial value (Lobón-Cerviá and Sanz 2017).

The following three sections compare the observed densities of the three size classes; adult (>200 mm), sub-adult (100-199 mm) and young-of-year (< 99mm) with expected densities predicted by the HABSCORE model. The HABSCORE model bases density estimates on a range of habitat variables. There is a consensus in the literature that the environmental variables and features of greatest importance to the density and distribution of salmonids are depth, current, substrate and cover (Milner 1982; Heggenes 1988; Armstrong et al. 2003; Riley et al. 2009). The HABSCORE model includes these habitat variables when producing density estimates of the three size classes of *S. trutta* (Milner et al. 1998) allowing the effect of organic pollution can be disentangled from habitat when comparing sites upstream and downstream of discharges. However, the discussion will nevertheless include reference to habitat variables, particularly those predicted by CCA to influence fish densities.

4.5.2.1 Adult *S. trutta* densities

On all three rivers, the mean densities of adult *S. trutta* were generally below predicted in the reaches immediately downstream of discharges on all rivers, though none were significantly lower

than estimates predicted by HABSCORE. On the Bourne, observed mean densities of adult *S. trutta* were marginally higher in the east channel than the west channel, with both sites having mean densities below expected. As adult *S. trutta* are highly territorial (Crisp 1993; Ayllón et al. 2010), the densities in the east channel may be depressed by the annual infilling of the channel with emergent macrophytes which would preclude the holding of territory within the channel over long temporal scales, so BRDS1 may be considered the downstream site. Here and further downstream in BRDS2 densities of adult *S. trutta* were above predicted and higher than both the east and west channels. Adult *S. trutta* densities tend to be higher around sources of cover which provide shelter from predation (Milner 1982). Canonical correspondence analysis showed large woody debris (LWD) to be a strong predictor of adult *S. trutta* density on the Bourne. This agrees with the findings of Langford et al. (2012) who found adult *S. trutta* at higher densities in LWD patches than other microhabitats. Submerged wood increases habitat heterogeneity and is often considered to directly increase localised abundances of fish by the 'condominium' effect (Dolloff and Warren 2003), whereby the fish utilise the three-dimensional matrix as cover and territory (Heggenes 1988; Armstrong et al. 2003; Langford et al. 2012). The site BRDS2 has undergone 'resnagging' whereby LWD is reintroduced into previously desnagged river channels to improve riverine habitat for fish (Roni et al. 2002; Sievers et al. 2017; Thompson et al. 2018). The associations between LWD, BRDS2 and adult *S. trutta* densities may suggest that resnagging efforts have been successful.

On the Crane, mean densities of adult *S. trutta* were below expected upstream of the discharge at CRUS and directly below it at CRDS1, while they were above expected densities further downstream at CRDS2 and CRDS3. The lower mean values at CRUS and CRDS1 largely resulted from an absence of adult *S. trutta* in both seasons in 2017. On the Frome, densities were above predicted upstream at FRUS and below for all downstream sites. Reaches under riparian canopy have been shown to be the most extensively utilised by adult *S. trutta* in Norwegian rivers (Heggenes 1988) and in Wyoming rivers (Binns and Eiserman 1979). Tree canopy may provide both shelter from predation and it can mitigate against unfavourable diel shifts in water temperature during the summer (Broadmeadow et al. 2011). The lack of tree canopy over FRDS1 may have played a role in the relatively low densities of adult *S. trutta* compared to FRUS. However, as HABSCORE takes into account riparian vegetation, the relative lack of riparian vegetation at these sites will be accounted for in producing the predicted values.

In contrast to the Bourne, which had large densities of adult *S. trutta*, on the Frome and Crane adult *S. trutta* were at much lower densities. It is likely that spawning age *S. trutta* do not remain in the Crane and Frome headwaters where the surveying took place, but rather migrate from lower reaches to spawn and/or are anadromous sea trout *Salmo trutta* (Mainstone et al. 1997). In

contrast, a culvert downstream of all survey sites effectively curtails seaward migration for the Bourne population.

In summary, adult *S. trutta* appeared to unaffected by discharges on the Bourne due to higher than predicted densities at BRDS1, while low mean densities on the Crane and Frome may be a function of migration.

4.5.2.2 Sub-adult *S. trutta* densities

Sub-adult *S. trutta* were at mean densities above predicted for every site on all rivers with the exception of BREC, where mean densities were marginally below those predicted by HABSCORE. However, BREC was infilled with macrophytes in the autumn when densities of sub-adult *S. trutta* at all other Bourne sites were highest. It is therefore possible that had the east channel been navigable for *S. trutta* and electric fishing surveys in autumn, mean densities of the size class may have been lifted above predicted densities. As mean densities were above predicted at BRDS1, this would suggest that discharges were not impacting on sub-adult *S. trutta* populations on the Bourne.

Observed densities of sub-adult *S. trutta* were higher in CRUS than in CRDS1, which may be linked to higher coverage of overhanging vegetation, which was shown by CCA to be a predictor of sub-adult densities. CRDS2 was the only site on the Crane where mean densities were significantly higher than predicted estimates. This was somewhat surprising as the habitat was not what is typically considered favourable for *S. trutta*, with highly silted substrates, low water velocity and low macrophyte coverage as indicated by the low predicted densities. However, LWD and tree canopy coverage were high, suggesting that these were important habitat variables that may have compensated for suboptimal substrate, velocity and macrophyte abundance. Observed abundances were highest of all at CRDS3, which was unsurprising as the site was the least modified and the most heterogenous and natural channel, providing the greatest capacity to support young salmonids (Crisp 1993; Langford et al. 2012)

On the Frome, mean sub-adult *S. trutta* densities were significantly higher than predicted for the upstream site FRUS. The high densities of the size class in FRUS may be linked to the channel profile of the site. CCA linked densities of the size class to shallow depth, and it is established that younger *S. trutta* utilise shallow areas (Armstrong et al. 2003; Riley et al. 2009).

In summary, the densities of sub-adult *S. trutta* were above predicted densities at all sites on all rivers. This would suggest that sub-adult *S. trutta* densities were not adversely affected by discharges from watercress farms.

4.5.2.3 Young-of-year *S. trutta* densities

The population dynamics of riverine *S. trutta* are known to be influenced by year-to-year variations in discharge levels (Solomon and Paterson 1980; Mann et al. 1989; Jensen and Johnsen 1999; Cattanéo et al. 2002; Daufresne and Renault 2006). The year 2015 preceding the survey, the first year of survey, 2016, and the final survey in 2018 were all very close to the average hydrograph for the preceding decade (see 2.7). The year 2017 had below average winter and spring discharge on all rivers, which led to river levels being unusually low during the spring surveys in that year.

The low discharge levels in winter 2016/17 were accompanied by abundances of young-of-year *S. trutta* well below predicted on both the Bourne and Crane for all sites, significantly so for all sites except BRWC. The Frome was not so adversely affected, perhaps due to its lower longitudinal position from the source, and the fact that flow to the north carrier is managed by an upstream gate which may have negated low flows. Extremes in water flow can negatively impact on salmonid recruitment and survival (Jonsson and Jonsson 2009). High flows occurring when recruits are emerging from gravel nests can cause displacement of emerging salmonid fry (Nehring and Anderson 1993; Nuhfer et al. 1994; Latterell et al. 1998; Jensen and Johnsen 1999; Cattanéo et al. 2002; Lobón-Cerviá and Rincón 2004). Low flows or drought conditions have been shown in some instances to decrease abundances of young-of-year salmonids, though the interaction between river levels and densities of young-of-year *S. trutta* is complex (Elliott 1987; Hendry et al. 2003; Elliott et al. 2006). In addition, as low winter flows may limit the numbers of salmonids returning to spawn (Jonsson and Jonsson 2009), the low abundances seen in the spring of 2017 may reflect low numbers of fish spawning the preceding winter. Marsh et al. (2019) noted similar low densities of young-of-year in autumn 2016 on the Frome, Dorset, which they attributed to high overwinter temperatures in 2015/2016 leading to recruitment failure.

Instream macrophyte coverage was dominated by water crowfoot (*Ranunculus spp.*), a submerged macrophyte common in chalk streams (Cotton et al. 2006). This macrophyte has been shown to support higher densities of young-of-year *S. trutta* through the support of high abundance of suitable macroinvertebrate prey (Wright et al. 2003; Riley et al. 2009) and due to the cover provided (Armstrong et al. 2003; Marsh et al. 2019). Canonical Correspondence Analysis showed young-of-year *S. trutta* densities on all three rivers to be strongly associated with high coverage of instream macrophytes. However, it was not possible to directly relate *Ranunculus* coverage to discharges as its growth is highly dependent on strong light intensity and they are therefore low or absent below riparian shading (Flynn et al. 2002). Moreover, its coverage is managed by weed cutting by riparian landowners to increase flows, and by fishery owners to improve fly fishing amenity (Flynn et al. 2002).

The densities of young-of-year on the Bourne and Crane when averaged across all surveys showed that densities were above predicted by HABSCORE upstream of discharges and below predicted in the sites immediately below them. On the Frome, where no salad washing occurred, there were no observable differences between up and downstream, indicating a possible impact on recruitment from salad wash effluent rather than watercress farming *per se*.

On the Crane, observed densities returned to above predicted at CRDS2 which was situated 460m downstream of the discharge and improved further by CRDS3 at 2.4km. Young-of-year densities did not return to above expected densities either on BRDS1 or BRDS2, which were situated 380m and 1km distance from the effluent discharge respectively, suggesting that any perturbation causing low young-of-year densities persisted for longer downstream on the Bourne sites than the Crane. The cause of the apparent increased longitudinal perturbation on the Bourne may arise for a number of reasons. The watercress farm on the Bourne covers a greater area and discharges considerably more effluent water than the facility on the Crane. The salad washing operation is larger in scale, both in the quantity of material washed and the frequency of operation which would output greater quantities of PEITC.

HABSCORE indicated that the physical characteristics of BREC should enable it to support the highest abundances of young-of-year trout, however there were no young-of-year *S. trutta* found in BREC at any time. This absence is unlikely to be a direct result of discharges from the watercress farm. A prior survey undertaken by the Environment Agency (EA) (Longley 2006) in 2006 found densities of young-of-year *S. trutta* in BREC to be 6.86 *n*/100m^2, significantly higher than the HQS for the site, and at higher densities than either BRWC, BRDS1 and BRDS2. However, as these data were obtained during a single survey and densities of young-of-year from year-to-year can be extremely variable (Mann and Blackburn 1991; Klemetsen et al. 2003) the results of Longley (2006) must be treated with caution. In contrast to 2016-2018 when the present surveys were conducted, the BREC channel at the time was kept clear of emergent macrophytes, allowing *S. trutta* access to the site to spawn. On all surveying occasions in the present study, by autumn the channel was completely infilled with emergent macrophytes, which were only cut back in the following spring. With the channel impassable during the *S. trutta* spawning season, it would have precluded any redd formation in BREC. As *S. trutta* juveniles remain close to their nest following emergence (Crisp 1995; Klemetsen et al. 2003), the logical explanation for the absence of young-of-year *S. trutta* in BREC would be the lack of spawning opportunity at the site. No such obstacles were present for spawning in the BRDS1, however, and mean densities were below predicted at this site immediately downstream of the confluence of BRWC and BREC which may indicate reduced spawning success, or that mortality rates of embryos or fry were high (Crisp 1995; Klemetsen et al. 2003). As the sites below salad wash effluent on the Bourne and Crane had below expected young-of-year densities,

but sub-adult and adults were not negatively affected, this suggests that salad wash effluent may be having a negative impact on spawning and/or early life stages.

4.5.3 *Cottus gobio* densities

C. gobio are listed on the Annex II of the EU Habitats Directive 92/43/EEC as considered threatened throughout Europe (JNCC 2017). However, *C. gobio* are particularly abundant in hard-water chalk streams in the UK (Mills and Mann 1983) and although widespread throughout the UK, their population trend is presently unknown (JNCC 2017). *C. gobio* densities at all sites were above the Natura 2000 target of 0.5 individuals per m^2 (Cowx and Harvey 2003), which would suggest that all sites held relatively good densities of the species.

On all the rivers surveyed, mean densities of *C. gobio* were higher at the sites immediately downstream of the watercress farm effluents than the upstream sites, which may suggest that the habitat for the species was improved by the discharges. *C. gobio* are crepuscular, spending most of their time hidden in crevices and beneath stones to avoid predation (Mills and Mann 1983). Laboratory choice experiments have shown them to seek out cobbles and boulders over other substrate types, including gravel and sand, as these would limit their ability to conceal themselves (Davey et al. 2005). Mark-recapture experiments in a Flemish river have shown the majority of individuals to remain at or within 10m of their initial capture site (Knaepkens et al. 2005). As *C. gobio* require hard surfaces such as cobbles and boulders on which to spawn (Knaepkens et al. 2004) and their movement is limited, they are likely to be found at higher densities where there are suitable spawning substrates.

The present study agreed with the findings of Davey et al. (2005) as CCA showed associations with *C. gobio* density and coarse substrate. However, *C. gobio* densities were greatest in downstream sites where there were greatest areas of silt. In the field, Davey et al. (2005) found that silt was shown to hold a slightly greater abundance of the fish than gravel which would provide a poor substrate for the fish to conceal themselves. The higher densities in the more silted downstream reaches may result from the limitations to capture efficiency of *C. gobio* (Mann 1971). Sampling efficiency is poorer for small benthic fish than for nectonic species (Polačik et al. 2008), and this is epecially true for those that lack swim bladders such as and gobiids, which may remain hidden beneith stones and in intestitial spaces in course substrates following immobilsation by electric fishing (Jude and DeBoe 1996; Cowx and Harvey 2003; Polačik et al. 2008). As silted substrates would provide both concealment and relatively easy capture by electric fishing methods, the higher densities at the more heavily silted downstream sites could be a function of easier capture, greater densities, or a combination of the two.

In chapter three, the macroinvertebrate assemblages at each site were surveyed to see if discharges were having an impact on potential prey species for chalk stream fish. *C. gobio* diet consists almost exclusively of benthic organisms, the diet composition of which shows considerable annual variation. Gammaridae, Asellidae and Molluscs form the largest part of the diet in winter months, while nymphs and larvae of aquatic insects dominate in the summer months, particularly Chironomidae (Mills and Mann 1983). A study which looked at the gut contents relative to environmental abundances of benthic taxa by Welton et al. (1983), revealed a significantly higher proportion of Asellidae than found in the environment. As there was an increase in the abundance of the preferred *C. gobio* prey Asellidae and Chironomidae in the downstream sites in all the rivers under study, the increased *C. gobio* densities may be a result of preferential foraging opportunities at these sites.

4.5.4 *Anguilla anguilla* densities

The European eel *Anguilla anguilla* is commonly encountered in chalk streams, but globally is placed on the IUCN red list as critically endangered (Jacoby et al. 2015). Despite their global decline since the 1980s, *A. anguilla* are ubiquitous in freshwater systems in the UK, and are undemanding in terms of preferred habitat characteristics (Laffaille et al. 2003). *A. anguilla* are well known for making long catadromous migrations. However, in freshwater systems they exhibit territoriality and have a very limited home range to which they exhibit homing behaviour (Feunteun et al. 2003). For example, 95% of *A. anguilla* recaptured after tagging were found four years later to be less than 100m from their original capture site (Guillouët et al. 2000). In the present study, *A. anguilla* were found in considerably lower densities than both *C. gobio* and *S. trutta*. Like the sedentary *C. gobio* and territorial *S. trutta*, their limited movement in rivers means that despite the low densities found, the relative distribution between upstream and downstream sites may reveal habitat preference to a greater degree than more 'nomadic' fish species. *A. anguilla* were present at all sites with the exception of BRDS1 and BRWC where they were absent on all sampling occasions. They were found at higher densities in sites immediately downstream of discharges than the respective upstream sites.

Previous studies have found *A. anguilla* densities to be higher in locations which provide optimal cover in the form of crevices which they can conceal themselves (Fischer and Eckmann 1997; Degerman et al. 2019). Degerman et al. (2019) studied *A. anguilla* habitat use in the south west of Sweden and showed that *A. anguilla* favoured large stones and boulders the most, which was speculated to be due to the shelter from predation they provide. Such rocky habitats were scarce in the sampling sites in the present study, but the positive association with *A. anguilla* density and overhanging vegetation and channel depth suggests that *A. anguilla* may have been using such

habitat features to remain inconspicuous to predators. Sites downstream of watercress farm discharges had greater areas of silt, a substrate type which Degerman et al. (2019) found predicted lower *A. anguilla* density than the gravel substrates which typify chalk stream substratum. The greater densities of *A. anguilla* in downstream sites are therefore likely to be due to habitat features other than substrate. As *A. anguilla* feed primarily on benthic macroinvertebrates (Prenda et al. 1997), a plausible explanation for the greater *A. anguilla* densities in downstream sites is improved foraging opportunities than upstream sites due to the higher macroinvertebrate abundances found in these locations. In terms of preferred prey items, analysis of stomach contents of 1348 *A. anguilla* in two chalk streams by Mann and Blackburn (1991), found that in spring they consumed mostly Ephemeroptera, Chironomidae, Simuliidae, Trichoptera and Gammaridae in descending order of presence in gut contents. During autumn they consumed the same diet but with the inclusion of large numbers of Asellidae, a macroinvertebrate which was more abundant in all downstream sites.

4.5.5 Densities of lesser-captured species

The Eurasian minnow *Phoxinus phoxinus* is a common small cyprinid in lakes and rivers of the palearctic region (Howes 1985). They are a hardy species with a wide geographical range, which has expanded through introductions where their plasticity and adaptability have enabled them to thrive (Museth et al. 2007). *P. phoxinus* were in high densities on the Frome, with just one individual caught on any other river. In common with other non-salmonids, this species was captured at greatest density downstream of the discharges in FRDS1, while the lowest densities were found in the fast flowing and shallow upstream site FRUS. The strong associations with depth revealed by CCA suggests that the upstream site may have been unfavourably shallow, while the deeper downstream sites provided more ideal habitat choice. Top-down predator-prey interactions can potentially affect distribution patterns of fish in chalk streams (Prenda et al. 1997). The low density of *P. phoxinus* in FRUS relative to FRDS1 may be associated with predation pressure from *S. trutta*, as both sub-adult and adult *S. trutta* prey on *P. phoxinus* (Museth et al. 2003, 2007) and both size classes of *S. trutta* were found in the greatest densities in FRUS and lowest densities in FRDS1. Some caution needs to be applied when discussing *P. phoxinus* populations surveyed using standard MP electric fishing techniques. Smaller species such as *P. phoxinus* are often targeted with specialist methods (Longley 2006), as capture efficiency with general electric fishing techniques can be low (Anderson 2004). The small size and shoaling behaviour of the *P. phoxinus* makes population density estimates by electric fishing difficult. For example, a large shoal may move in and out of a delineated reach passing through stop nets, resulting in a large number captured if a shoal is encountered, and none at if not encountered which may heavily bias estimates (Mann 1971).

Stone loach *Barbatula barbatula* are a small benthic fish belonging to the Balitoridae, which shelter within the substratum during the day and emerge to feed at dusk (Philipp Fischer 2000). Captures of stone loach in the present study were limited to sites on the Frome where they appeared at all sites but in relatively small numbers; between nine and 22 individuals in total at each site. Densities were marginally higher in FRDS1 than FRUS. However, due to the low capture efficiency of the species using electric fishing due to their nocturnal habits (Reyjol et al. 2005) and the low number of individuals captured, it is possibly unwise to draw conclusions regarding distribution related to watercress farm discharges from the dataset in the present study.

Grayling *Thymallus thymallus* are a salmonid of central, northern and north eastern Europe (Smoliński and Glazaczow 2019) which are typically found in the middle reaches of chalk streams rather than the headwaters (Huet 1959; Mainstone et al. 1997). The longitudinal distribution explains their presence in the Frome sites and absence in the headwater sites of the Bourne and Crane. Like *S. trutta*, the other salmonid captured, densities were greater in the upstream reaches. They were at their highest density in FRUS, with six individuals captured over the duration of the study, while none were captured at in FRDS1. Densities and numbers of individuals captured increased downstream with and two and four captured in CRDS2 and CRDS3 respectively.

The appearance of roach *Rutilus rutilus* and tench *Tinca tinca* in CRDS3 appears to be an anomaly for a chalk stream headwater as they are typically found in lower sluggish reaches of chalk rivers (Mainstone 1999). It was suggested by members of a local angling club that their presence in CRDS3 resulted from accidental introductions from a coarse fishing lake situated next to the site. The lake has an overflow drain leading into CRDS3, where discharges containing fish may occur following heavy rainfall events. These escaped fish would not be able to navigate a weir between CRDS3 and CRDS2, and so they were not found populating reaches upstream of CRDS3.

Brook lamprey *Lampetra planer*, stickleback *Gasterosteus aculeatus*, pike *Esox lucius* and perch *Perca fluviatilis* were captured rarely and with their densities combined contributed to <1% of the cumulative differences between sites. These species contributed to diversity metrics, but with infrequent captures and the minimal between-site differences in density, their relative densities between sites has little power to distinguish sites.

4.5.6 Physicochemistry, water velocity and substrate

Chapter three presents physicochemistry measurements taken during the electric fishing surveys. The determinands measured were included with habitat variables and macroinvertebrate abundances in the multiple stepwise regression of fish densities. None of the physicochemistry determinands included were found to significantly predict fish densities, most likely due to the small

between-site differences and all parameters being well within tolerance ranges for chalk streams. Non-salmonid species were typically found in greater densities below discharges and have a wider tolerance of a range of water quality parameters. Salmonids have a narrow tolerance threshold, which is of particular import for egg incubation (Crisp 1993; Sternecker et al. 2013). As young-of-year *S. trutta* were found at below predicted densities below salad wash on the Bourne and Crane, the physicochemistry and substrate conditions relating to the species and size class requires specific attention.

The discharge from watercress farms, in terms of the DO, suspended solids, pH and conductivity in the immediate downstream sites, did not appear to make them unsuitable for sustaining salmonid populations. The site BREC had the lowest mean DO and highest suspended solids of all sites. However, the observed dissolved mean oxygen levels in BREC and those recorded at the outflow by the Environment Agency were well above the minimum of 5mg/L required for *S. trutta* to flourish (Crisp 1993). Similarly, the recorded mean suspended solid levels of 2.12 mg/L were well below the <25 mg/L considered ideal, and <80 mg/L considered acceptable for *S. trutta* (Crisp 1993). pH and conductivity were found to be well within tolerability for all *S. trutta* life history stages (Crisp 1993; Hendry et al. 2003).

Water velocity is important for spawning in riverine *S. trutta* populations, and all sites bar one had mean velocities above the lower limit of 0.15-0.2 m/s below which *S. trutta* prefer not to spawn (Crisp and Carling 1989). The only site to fall below this was the heavily silted site CRDS2 with a mean velocity of 0.11 m/s. However, this site held above predicted densities of young-of-year trout, indicating that spawning may have taken place despite the silted substrates and low velocities. This may be explained by the upper 20 m having a faster flow than the mean, before a culvert diverts some of the flow leading to the reduced mean flow and sedimentation in the lower 30 m of the site. In terms of water velocity, the top 20 m of CRDS2 and all other sites would provide suitable spawning habitat for *S. trutta* regardless of discharges from watercress farms.

Water velocity typically determines substrate characteristics, with high velocities leading to eroding substrates and the clean gravel beds are a requisite for successful salmonid spawning (Turnpenny and Williams 1980; Heywood and Walling 2007; Soulsby et al. 2001; Hauer et al. 2020). The present study found greater areas of silted substrates below watercress farm discharges than upstream, which may have led to decreased spawning success at immediate downstream sites (Sear et al. 2016). However, young-of-year *S. trutta* were only below predicted densities on the Bourne and Crane downstream sites. Increased sedimentation occurred at FRDS1, yet young-of-year were above predicted densities, suggesting salad wash may be responsible for low densities on the Bourne and Crane. While there were greater areas of silt in sites immediately downstream of discharges, these were largely limited to channel margins which is a natural facet of chalk stream

substrate topography (Mainstone 1999). The dominance of gravels and cobbles in the central channels of these sites suggest that the availability of suitable spawning sites would not be adversely compromised by watercress farm discharges. As the present study surveyed only the percentage of visible surface silt, and not the quantity of interstitial sediment within gravel beds, it is possible that interstitial fines in sites downstream of discharges may have rendered the substratum suboptimal for salmonid egg incubation. Future studies should utilise freeze-core techniques (eg. Rood and Church 1994) to quantify the fraction of fines within the substrate to fully assess the suitability of the substrates for spawning.

4.5.7 *Salmo trutta* condition (W_r)

Condition, expressed as mean W_r was found to be significantly higher in spring than autumn on both Bourne and Crane, which may be explained by decreased foraging opportunities and rates by *S. trutta* during the winter (Bremset 2000). Such seasonal variation was not apparent on sites on the Frome. There may be many environmental and biological variables acting synergistically which maintained a lower condition in *S. trutta* on the Frome which were not accounted for. However, two possible explanations arise from the surveying schedule. Firstly, the Frome could only be surveyed in one spring season and this season may have been an outlier. Secondly, the survey took place at the end of April, which was earlier in the year than for any Bourne and Crane spring surveys. This survey date was before peak densities of macroinvertebrates appear in the water column and when feeding activity is most intense (Klemetsen et al. 2003). Moreover, spring sees an increase in the availability of terrestrial invertebrates which can range between 50 - 80 % of adult *S. trutta* diet biomass in the spring and summer (Wipfli 1997; Bridcut 2000). Therefore, it is likely that *S. trutta* on the Frome in spring were still in low condition from the previous winter, having not had the opportunity to build up fat reserves.

On the Bourne, the downstream site BRDS1 had the highest W_r, with the upstream site BRWC and the further downstream site BRDS2 being lower and broadly similar. On the Crane, both CRUS and CRDS1 had significantly higher W_r than the two downstream sites. As mentioned in chapter three, the upstream site CRUS was likely to be impacted by an STW 1.2 km upstream which may have masked some differences between CRUS and CRDS1. It would appear, however, that condition was improved by discharges on both the Bourne and Crane. A study of *S. trutta* in an Arkansas river found similar results, whereby a heavy metal polluted reach contained fish with higher condition factors than an unimpacted upstream reach (Clements and Rees 1997). The authors of the study found the results surprising and speculated that condition was improved by increased macroinvertebrate abundances in the polluted reach. The strong and significant positive correlation between condition and macroinvertebrate abundance in the present study suggests that the higher

macroinvertebrate abundances in the sites immediately downstream of discharges was likely to have improved foraging opportunity and ultimately their condition (Archer et al. 2020).

S. trutta are generalist feeders on aquatic and terrestrial invertebrates and feed chiefly on mid-water and surface prey (Giller and Greenberg 2015). Several studies have shown that there is an ontogenetic niche shift from the consumption of benthic macroinvertebrates to terrestrial invertebrates as *S. trutta* increase in size (Montori et al. 2006; Teixeira and Cortes 2006; Dineen et al. 2007; Gustafsson et al. 2010). As terrestrial invertebrate availability is lower in summer, Gammarids are considered to be particularly important to salmonids during winter (Macneil et al. 1999; Giller and Greenberg 2015). The low historical abundances of this common macroinvertebrate below watercress farms has been a cause for concern among trout fishery owners. Abundances of this important winter forage were reduced in immediate downstream sites compared to upstream on all river systems, but this did not result in reduced condition of *S. trutta*. Two other important winter forage taxa are Chironomidae and Trichoptera (Kelly-Quinn and Bracken 1990; Klemetsen et al. 2003; French et al. 2016) of which Chironomidae were increased below discharges and Trichoptera reduced. The improved *S. trutta* condition downstream of salad wash discharges may therefore have been sustained by the high abundances of Chironomids at those sites. Chapter three describes the lower biotic index of macroinvertebrate communities in the immediate downstream sites relative to upstream sites as measured using the WHPT metric. While this is of concern for the diversity of pollution-sensitive macroinvertebrate taxa, measures of *S. trutta* condition showed a significant negative correlation with WHPT scores, suggesting that these generalist feeders are able to exploit food in sites affected by organic pollution. The dietary flexibility of *S. trutta* enables them to exploit a range of prey, and in sites polluted by heavy metals and low in macroinvertebrates have been demonstrated to feed more heavily on terrestrial invertebrates (Kraus et al. 2016). Indeed in, Kraus et al.'s (2016) study in Rocky Mountain streams in the USA, *S. trutta* size, condition and quantity of food in stomach was driven more by fish density than magnitude of pollution. In the present study, the lower *S. trutta* densities downstream of discharges may have increased feeding opportunities due to lessened resource competition, leading to higher condition. Studies have shown that the growth and condition of *S. trutta* can be negatively correlated with stocking density, with high densities of *S. trutta* having smaller territories which require increased energy expenditure to defend, with less available food and fewer opportunities for foraging (Jenkins et al. 1999; Bohlin et al. 2002). Kobler (2004) noted faster growth rates in *S. trutta* below wastewater treatment plants in central Switzerland than sites above the discharges and linked the differences to lower fish densities and greater food resources. It is therefore possible that the lower *S. trutta* densities in the sites immediate downstream of salad wash discharges may have resulted in greater opportunities to forage and build up fat reserves and

led to improved condition. However, in contrast to macroinvertebrate abundance, no correlation was found between *S. trutta* density and condition, so improved forage potential remains the most plausible explanation for higher condition below discharges.

Young-of-year fish were not included in condition assessment due to the inherent difficulties in accurately measuring the weight of small fish in the field where water on the balance and wind can affect readings. In addition, there are greater morphological changes that occur in the age group. Unlike larger size classes, young-of-year are reliant on aquatic macroinvertebrates across all seasons (Dineen et al. 2007). Young-of-year *S. trutta* have been found to feed predominantly on the most abundant small aquatic macroinvertebrates such as Chironomidae and Baetidae nymphs (Skoglund and Barlaup 2006; Teixeira and Cortes 2006). As these taxa, and macroinvertebrates in general, were more abundant in the sites immediately downstream of watercress farm discharges it can be assumed that in terms of foraging, these sites would contain a plentiful supply of food for young-of-year *S. trutta* and may be preferable feeding grounds. With more potential prey items below discharges than above, the lower than predicted densities of young-of-year *S. trutta* at sites immediate downstream of salad wash effluent cannot be attributed to lack of foraging opportunities. Indeed, the significant correlation between condition and invertebrate abundances would suggest that feeding opportunities for young-of-year *S. trutta* should be improved below discharges. Askey et al. (2007) found increased macroinvertebrate abundance and concurrent and increased abundance of *S. trutta* and other fish species in wastewater enriched river in Alberta. Similarly, the present study found densities of non-salmonids were higher below the salad wash discharges and sub-adult and adult *S. trutta* to be little affected. This suggests that salad wash discharges are having an impact on young-of-year densities unrelated to prey availability.

4.5.8 Spot recognition of *Salmo trutta*

Site fidelity as expressed as the percentage of *S. trutta* that were re-captured at the same site on each river rather than elsewhere during the study period was similar for the Bourne (94.59%) and the Crane (95.69%). These figures are similar to a study by Knouft and Spotila (2002) that used mark recapture in tandem with telemetry on *S. trutta* in a creek in Pennsylvania, USA, which found that 95.5% of all recaptures and contacts occurred within 800m of initial tagging sites. While the distances monitored (800 m) was larger than the 100 m sites in present study, *S. trutta* movement is usually limited to no more than a few hundred metres in smalls streams (Heggenes 1988), with some populations having smaller ranges still. For example, Aparicio et al. (2018) found mean dispersal distances in small rivers in the Ebro basin (NE Iberian Peninsula) to range from c 21-45 m, and mean movement in southern Irish streams was up to 20 m (Bridcut and Giller 1993). In this respect, site fidelity by *S. trutta* on the Bourne and Frome appears to be typical for the species. Site

fidelity on the Frome was 100%, but the number of recaptured individuals was only seven, six of which were recaptured on the upstream site FRUS. With such a small number of recaptured individuals, it would be unwise to draw the conclusion that site fidelity was higher on the Frome. The Frome is known to support an anadromous population of *S. trutta* (Goodwin et al. 2016) so the lower recapture rate may be a function of seaward migration of smolts. Some of the fish were silvery and had few, if any, melanophores, so these fish were unable to be used for spot recognition. The absence of melanophores on some of these silvery Frome *S. trutta* form part of the morphological changes that occur in migratory sea *S. trutta* prior to smolting (Ferguson et al. 2019). The spot recognition method is best suited to non-migratory, non-anadromous *S. trutta* populations.

Mark-recapture experiments where sampling is done at or near the initial site, usually have low recapture rates; returns greater than 25% are rare in studies lasting over 90 days (Knouft and Spotila 2002). Overall, 12.4% of the 1098 photos taken were matched. There was considerable variation in matching between sites, ranging from 0% in FRDS1 and FRDS2, to the highest percentage of matching in FRUS (35.1%) and CRDS3 (26.6%). These differences may be explained by physical attributes of the channels. FRUS and CRDS3 were the most heterogeneous habitats, with abundant riffle and pool complexes, overhanging vegetation, LWD and natural topography. In contrast, FRDS1 and FRDS2 were artificially straightened channels of relatively uniform dimensions and poor in instream features. Habitat heterogeneity introduces spatial complexity, which increases the quantity of territory and the sizes of territory (Heggenes et al. 2007). This leads to decreases in mobility in *S. trutta* (Heggenes et al. 2007) which may account for the disparity between recapture rates between the most heterogeneous sites and the least heterogenous sites.

In the present study, too few individuals from each site spanning the same timeframe were recaptured to make statistical comparisons of growth rates between sites. The use of data loggers to monitor temperatures at each site over the duration of the study would have allowed the use of degree days in place of time in the growth equation, which would have allowed growth rates at each site from differing timespans to be pooled. This would have increased the replicates for each site allowing statistical comparisons, and so this approach would be recommended in future studies. In addition, future use of spot recognition for growth analysis could aim to increase sample size by sampling more *S. trutta* at each survey by increasing the site dimensions.

There was a great deal of variation between Jolly-Seber (J-S) population estimates and Carle and Strubb (C&S) depletion estimates. The J-S population estimates most closely matched the C&S depletion estimates for CRDS3 on the final sample. CRDS3 had the largest sample size and the by far the largest number of recaptures; 70 individuals compared to the next highest site where just 17 individuals were recaptured. Krebs (1999) states that high capture probabilities are needed for

reliable J-S population estimates. This suggests that recapture rates for all but CRDS3 were potentially too low for accurate J-S estimation. Further, accurate estimates of population densities using mark-recapture would have been hampered by a variety of instances where fish were not 'marked' or would have been unable to be 'read' following recapture. Some of these were the result operator error and could be mitigated with increased experience. These included the photographing of the wrong flank in some of the earliest surveys, and problems with image quality in some cases either due to too much reflection on the fishes' flank or not being sufficiently focussed to clearly distinguish all melanophores. In other instances, the morphology of the fish rendered the software unusable as there were too few melanophores for spot recognition to be applied, such as on the Frome. Such instances may have missed potential recaptures, resulting in a scenario similar to the loss of a conventional tag. McDonald et al. (2003) modelled the effect of tag loss on J-S population estimates and found that such scenarios lead to overestimation of the population size. In the present study, the population density estimates generated using the J-S were inflated by an average of 15% over results obtained using C&S depletion methods. This suggests that failure to 'recapture' marked individuals due to the aforementioned imaging issues may have led to the inflated J-S estimates.

Using spot recognition software in mark-recapture studies shows some promise as a low-cost non-invasive technique in studies of non-migratory *Salmo trutta*. However, particularly before attempting to estimate population sizes, it is recommended that spot recognition of *S. trutta* is trialled in a closed population in concert with conventional tagging so that absolute efficiencies of the software and human operator to identify individual fish is assessed. It should also be noted that the processing of images and matching of individuals is labour-intensive when there are large numbers of individuals. The photographing of fish in the field may be quicker than physical tagging where anesthetisation is required, which is beneficial from a welfare standpoint. However, it should be noted that the processing of images for spot recognition is a more time-consuming process than the recording of physical identity tags. However, due to the lack of specialist equipment and licensing needed to 'mark' and recapture fish, its application has a wider reach than use by fisheries specialist alone. For example, images taken by sport anglers, in concert with location and length and weight metrics could be used to develop databases to monitor fish movements and growth. The use of such citizen science data gathering over the last decade has grown immensely (McKinley et al. 2017). The analysis of publicly-sourced images of whale sharks taken by tourists using I^3S was demonstrated by Davies et al. (2012) to be useful in mark-recapture studies of resident fish. Applying the technique to *S. trutta* could prove useful for tracking the movement and growth of stocked fish into sport fisheries, which has previously relied on costly invasive tagging methods and manual tracking (Bettinger and Bettoli 2002; Aarestrup et al. 2005; Flowers et al. 2019).

4.6 Conclusions

There were a number of key differences between fish populations in sites immediately downstream of watercress farm discharges relative to sites upstream of the effluent and to those further downstream. The extent of the changes varied from farm to farm and mirrored the extent of salad washing activity on the farm as described in chapter two, whereby greatest effects were seen below discharges on the Bourne which had the most extensive salad washing activity, followed by the Crane which washed only intermittently and the least on the Frome which did not salad wash. Relative to upstream sites, the immediate downstream sites generally had:

- Higher Shannon index of fish, though species richness was variable.
- Higher densities of non-salmonids, possibly resulting from improved foraging opportunities afforded by increased benthic macroinvertebrate abundances
- Higher relative condition of *Salmo trutta*, which was significantly correlated with greater abundances of macroinvertebrate prey.
- Lower densities of young-of-year *S. trutta* than predicted by HABSCORE on the Bourne and Crane, while sub-adult and adult densities remained largely above predicted abundances

The lower than predicted densities of young-of-year *S. trutta* below watercress farm discharges on the Bourne and Crane indicate possible negative impacts on recruitment from the discharge. This may take the form of either chemical compounds within the discharge or sedimentation of spawning gravels. Silted substrates impeding embryonic survival or spawning success cannot be ruled out. However, while fine sediment was increased downstream of discharges on all sites, on the Frome where no salad washing occurs on the watercress farm, young-of-year *S. trutta* densities were higher than predicted. The lower than predicted densities of young-of-year *S. trutta* in sites downstream of salad washing effluent on the Bourne and Crane suggest that a component of salad wash effluent such as PEITC may have negatively impacted recruitment. In the next chapter, laboratory ecotoxicology trials are used to assess the embryotoxicity of PEITC to assess the potential for its release from salad washing processes to impact on recruitment.

4.7 Further research and limitations

There was a high degree of temporal variation in densities of fish in the present study. As fish populations are known to be experience wide annual fluctuations, future studies might aim to increase the duration of surveying work to encompass a greater number of years, or to add to the datasets contained within this research. By spanning a greater number of years, interannual variations in fish populations would be better accounted for which would increase the power of the data.

It was not possible to age fish from scale samples in the present research as it is an invasive process that requires a Home Office licence. This limited the resolution to distinguish between young-of-year *S. trutta* and those born in the previous season, which may have led to over or underestimates of the young-of-year populations. This may be addressed in future studies with appropriate licensing.

Small benthic fish such as *C. gobio*, stone loach and lamprey are best sampled using specialised techniques as standard electric fishing is not as suitable for their capture as it is for pelagic species. Future studies may choose to specialise in surveying specific benthic fish to increase catch efficiencies and resolution into how watercress farm discharges affect their population structures.

An avenue for further research raised in chapter three (section 3.7) is also applicable to the present chapter, the statistical benefit of increasing the number of watercress farms studied with salad washing facilities. By allowing data from upstream and downstream of salad wash discharges to be pooled, mixed effect models with site identity held as a random factor could be employed. This would avoid the pseudoreplicaton inherent in assessing upstream and downstream effects of sites on individual rivers.

ECOTOXICOLOGY OF PEITC ON EARLY LIFE STAGES OF FISH

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5.1 Introduction

The introduction of contaminants to an aquatic environment can have obvious impacts on individuals and populations, such as mass mortalities and/or reduced fecundity, both of which may lead to extirpation or even extinctions of species (Wódz 1992; Bird et al. 1995; Kasuya 2002; Allenbach 2011). Toxicological research has recently shifted from examining lethal effects of contaminants on organisms, to encompass sub-lethal effects (Scott and Sloman 2004; Sfakianakis et al. 2015), such as reduced growth, increased susceptibility to disease, increased morphological anomalies and altered behaviours, which may lead to alterations of natural population structures (Bird et al. 1995; Baumann et al. 1996; Galloway et al. 2004; Scott and Sloman 2004).

As fish increase in size, susceptibility to toxins lessens, as body size has been shown to be negatively related to toxic response (Anderson and Weber 1973; Tsai and Chang 1981). Additionally, it is well understood that toxicants have a more pronounced impact on early life stages of fish than adults (Weiss 1989; Kristensen 1994), with early life stages undergoing developmental phases sensitive to disruption from a wide range of chemicals (McKim 1977; Belanger et al. 2010). Consequently, fish embryos and eleutheroembryos (yolk fry/alevins) have been widely adopted as models for ecotoxicology studies. When naturally-occurring or anthropogenic stressors are experienced by fish during development, the homeostatic control of morphological development may be disturbed, resulting in abnormal phenotypes (Allenbach 2011). Many studies of toxins and pollutants on early life stages of fish report higher rates of morphological deformities (Gjerde et al. 2005; Eissa et al. 2009; Jezierska et al. 2009). Some of the most common deformities are found in the vertebral column, most frequently in the form of lordosis (inward curvature of the spine), kyphosis (outward curvature of the spine) and scoliosis (lateral curvature) (Sfakianakis et al. 2015). Such deformities can impair predator avoidance and foraging capabilities (Kroger and Guthrie 1971), which can lead

to early mortality and so deplete populations of older and more fecund individuals (Tutman et al. 2000; Messaoudi et al. 2009). Jezierska et al. (2009) reviewed toxicant exposure on early life stages of fish reporting high mortality rates, hatching delay and morphological deformities. Toxins such as heavy metals can increase energy expenditure on detoxification processes, resulting in stunted growth (Sfakianakis et al. 2015). For example, Osman et al. (2007) exposed embryos of the African catfish (*Clarias gariepinus*) to 100, 300 and 500 µg/L lead nitrate and found a progressively longer delay in hatching with higher exposure levels. Successful hatching ranged from 75% in the control group to 40% in the highest exposure treatment and frequencies of deformities (spinal deformities, yolk sac edema and irregular head shape) significantly increased with increasing lead concentrations. Ren et al. (2019) exposed embryonic-larval stages of a marine flounder (*Paralichthys olivaceus)* to Methylmercury (MeHg) concentrations from 0-15 µg/L. Exposures ≥ 13 µg/L caused dose-dependent increases in mortality, morphological deformities and yolk absorption rate.

5.1.1 Behavioural responses to toxin exposure

Many contaminants disrupt fish behaviour at exposures far lower than those that cause significant mortality (Scott and Sloman 2004; Nassef et al. 2010) or physiological and morphological anomalies (Sloman and Mcneil 2012). As such, behaviour is considered a highly sensitive indicator of the condition of animals and their environment (Kasumyan 2001). For example, rainbow trout (*Oncorhynchus mykiss*) exposed to the herbicides tributyl phosphorotrithioate and 2,4-dichlorophenoxyacetic acid, had alterations in swimming behaviour at concentrations as low as 0.7 to 5% of their LC50 values (Little and Finge 1990). There have been studies which have shown predator-avoidance responses in fish diminished following exposure to sub-lethal levels of toxicants (Weiss 1989; Little and Finge 1990; Zhou and Weis 1999). Carlson et al. (1998) performed simultaneous electrophysiological and behavioural studies on 21–32 day old juvenile medaka (*Oryzias latipes*) and found a neurological suppression of the startle response in fish subjected to sub-lethal concentrations of organic toxicants (chlorpyrifos, carbaryl, fenvalerate, endosulfan, phenol, 1-octanol and DNP) which would leave them at increased risk of predation. In addition to the avoidance of predation, efficient foraging activity is key to survival. Disturbances in foraging behaviour by exposure to chemical pollutants can occur through suppression of appetite or through structural and functional changes in sensory systems (Kasumyan 2001). Most sensitive to low concentrations of toxicants are alterations of the sensory systems that impact the ability of fish to perform the search for food, pursue and grasp prey, and estimate its palatability (Kasumyan 2001). Inappropriate behavioural responses to environmental and physiological stimuli due to toxic effects of contaminants can have deleterious effects on survival (Weber and Spieler 1994).

5.1.2 Phenethyl isothiocyante

Phenethyl Isothiocyanate (PEITC) is a secondary metabolite produced by brassicas (e.g broccoli, Brussels sprouts, cabbage, cauliflower, kale and watercress) in response to, and as a defence against herbivory (Fenwick et al. 1983). The synthesis of PEITC via the glucosinolate-myrosinase pathway and its production, its toxicity to macroinvertebrates and its release through watercress farming and salad washing processes are described in section 1.5.1.4. In field studies in chapters three and four, watercress farm discharges from two farms that washed salad crops on site were observed to exhibit impacts that were not apparent on a farm on which no salad washing took place. In chapter three, this took the form of altered macroinvertebrate assemblages, resulting in lower abundances of pollution-sensitive species and greater abundances of pollution-tolerant species. In chapter four, they took the form of below expected densities of young-of-year *Salmo trutta*, while larger fish were generally at greater than expected densities, pointing to a potential impact on recruitment from PEITC. It has been hypothesised that salad washing process would liberate higher concentrations of PEITC than watercress production alone (Dixon 2010) and the results of chapters three and four would suggest this may be the case. Dixon (2010) calculated an estimate of the PEITC concentration found in salad wash water from the watercress farm on the Bourne Rivulet to be between 600-1040 ug/L PEITC, which was higher than the estimate of 0.32-0.59 mg/L from release by harvesting calculated by Worgan and Tyrell (2005) (see section 1.5.1.5).

Watercress farming, and salad washing in particular, are not the only route by which PEITC may enter waterbodies and lead to exposure by teleost fish. The increasing demand for organic produce has led to a large and growing body of research into the use of non-synthetic pest control practices such as biofumigation (Matthiessen and Kirkegaard 2006; Gimsing and Kirkegaard 2009; Ntalli et al. 2017). Biofumigation is the application of specific brassicas which are macerated to activate the glucosinolate-myrosinase system and incorporated into soils as 'green manures' (Ntalli et al. 2017). A range of isothiocyantes are produced, including PEITC, which control soilborne pathogens, nematodes and weeds (Petersen et al. 2001; Smith and Kirkegaard 2002; Rumberger and Marschner 2003). Laboratory trials showed that following biofumigation, isothiocyanates may be washed down into soil to a depth of 1 m during a heavy rainstorm (Laegdsmand et al. 2007). The study estimated leachate from soil could enter waterbodies located close to fields where applications took place at PEITC concentrations of up to 50 µg/L. The extent of the application of biofumigation in practice is at present not known, but there is a risk that PEITC-containing leachate could enter ponds, lakes, rivers or streams.

5.1.3 Fish species

Three fish species, brown trout (*Salmo trutta*), common carp (*Cyprinus carpio* L.) and zebrafish (*Danio rerio* Hamilton) were used in PEITC ecotoxicology trials in the present study. For each species there follows a description of the range, habitat and spawning requirements and rationale for their inclusion in the ecotoxicology trials.

5.1.3.1 Brown trout (*Salmo trutta*)

S. trutta are an indigenous salmonid of Europe, North Africa and Western Asia. *S. trutta* are an ecologically and commercially important species in chalk streams (Mann 1971; Power 1994). Their spawning and subsequent embryo incubation in the chalk stream headwaters where watercress production typically occurs (Mainstone 1999) makes them a key species of interest for the present study. In UK chalk streams, spawning typically occurs in late December/early January. The female fish excavates a depression in suitably silt free gravel into which she deposits her eggs. The male fish then fertilizes the eggs with milt (sperm) before the female backfills the depression with gravel to cover the embryos (Crisp 1993). The fertilized embryos typically remain incubating within the gravel nest, or redd, for several months. Three important events are recognised to occur during intra-gravel development. The first is when the eye pigment of the embryo becomes visible through the chorion (egg casing) which is referred to as 'eyeing'. Later, the chorion is shed during hatching into first free-swimming stage the alevin which remains within the gravel nest subsisting on the yolk-sac. When the yolk-sac is close to exhaustion, the alevin acquires skin pigmentation and emerges from the gravel in the stage known as 'swim-up'. At this stage, the fish commences exogenous feeding and is no longer classed as an alevin but as a fry (Crisp 1993). Emergence from gravels typically occurs in April in UK waters (Mann et al. 1989). The early life stages of *S. trutta* are especially sensitive to xenobiotics (Power 1994; Finn 2007), where the large size of *S. trutta* eggs (>5 mm), and the long developmental period in the redd, may favour increased uptake of toxicants during the incubation period (Kristensen 1994; Schubert et al. 2014).

The thermal tolerance of *S. trutta* embryos is well-established. Ojanguren and Braña (2003) found maximum survival rates occurred between 8 and 10° C, while Crisp (1993) reports 95% survival rates between 0-10 °C, at least 50% mortality at temperatures greater than 12 °C, and 100% mortality above 15.5 °C. Alongside embryonic survival, the duration of embryonic development of *S. trutta*, from fertilisation to hatch, is strongly influenced by the ambient water temperature. Figure 65 reproduced from (Ojanguren and Braña 2003) highlights the influence temperature has on hatch timing in *S. trutta*.

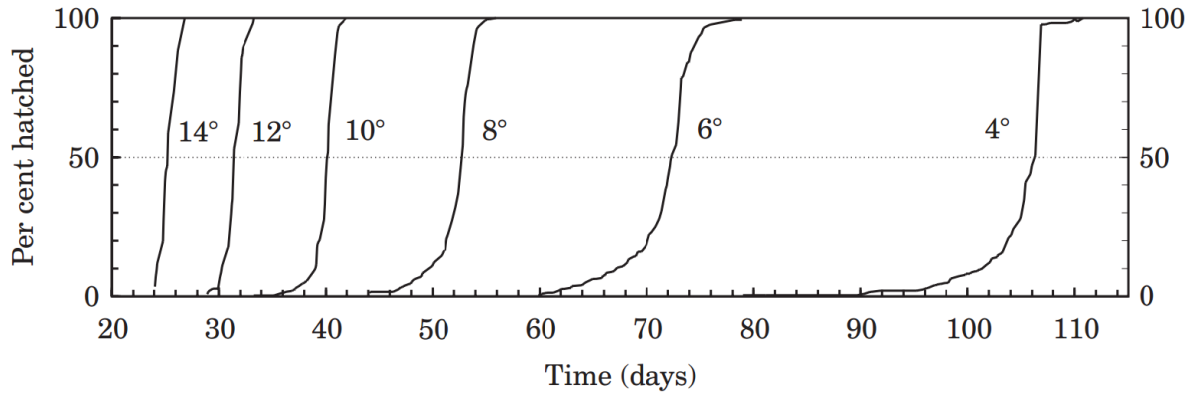

Figure 65. Percentage of hatched *S. trutta* embryos over various incubation temperatures as a function of time from fertilisation (Ojanguren and Braña 2003)

Oxygen consumption by *S. trutta* embryos varies with development stage, and it broadly increases through development (for review, see Greig at al. 2007). Davis (1975) reports that levels of DO in redds greater than 5 mg/L are critical for successful embryonic development of salmonids. Using study sites on the River Rhine, Ingendahl (2001) found that *S. trutta* fry did not emerge from redds where the mean dissolved oxygen levels were below 6.9 mg/L.

5.1.3.2 Common carp (*Cyprinus carpio*)

C. carpio is a cyprinid originally native to temperate Eurasia, that through human introductions is now found on every continent except Antarctica (Bíró 1995). The global distribution has been principally driven by aquaculture, and it now has naturalised populations in the UK and many other parts of the world (Lever 1996). In some locations it is considered an invasive species, having a place on the IUCN list of the world's worst 100 alien invasive species (Lowe et al. 2000). *C. carpio* is not found in chalk stream headwaters where watercress farming occurs, but does have populations in the lower reaches of chalk rivers (Mainstone 1999) and could be affected by PEITC in leachate from biofumigation. In addition, they are present in ponds and lakes that receive watercress farm discharges, such as Old Arlesford Pond in Hampshire, a SSSI site (Natural England 2017) which receives outflows from four watercress farms. In contrast to *S. trutta*, *C. carpio* is regarded as tolerant in terms of habitat quality, which accounts for its extremely widespread distribution and status as an invasive species (Lever 1996). *C. carpio* has a high fecundity, ranging from 100,000 to 300,000 eggs per kg body weight per annual spawning cycle (Linhart et al. 1995). In European waters, *C. carpio* commence spawning when water temperatures rise to 18° C , typically during the months of June and July (Yaron and Levavi-Zermonsky 1986) when watercress harvesting is at its peak (Cox 2009). The incubation period for *C. carpio* is temperature-dependent, with higher temperatures promoting more rapid embryonic development. At 32° C hatching may occur between 1-2 dpf, while at 20° C it may take between 4-5 dpf. (Korwin-Kossakowski 2008). In

aquaculture, *Cyprinus carpio*are commonly raised through embryo development between 18-22° C (Korwin-Kossakowski 2008). The inclusion of this hardy cyprinid in the study expands our knowledge by encompassing a pollution-tolerant family to gain a wider understanding of the impact of PEITC exposure on teleost embryonic development.

5.1.3.3 Zebrafish (*Danio rerio*)

D. rerio are indigenous to South Asia, with a distribution which encompasses parts of India, Pakistan, India, Nepal and Myanmar (Lawrence 2007). This small cyprinid has become the pre-eminent vertebrate model for scientific research in an ever-increasing range of fields including behavioural research in toxicology (Franco-Restrepo et al. 2019) and drug development (MacRae and Peterson 2015). It is estimated that over 5 million *D. rerio* are used in research annually with over 3250 institutes working with the species (Kinth et al. 2013). They are a hardy fish, which exhibit a wide range of thermal tolerance, with laboratory studies suggesting a maximal thermal tolerance between 6.7 - 41.7 °C (Cortemeglia and Beitinger 2005). However, in its home range, such extremes in temperatures are unlikely to be encountered. Spence et al. (2006) recorded temperatures between 16.5 to 33 °C and a mean pH of 8.0 from surveying nine *D. rerio* sites in Bangladesh. *D. rerio* are asynchronous spawners that spawn continuously once sexually mature. The resultant eggs are scattered over the substratum and the adults exhibit no parental care (Lawrence 2007). In a wild habitat eggs typically hatch within 4-7 dpf, while in the laboratory temperatures are set to minimise incubation duration (typically 28.5 °C) whereby they hatch between 2.5-3 dpf (Westerfield 1995). The inclusion of *D. rerio* in the ecotoxicology trials was due firstly to their status as a model organism, used widely in ecotoxicology research (Bambino and Chu 2017). Secondly, as the use of *D. rerio* in research is widespread, laboratory produced embryos are available year-round, in contrast to both *S. trutta* and *C carpio* for which eggs are available on a seasonal basis.

5.2 Aims

Discharges from salad washing processes on watercress farms and leachate from biofumigation are potential routes for teleost embryos to become exposed to PEITC in receiving waterbodies. The purpose of this laboratory study was to examine the effects of PEITC exposure on developing fish embryos. Developing *S. trutta*, *C. carpio*, and *D. rerio* embryos were exposed to controls and increasing PEITC concentrations to gain insight into environmental PEITC concentrations that may impact on recruitment and survival in natural populations. In addition, *D. rerio* embryos were exposed to a watercress assay designed to approximate salad wash effluent to observe if results obtained using analytic grade PEITC are replicated, thereby establishing a link between PEITC and

salad wash effluent. Using a series of trials on the three species, this study aimed to address the following aims:

I. To discover the PEITC concentration threshold that results in complete mortality of all embryos and sublethal concentrations that result in no increases in mortality
II. To discover if PEITC affects hatch timing, either by delaying hatching or promoting earlier hatching
III. To investigate any teratogenic impacts on surviving eleutheroembryos from sublethal dosing, including changes in body size, weight and incidence of spinal deformities
IV. Using an automated behavioural chamber, to investigate alterations in motor responses to stimulus in eleutheroembryos following sublethal embryonic PEITC exposure

5.3 Methods

5.3.1 Ethics

The methodology and use of *S. trutta*, *C. carpio* and *D. rerio* embryos were reviewed and approved by the University of Brighton's Animal Welfare & Ethics Review Board (AWERB). Due to the life stages involved in the experiment (pre-independent feeding), the procedure did not enter the regulatory framework of the Animals (Scientific Procedures) Act 1986 Amendment regulations (SI 2012/3039) and experiment time-frames were designed around this (Strähle et al. 2012).

All procedures were carried out on fish embryos, larvae and alevins prior to their ability of perform exogenous feeding, as outlined as an exemption from licence by the UK Government Home Office under The Animals (Scientific Procedures) Act 1986 and EC Directive 2010/63/EU (Commission of the European Communities 2008).

5.3.2 Production of phenethyl isothiocyanate stock solution

To ascertain phenethyl isothiocyanate (PEITC) embryotoxicity, analytical grade PEITC (99%) (Sigma-Aldrich, Darmstadt, Germany) was used for dosing. Using the highest purity PEITC available increased dosing accuracy, as in the absence of facilities such as gas chromatography-mass spectrometry (GC-M)S to analyse treatment concentrations, dosing concentrations were achieved using serial dilution. PEITC is immiscible in water so requires dissolving in a solvent prior to introducing into an aqueous solution for use in ecotoxicology experiments. Dimethyl sulfoxide (DMSO) was selected for its relatively low toxicity to fish (Hallare et al. 2006; Maes et al. 2012).

While no studies on salmonid embryonic exposure to DMSO could be found, work on the embryos of *D. rerio* indicate that levels of 1% volume per volume (v/v) or lower may be suitable for toxicology experiments with hatch rate, morphological development and survival as endpoints, while Chen et al. (2011) discovered *D. rerio* behaviour may be altered above 0.01%. Benville et al. (1968) found no distinct pathology in adult salmonids to DMSO concentrations below 2% v/v.

No reference to the ratio of DMSO to PEITC required for successful dissolution of PEITC could be found in the literature. To minimise the concentration of solvent used in the assays while ensuring enough DMSO was present to fully dissolve PEITC, a trial to find the minimum ratio was conducted. PEITC was added to DMSO (78.13 g/mol) (Sigma-Aldrich, Darmstadt, Germany) over an increasing range of ratios, with the resultant assay observed under a stereo microscope for persistence of oil droplets. PEITC was fully dissolved at a ratio of 1µg PEITC in 1000µl DMSO. The highest concentration of PEITC in the experimental design was set at 1µg/L requiring 1ml/L of DMSO to fully dissolve it in the assay. Therefore the highest DMSO concentration in any treatment was 0.001% v/v, which according to the literature is far below levels shown to cause pathology in *D. rerio* embryos (Hallare et al. 2006; Chen et al. 2011; Maes et al. 2012). A DMSO solvent control was included in addition to a water control in all trials.

5.3.3 Embryo rearing

In order to maintain fish embryos for ecotoxicological studies, a closed recirculation system was constructed in the laboratory which allowed for the suspension of embryo-containing beakers in temperature-controlled water (Figure 66). The system consisted of a series of nine 30 x 30 x 30 cm aquaria drilled at the top rear with an outflow pipe installed. Water circulates from a sump below the tanks via a pump (Eheim universal 1260 centrifugal pump, Germany) into the aquaria via overhead piping terminating in a valve to control water velocity entering the aquaria. Water then returns to the sump via outflow pipes from the aquaria. Within the sump, an identical Eheim pump circulates sump water through a thermostatically controlled refrigeration unit (DC-750 refrigerated cooler, D-D The Aquarium Solution Ltd., UK) before being returned to the sump. An early test found that this system was able to maintain a consistent temperature in the aquaria 3 degrees below the lowest temperature desired for the experiments. A heater stat (Interpet 300w) placed within the sump allowed for control of temperatures above ambient for trials using *Danio rerio*.

Each aquarium was able to hold up to five 400 ml glass beakers, which were suspended in the temperature-controlled water via a 30 x 30 cm polystyrene tile floating on the surface with holes cut to the exact diameter of beakers. The round holes in the polystyrene tile were cut to be a close fit for stability and so that the lip of the beakers could not pass through.

To replicate the dark conditions of a redd when incubating *S. trutta*, black adhesive vinyl sheeting was applied to all surfaces of the aquaria bar a gap for viewing at the front (Figure 66). Additionally, blackout curtains encircled the aquaria hanging from the shelf above the aquaria to effectively black out all light. The front blackout curtain could be raised to enable access for dosing and water changes which were undertaken with the room darkened. *C. carpio* and *D. rerio* were incubated without blackout measures so that light from a nearby window provided the natural photoperiod.

Figure 66 The embryo rearing system at the Hastings laboratory in use incubating *Salmo trutta* embryos. The front black out curtain is lifted to show three semi-blacked out aquaria with airlines running into beakers suspended into chilled re-circulating water

Into each beaker ran a metre of airline tubing, fastened to the inside lip of the beaker with aquarium grade silicone sealant (King British aquarium sealant, UK), terminating in an airstone at the bottom of the beaker. The airline from each beaker ran into a manifold that allowed the aeration of the beakers to be balanced. Air was supplied via an air pump (Hailea ACO-9630, China). Gentle aeration was employed to ensure a slow steady movement of water over the embryos and to ensure dissolved oxygen levels are maintained. Fastening the airline to the beaker with silicone sealant ensured that the airstone remained at the base of the beaker to maintain a consistent aeration and ensure any movement of the airline during water changes and dosing did not cause the airstone to knock into the delicate embryos. A small square of Parafilm® (Bemis, NA, USA) was placed over the top of each beaker to minimise water loss and prevent the access to airborne pathogens and the accumulation of dust.

Water quality analysis was carried out daily on a randomly selected beaker within each of the aquaria. Dissolved oxygen (DO mg/L) and temperature (°C) were measured using a Hanna H19142 probe (Hanna, USA), and pH was measured with a Hanna HI98107 pocket pH tester (Hanna, USA).

5.3.4 Gametes and fertilisation

5.3.4.1 Gametes and fertilisation *Salmo trutta*

Salmo trutta trial one utilised triploid *S. trutta* eggs and milt sourced from Allenbrook Trout Farm, Dorset, UK. The same farm supplied gametes produced by photoperiod manipulation for trials three and four. Gametes for trials five and six were supplied from The Berkshire Trout Farm, Berkshire, UK. In all instances, eggs from two females and milt from a single male were stripped and immediately returned in a cool box to the laboratory in Hastings by road, a journey of 3 to 4 hours. Fertilisation was initiated immediately on arrival in the laboratory. The milt was introduced to the eggs and stirred. Matured tap water at 10 °C was then added to activate the spermatozoa, left for two minutes while fertilisation occurred. Following this period, the excess milt was rinsed away with matured tap water.

5.3.4.2 Gametes and fertilisation *Cyprinus carpio*

Cyprinus carpio embryos were fertilised at 23° C using the facilities at V. S. Fisheries, West Sussex, before being immediately bought back to the laboratory, a journey of two hours. The bag containing the embryos was placed into a water bath in the Hastings laboratory and allowed to cool down over six hours to 18 °C. Fifty viable embryos were then counted into each replicate beaker and placed into the rearing system set to 18 °C. 12 hours later, the temperature was lowered to the target experimental rearing temperature of 15.5 °C over the course of 6 hours. The temperature was set relatively cool at 15.5 °C in order to maximise the incubation period and PEITC dosing days, yet still reflect temperatures that may be experienced by embryos in wild populations following a drop in water temperature post spawning (Simon Scott (V.S. Fisheries), pers. comm. 2017).

5.3.4.3 Gametes and fertilization of *Danio rerio*

Danio rerio embryos of the Ekwill strain were obtained from University College London (UCL) Zebrafish Facility. The embryos were fertilised at typical zebrafish laboratory rearing temperature of 28 °C, a temperature which minimises the incubation period and hastens hatching. By the time the embryos had reached the laboratory some 5 hours later, the water temperature had dropped to 25 °C. To increase the duration of the trials and available dosage days, the embryos were maintained below the typical laboratory temperature, but at a temperature which is found in their

natural habitat. The target temperature was set to 24 °C, which increased the incubation period to reflect the upper end of the natural incubation period found in wild populations.

5.3.5 PEITC dosing regimen and incubation maintenance

Matured water was used for daily water changes and dosing of PEITC, created by aerating tap water in beakers for 24 hours in a water bath set at the target temperature for the particular trial. This ensured that chlorine had gassed-off and the water was at the correct temperature for water changes to avoid thermal shock to the embryos.

For each of the trials conducted, 50 newly fertilised embryos were placed into each of the 15 beakers which contained 250ml of matured tapwater at the required temperature for the species. The 15 beakers were assigned a number from 1-15 and a random number generator was used to allocate the replicates throughout the embryo rearing system. The beakers were then immediately placed into the embryo rearing system where they were aerated for 24hrs prior to the first dosing of PEITC. Each replicate 400ml beaker was maintained with 250ml of water. Water changes were performed by removing the airline from the manifold and syphoning off 200ml through the airstone fixed into the beaker, leaving 50ml remaining to keep the embryos immersed. 200ml of matured tap water was gently poured down the outside of the airline onto the airstone. These two measures ensured minimal disturbance to the developing embryos. On the days requiring PEITC dosing, the beaker was syphoned as usual and topped up with matured water containing PEITC/DMSO stock, or matured water and DMSO for a solvent control. The concentrations were 0.01, 0.1 and 1µg/L plus a solvent control to replicate the highest concentration in any treatment, which was 1 ml/L in the 1 µg/L treatment (Table 39). Alongside a solvent control, a water control of matured tap water was used.

For each treatment, PEITC/DMSO stock was pipetted into 2 L of matured water to obtain the desired concentration and vigorously mixed with a glass stirrer. The desired concentration was 25% stronger than the required concentration in each beaker to allow for dilution in the 50ml of water remaining in each beaker.

Table 39. Quantities of stock solution and the concentrations of PEITC and DMSO used to make up 2 L of water for each treatment. Concentrations in 2 L water for changes are 25% higher than in the final concentrations in the beakers to account for dilution when adding to the remaining water in the beakers

Treatment	*Final volume*	*Stock volume (µl)*	*PEITC µg/L*	*DMSO ml/ L*
Water control	2 L	0	0	0
DMSO control	2 L	n/a	0	2.5
1 µg/ L	2 L	2500	2.5	2.497.5
0.1 µg/ L	2 L	250	0.25	0.24975
0.01 µg/ L	2 L	25	0.025	0.024975

5.3.6 Trials

A series of seven trials were undertaken in the Hastings laboratory; five on *S. trutta*, and one each on *C. carpio* and *D. rerio* (Table 40). As can be seen from the table, certain toxicological endpoints were not examined in all trials. The reasons are discussed under the trial descriptions in sections 5.3.6.1 to 5.3.6.3. The final trial using *Danio rerio* embryos, trial seven, is the only trial to include a watercress assay in addition to PEITC dosing. This was made possible by an expansion of the experimental set up and was not possible in earlier trials due to space limitations.

Prior to the first set of *S. trutta* trials, a dosing regimen was trialled at Sparsholt College using a species closely-related to *S. trutta*, the rainbow trout (*Oncorhynchus mykiss*) which spawn earlier in the season than *S. trutta*. The trial was performed to gain an understanding of the concentration of PEITC that might cause total mortality to the closely related *S. trutta*, so that sub-lethal dosing could be achieved. The pilot trial on *O. mykiss* used a regime of 12 hours of PEITC exposure followed by 12 hours in water. Treatments consisted of a water control, a solvent control and three PEITC treatments at 0.5, 1 and 2 µg/L. Embryo mortality in all PEITC treatments reached 100% by 14 dpf, indicating that PEITC concentrations used were too high to investigate sub-lethal effects. These results informed a revised dosing regimen for the subsequent *S. trutta* trials, which would use logarithmic concentrations of 0.01, 0.1 and 1 µg/L PEITC and dosing every third day rather than daily.

Table 40 Summary of the ecotoxicology trials on brown trout (*Salmo trutta*), common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) showing the toxicological endpoints examined in each trial. VCD; vertebral column disorder

		Toxicological endpoints assessed					
Trial no.	*Species*	*mortality*	*hatch*	*VCD*	*length*	*weight*	*behaviour*
1	*Salmo trutta*	✓		✓			
2	*Salmo trutta*	✓	✓	✓			
3	*Salmo trutta*	✓	✓	✓			
4	*Salmo trutta*	✓	✓	✓	✓	✓	✓
5	*Salmo trutta*	✓	✓	✓	✓	✓	✓
6	*Cyprinus carpio*	✓	✓	✓	✓		✓
7	*Danio rerio*	✓	✓	✓	✓		✓

5.3.6.1 Salmo trutta

During all *S. trutta* trials, a 24hr dark photoperiod was used to replicate conditions within a redd. The first PEITC dosing was performed at 1 dpf and was repeated every third day for the duration of the trial until the first alevin hatched. At each daily water change and dosing, dead eggs - identified by a cloudy white appearance - were removed and counted. Water quality parameters for each of the five trials are presented in Table 41. A target temperature of 10 °C was aimed for, a temperature which is used in the aquaculture of *S. trutta* (Trevor Whyatt (Allenbrook Trout Farm) pers. comms. 2016) and typical of springs feeding chalk streams (Mainstone 1999).

Table 41 Mean (±SD) water quality parameters; temperature, pH and dissolved oxygen (DO) recorded over the duration of phenethyl isothiocyante exposure trials one to five on *Salmo trutta* embryos

Trial	*n*	*Temp (°C)*	*pH*	*DO (mg/L)*
1	123	10.4 ± 0.3	7.4 ± 0.2	11.1 ± 0.5
2	126	10.6 ± 0.21	7.86 ± 0.12	11.28 ± 0.52
3	126	10.4 ± 0.25	8.10 ± 0.14	9.56 ± 0.31
4	126	11.32 ± 0.19	8.17 ± 0.15	9.24 ± 0.42
5	126	11.06 ± 0.20	8.19 ± 0.12	9.56 ± 0.27

5.3.6.1.1 Salmo trutta: trial one

By 42 dpf the Hastings laboratory was closed for the Christmas period. The embryos were relocated to a local public aquarium whose staff undertook daily water changes, removed and counted mortalities, and estimated the number of hatches from each treatment. All alevins hatched during this period and were collected and bought back to the laboratory 53 dpf. Motor activity assay trials commenced at 54 dpf on 24 randomly selected alevins from each treatment. Following the activity

trials, the alevins were first euthanised via an overdose of 2-Phenoxyethanol (Aqua-Sed; Vetark Professional, UK) before preserving in 4% formalin (Sigma-Aldrich, Darmstadt, Germany), prior to morphological analysis.

As it was discovered following this first trial that PEITC exposure caused delayed hatching relative to controls, the controls and animals exposed to lower concentrations would be, on average, older post-hatch than those exposed to high concentrations. The discrepancy in age post hatch between treatments would be a factor affecting endpoints pertaining to length, weight and behaviour. As such, only data pertaining to mortality rates and incidents of spinal abnormalities are included for analysis. Subsequent trials addressed this issue.

5.3.6.1.2 Salmo trutta: trials two and three - photoperiod gametes

Trials two and three utilised gametes produced using broodstock induced to spawn by the use of artificially manipulated daylength, also known as photoperiod manipulation (Bonnet et al. 2007). This enables viable gametes to be produced independent of season. Using such gametes circumvented the issue of the Christmas laboratory closure which resulted in alevins of unknown age post hatch in trial one and increased the number of *S. trutta* trials possible during the duration of the research.

The dosing frequency was the same as trial one, but unlike trials one, PEITC/DMSO stock was created before the trials commenced and was frozen pre-measured for each dose into centrifuge tubes at -20°C. This was to streamline the dosing process, enabling pre-measured stock to be thawed at room temperature prior to use before adding to matured tapwater to create the treatments.

Newly hatched alevins were removed daily from each treatment at ≤ 1 dph and euthanised until the day there were at least 24 hatched alevins per treatment to use in trials. Removing all early hatched alevins ensured that the larvae used in the trials were of consistent age, so minimising the age-related variation in behaviour and morphology in trial one. Once there were a minimum of 24 alevins, they were allowed to develop for one day prior to use in the motor activity assay. This ≤ 1 dph development period was revised to 2-4 dpf in subsequent trials when the author became aware of literature which pointed to a lack of photonegative response in newly-hatched alevins (Stuart 1953).

As sublethal teratogenic effects of PEITC exposure did not reproduce the trends in previous trials (see section 5.4.1), it was speculated that effective dosing had not been achieved in trials two and three. Two possible factors were considered:

1. The PEITC used was from a bottle opened some months prior for use in a previous trial. As PEITC is volatile (Chen and Ho 1998; Doheny-Adams et al. 2018), the act of opening and closing the bottle and its length of storage may have caused the product to degrade.
2. The PEITC was frozen prior to use, which may have caused changes to the compound.

Factor one was considered the most likely explanation, but to address both possibilities, subsequent trials utilised freshly opened stock and did not freeze before use.

Mortality, hatch timing and spinal malformations for trials two and three are reported in the results section. Morphometrics such as length and weight measurements are very time-consuming to obtain, so these were not gathered when it became apparent from the lack of hatching delay and morphological anomalies that effective PEITC dosing had not been achieved. In addition to the failure to effectively dose PEITC, the motor activity assay was conducted on alevins before onset of photonegative behaviour so has not been reported.

5.3.6.1.3 Salmo trutta: trials four and five

On each dose day, fresh PEITC/DMSO stock solution was prepared before dosing. Every three weeks a new bottle of PEITC was opened and the old PEITC discarded in order to maintain freshness. Newly hatched alevins were removed daily from each treatment at ≤ 1 dph and euthanised until the day there were at least 24 hatched alevins per treatment, at which point these were set aside and allowed to develop for four days (trial four) and two days (trial five) for the motor activity assay. Morphometric analysis for each treatment was carried out on the *n* = 24 alevins used in the motor activity assay at 4 dpf in trial four, and 2dph trial five. In addition, 30 randomly selected alevins from the day of peak hatch (the day that > 50% hatched) and all subsequent later hatches were euthanised daily at ≤ 1 dph and preserved in formalin for morphological analysis.

5.3.6.2 *Cyprinus carpio* trial

The experimental set up was identical to that described for *S. trutta* in trial one, but with three key adjustments for *C. carpio*. The frequency of dosing was raised to once every 24 hours to better replicate exposures in a lake fed by multiple watercress farm discharges such as Arlesford Lake. The temperature was adjusted with a target of 15.5 °C (see 5.3.4.2), resulting in mean values over the duration of the study from 1dpf of 15.5 ± 2 °C, with DO of 9.8 ± 4 mg/L, and a pH of 7.6 ± 0.2. The tanks were not blacked out but were allowed natural light from nearby windows to enter, thereby replicating the natural photoperiod that *C. carpio* would experience during embryogenesis in the wild.

Newly hatched fry were counted and those that hatched early were removed and euthanised. The day on which at least 50% of eggs from a given treatment had hatched was considered the median hatch day, and on this day *n*= 36 of these larvae per treatment were kept for the behaviour trials and morphological analysis. Due to the very small mass of *C. carpio* larvae, unlike *S. trutta*, no weight measurements were possible.

5.3.6.3 *Danio rerio* watercress assay trial

The *D. rerio* trial used non-blacked out tanks as per the *C. carpio* trial. The temperature was set at 24 °C, resulting in mean temperature of 23.8 ± 0.3 °C, with a pH 8.01 ± 0.17 and DO of 8.5 ± 3 mg/L. Dosing was performed daily due to the short incubation period of the species and utilised the same PEITC treatments and solvent and water controls used in all previous trials. In addition to these, an expansion of the laboratory rearing system capacity allowed for the inclusion of more replicates. This capacity was used to house four additional treatments comprising of three replicates of watercress assay. The purpose of the watercress assay was to approximate salad wash effluent from the watercress farm on The Bourne Rivulet (see 2.6.1) to investigate if similar embryotoxic responses to PEITC in earlier trials were observed which may establish a link between PEITC embryotoxicity and salad wash effluent. It should be noted that in the absence of analytical methods to measure PEITC concentrations, actual PEITC concentrations in the watercress assays were unknown.

The salad wash process on the Bourne aims to wash 1kg leaf in 50 L water. An approximately 5% maximum of the leaf tissue becomes loose and is macerated by water pumps and enters the discharge (Steve Rothwell, pers. comms 2018). With 5% as a maximum estimate, it would be expected that up to 1 g wet weight of watercress tissue would potentially be macerated by pumps per litre discharged. As this is a maximum estimate, 1 g/L has been chosen as the highest nominal concentration. PEITC release through bruising and tumbling of watercress tissues during washing is likely to be less than released through maceration of tissues by the pumps, so the 5% estimate could be considered to include release from bruising and tumbling (Steve Rothwell, pers. comms 2018).

To replicate salad wash, 1g of watercress tissue containing leaf and stem was freshly picked from a living potted watercress plant and was blended in a falcon tube with 50 ml of matured tap water for 5 seconds using a homogeniser. To remove debris, the homogenate was poured through aquarium filter wool placed in an aquarium hand net and the resultant filtered liquid added to 950ml of matured tapwater at 24 °C. Logarithmic serial dilutions were performed to produce a

further two concentrations, resulting in treatments of 1, 0.1 and 0.01g wet weight watercress tissue per litre (WX1, WX 0.1 and WX 0.01 respectively).

To isolate the action of PEITC in salad wash discharge, a further treatment (WXD) was produced by boiling 1g of watercress tissue for 10 minutes to denature the enzyme myrosinase which catalyzes the hydrolysis of glucosinolates to form PEITC (Traka and Mithen 2009). Doing so produced watercress tissue that had inhibited capacity to produce PEITC when subsequently homogenised. This thermally deactivated myrosinase watercress assay was filtered as before into a 1 g/L assay, but with no serial dilutions due to space limitations in the laboratory.

Thirty-six *D. rerio* larvae were randomly picked from each treatment at ≤ 1 dph during peak hatching for the motor activity assay. Following the motor activity assay, they were euthanised and fixed in formalin as in prior trials before morphological analysis. Due to the small mass of *D. rerio* individuals, weight measurements were not possible.

5.3.7 Embryo mortality and hatch timing

Daily cumulative tallies were maintained for mortalities for all trials. Mortality was expressed as the number of expired embryos, the percentage expired and the daily mortality rate (DMR), calculated as per Réalis-Doyelle et al. (2016), where:

DMR = ((Number of dead individuals over a period of time / number of days within this period of time) x 100 / Total number of individuals)

Accurate records of daily hatches were maintained for all trials except trial one, in which visual estimates were made as described in section 5.3.6.1.1. Mean and median hatch times were obtained, and significance testing performed, on the dpf hatch values for every individual in a treatment. It was not possible to statistically analyse the hatch timing of the *S. trutta* during trial one due to the lack of precise hatch numbers for each day.

5.3.8 DanioVision™ behavioural study

Activity levels under light and dark conditions were investigated using a motor activity assay in a DanioVision™ Observation chamber (Noldus, Wageningen, Netherlands). This closed system consisted of an infrared Basler acA1300-60 GenICam camera (Basler AG, Ahrensburg, Germany) fitted above a chamber which tracked movements of fish placed in microtiter plates. 96-well microtiter plates were used for *C. carpio* and *D. rerio* and 6-well plates for the larger *S. trutta* alevins (Figure 67). Water at the respective rearing temperatures was circulated underneath the plates to maintain consistent temperature in the wells (arenas).

Figure 67. 6-well microtiter plate containing 4dph *S. trutta* alevins situated in the DanioVision™ observation chamber

5.3.8.1 *Salmo trutta* trials

From each treatment, 24 alevins were randomly selected by combining the three replicates into one beaker which was gently swirled while pouring into an aquarium hand net until the correct number of alevins were available. These were transferred into individual arenas using a plastic 3ml pipette, prepared by cutting off the narrowed tip. Each 6-well plate contained alevins from the same treatment, with the order of trial randomised. Each arena was filled with 10 ml of water taken from their respective housing beakers. The trials were conducted in a dimmed room, with the alevins transferred rapidly into the DanioVision™ Observation Chamber to minimise light exposure. The alevins were first allowed to acclimatise for four minutes in darkness prior to recording a 10-minute cycle of two minutes of dark/light/dark/light/dark. The cycle times were set at two minutes, similar to the studies by Hua et al. (2014) and Bossus et al. (2014). The cycle for *S. trutta* was started with darkness to replicate the conditions alevins would find in a redd, with the light part of the cycle mimicking a disturbance to the redd.

5.3.8.2 *Cyprinus carpio* trials and *Danio rerio* trials

The behaviour trials for both *C. carpio and D. rerio* were similar to one another but distinct from the *S. trutta* trials largely due to the smaller size of the larvae and the different light requirements. Both *C. carpio and D. rerio* trials commenced in the afternoon when the larvae were acclimatised to light. Replicates of each treatment were first combined before $n = 36$ larvae from each treatment were picked out at random with a Pasteur pipette and placed into 96-chambered well plates along with water from the respective treatment. The placement of larvae of each treatment in the well plate was randomised by row number. *C. carpio* larvae were given two minutes of acclimatisation in the behavioural chamber in the light, before the recorded trial started. The trial consisted of six minutes of light, four minutes of dark, followed by four minutes of light. *D. rerio* larvae were allowed four minutes acclimatisation followed by four minutes of light/dark/light dark.

5.3.8.3 Data analysis

At the onset of recording, the position of fish were recorded every 0.04 seconds using EthoVision XT V11 software (Noldus, Wageningen, Netherlands) with the software recording the activity in terms of velocity (cm/s), distance moved (mm), duration of movement (s) and duration of inactivity (s) of each subject.

The measurements of activity produced near identical trends during all trials of the present study, so just one endpoint has been chosen for analysis and reporting; the distance moved (mm) per unit time. For each treatment, the distance moved by all fish of each treatment between each 0.04 s frame was averaged. These averages were subsequently averaged into 10 s time bins for analysis of overall activity levels during the trial and activity levels in light and dark conditions. The data for each 10 s bin summarised the distanced moved over the preceding 10 s.

The data from two arenas in trial five were removed as outliers (one 0.01 µg/L PEITC and one water control treatment) and four from trial seven (two individuals in WX 0.1 and one each in WC and WX 0.01 treatments) due to exceptionally high locomotory readings. The video playback of the trial revealed that the software had not successfully tracked the fish, with the tracking points moving rapidly and independently of the animal. In trial seven, three further outliers were removed from the 0.1 ug/L PEITC treatment as the fish did not move throughout the trial and were presumed dead.

5.3.9 Morphometrics

All eleutheroembryos were removed from the formalin preservative and briefly rinsed in fresh water prior to photographing.

S. trutta alevins had the formalin-hardened yolk dissected, and both the yolk and body were blotted dry of moisture. The body, including the membrane surrounding the yolk, and the yolk itself were then separately weighed to four decimal places on a Sartorius AZ124 analytical balance (Germany). The alevins were photographed laterally on both sides and dorsally using a Fujifilm x70 camera (Fujifilm, Tokyo). Photography was performed after the removal of the yolk sac, allowing the alevins to lie flat to increase accuracy of image analysis. A graticule was included for scale.

C. carpio and *D. rerio* larvae were imaged using a Motic SMZ-168 series zoom microscope with a Moticam 5 attachable C-mount camera and Motic Images Plus 2.0 software (Motic Asia, Hong Kong). The fish were placed on a slide before excess water was blotted off with a tissue, allowing the vertebrae of the fish to adopt its natural resting position. Larvae were imaged laterally on both sides, and dorsally where there was scoliosis (lateral curvature of the spine). A graticule was included in the images for scale. Due to the very small mass of *C. carpio* and *D. rerio* larvae, weighing individuals proved to be impractical on a 4 decimal place balance, so no body weight data was collected.

All image analysis was undertaken in ImageJ (https://imagej.nih.gov/ij/), with length measurements in mm to three decimal places. Total length was recorded for *S.trutta* alevins, running from the front of the head to the end of the caudal fin (Figure 68). *C. Carpio* and *D. rerio* larvae had translucent and indistinct caudal fins, so standard length was recorded, being the length from head to the posterior of the caudal peduncle (Figure 68). In instances of scoliosis, total length was either measured from the dorsal image, or with *S. trutta* by flattening the alevin down with forceps during photographing. Instances of lordosis (excessive inward curvature), kyphosis (excessive outward curvature) or scoliosis (abnormal lateral curvature) were recorded as presence or absence following visual identification (Figure 69). In addition, some individuals exhibited a strong anterior-posterior compression of the vertebrae, and these were recorded as ‘stump body’. Lastly, any animal displaying any one or combination of the aforementioned spinal abnormalities was recorded as displaying vertebral column disorder (VCD).

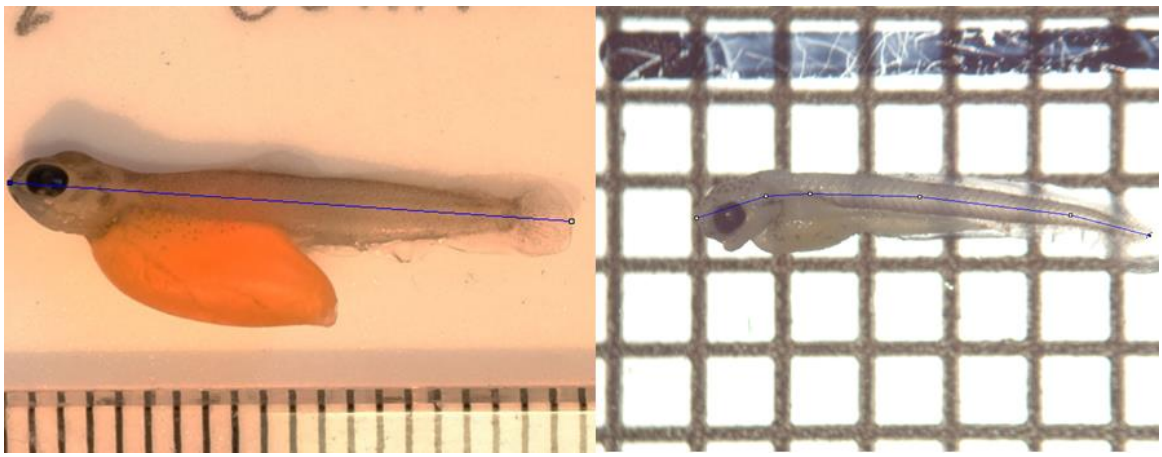

Figure 68 Measuring standard length in *S. trutta* (left) and standard length in *C. carpio* (right) using imageJ. *S. trutta* alevins were considerably larger than *C. carpio* and *D. rerio* larvae so were photographed using digital camera with a ruler to calibrate the scale in mm. *C. carpio* and *D. rerio* were photographed under the magnification of a dissecting microscope placed on a graticule slide with 1mm gridlines for calibration

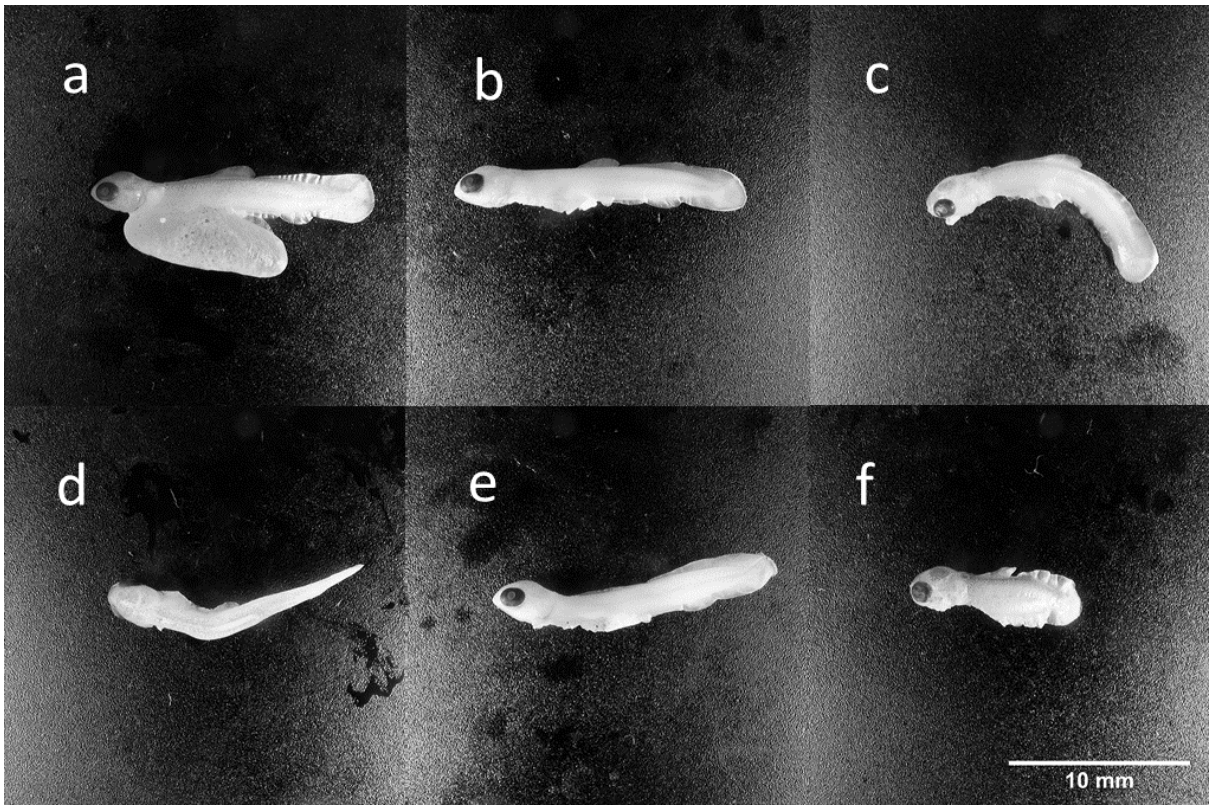

Figure 69 *Salmo trutta* alevin morphology, examples of a) normal alevin before removal of yolk sac; b) normal alevin after removal of yolk sac; c) lordosis; d) scoliosis; e) kyphosis and f) stump body

5.3.10 Statistical analysis

Data for morphometrics and hatch timing were tested for normality using the Shapiro-Wilk test, and homogeneity of variance evaluated using the Levene test. Where data were normally distributed and exhibited homogenous variance, a one-way ANOVA was performed, followed by Tukey's post-hoc test (95% CI). In converse cases, data were analysed using the Kruskal-Wallis test, with significant differences in medians explored using pair-wise Mann-Whitney tests. In the *D. rerio* trial where more than four treatments were compared, significant differences were explored using the Dunn test.

There was no significant difference between the solvent control and water control for the majority of endpoints so, following OECD protocol (OECD 1992; Green and Wheeler 2013), the data for both controls were pooled to increase statistical power. As recommended in the OECD protocol, in cases where there was a significant difference between the controls, the solvent control only was used in the analysis.

The motor activity assay data were analysed using Repeated Measures ANOVA with individuals set as a random factor and treatment and condition as set as fixed factors.

Statistical analysis for all but the Dunn test were performed using Minitab 18 (Minitab, Inc, USA) with significance set at $p < 0.05$. The Dunn test was performed in R, using the package "FSA" and used the Bonferroni method to control for family wide error rate.

5.4 Results

5.4.1 Salmo trutta

5.4.1.1 Embryo mortality following embryonic exposure to PEITC

Exposure during embryonic development to the highest PEITC concentration resulted in 100% mortality in all five *S. trutta* trials (Table 42) corresponding with four dose days of PEITC exposure at 1 µg/L. 100% mortality was reached by 11 dpf in trial one, 12 dpf in trials four and five and more slowly in the photoperiod trials two and three (14 and 13 dpf respectively). Lower concentrations of PEITC did not result in total mortality, but exposure to 0.1 µg/L PEITC raised daily mortality rates (DMR) and percentage mortality fourfold over other treatments in trials one, four and five. Exposure to 0.1 µg/L PEITC in trials two and three using photoperiod embryos did not increase DMR and percentage mortality rates compared to the controls. Exposure to 0.01 µg/L PEITC increased DMR over the controls in trial one but was on par with controls for all other trials.

5.4.1.2 Hatch timing following embryonic exposure to PEITC

Exposure to 0.1 µg/L PEITC delayed approximate hatching times in trial one where significance testing was not possible (Table 42). In trial four, exposure to 0.1 µg/L PEITC significantly delayed the median hatch relative to both the control ($W = 49062.00$, $p < 0.001$) and the 0.01 µg/L PEITC treatment ($W = 56975.5$, $p < 0.001$). Similarly, in trial five there was a significant delay in median hatching for the 0.1 µg/L PEITC treatment over the control ($W = 52196.5$, $p < 0.001$) and the 0.01 µg/L PEITC treatment ($W = 16593.5$, $p < 0.001$). In both trials, the 0.01 µg/L PEITC treatment did not hatch significantly later than the controls (trial five $W = 56975.5$, $p = 0.440$; trial six $W = 62775$, $p = 0.314$).

There were significant differences in hatch timing for photoperiod trials two and three, but in contrast to other trials, there were no consistent patterns between PEITC dose and hatch timing.

Table 42 Mortality and hatch summary data of *Salmo trutta* exposed during embryonic development to 0.01, 0.1 and 1 µg/L PEITC and water control (WC) and solvent control (SC). Showing duration to hatch in days post fertilisation (Dn), actual mortality (Mort) and percentage mortality (% Mort), daily mortality rate (DMR), mean hatch (Mn) and median hatch (Md). Different letters in hatch groups refer to significant differences in hatch timing between treatments (Mann-Whitney with 95% CI). Asterisks indicate significant differences; *: *p < 0.05, **: p <0.01, ***: p <0.001*

Trial	*Treatment*	*n*	*Dn*	*Mort*	*% Mort*	*DMR*	*Mn*	*SD*	*Md*	*Hatch group*
	WC	150	45	24	16.00	0.372	n/a	n/a	43	n/a
	SC	150	48	13	8.67	0.193	n/a	n/a	45	n/a
Trial one	0.01 µg/L	150	51	43	28.67	0.597	n/a	n/a	48	n/a
	0.1 µg/L	100	51	61	61.00	1.271	n/a	n/a	48	n/a
	1 µg/L	150	11	150	100.00	9.091	n/a	n/a	n/a	n/a
	WC	148	42	39	26.35	0.627	41.03	1.286	41	a
Trial two	SC	147	43	31	21.09	0.490	41.09	0.654	41	a
(photoperiod	0.01 µg/L	148	43	38	25.68	0.597	41.31	0.973	41	b***
embryos)	0.1 µg/L	149	43	33	22.15	0.515	40.18	1.495	40	c***
	1 µg/L	150	14	150	100.00	7.143	n/a	n/a	n/a	
	WC	130	47	14	10.77	0.229	42.70	1.175	43	a***
Trial three	SC	127	46	12	9.45	0.205	43.21	0.874	43	b***
(photoperiod	0.01 µg/L	142	47	13	9.15	0.195	42.16	1.058	42	c***
embryos)	0.1 µg/L	136	47	16	11.76	0.250	42.88	0.942	43	d***
	1 µg/L	150	13	150	100.00	7.692	n/a	n/a	n/a	
	WC	145	42	6	4.14	0.099	40.48	0.640	40	a
	SC	142	42	6	4.23	0.101	40.33	0.560	40	a
Trial four	0.01µL L	143	42	6	4.20	0.100	40.34	0.561	40	a
	0.1µL L	143	42	23	16.08	0.383	40.88	1.036	41	b***
	1µL L	150	12	150	100.00	8.333	n/a	n/a	n/a	
	WC	150	43	2	1.33	0.031	41.65	0.667	42	a
	SC	150	43	4	2.67	0.062	41.72	0.653	42	a
Trial five	0.01µL L	149	43	4	2.68	0.062	41.76	0.639	42	a
	0.1µL L	150	43	16	10.67	0.248	42.53	1.098	42	b ***
	1µL L	150	12	150	100.00	8.333	n/a	n/a	n/a	

5.4.1.3 Motor activity assay

In trial four at 4dph (Figure 70a), Repeated measures ANOVA showed significant differences between treatments ($F_{3,232}$ = 9.05, p < 0.001), condition (light and dark) ($F_{1,232}$ = 57.41, p < 0.001) and the interaction between both factors ($F_{3,232}$ = 2.65, p = 0.049). Mean movement during periods of illumination was significantly greater than darkness for the water control, solvent control and the 0.01 µg/L treatment (Bonferroni post-hoc analysis; p < 0.001, p = 0.001, p = 0.028 respectively). There was no significant difference in distance moved between light and dark phases for the 0.1 µg/L treatment (p = 1). Total combined movement in both light and dark periods was significantly higher in the water control than the 0.01 µg/L and 0.1 µg/L treatments (p = 0.001 and p <0.001 respectively). There was no significant difference in total movement observed between all other pairwise comparisons (water control vs. solvent control (p = 0.069); solvent control vs. 0.1 µg/L (p = 0.103); solvent control vs. 0.01 µg/L (p = 1)). The mean alevin activity over time is presented in Figure 71. All treatments respond in with increases in activity in response to light, though the 0.1 µg/L treated alevins tended to respond less strongly to the stimulus. Moreover, they were less active following the initial change in condition.

In trial five at 2dph (Figure 70b), there were significant differences in mean distance moved between treatments ($F_{3,232}$ = 12.10, p = < 0.001) and between light and dark conditions ($F_{1,232}$ = 35.88, p < 0.001) but not in the interaction of the two ($F_{3,232}$ = 0.95, p = 0.419). During illumination, movement was significantly greater in the water control (p= 0.014) and the solvent control (p < 0.003), but not significantly greater for the 0.01 µg/L PEITC (p = 1) or the 0.1 µg/L PEITC treatments (p = 0.195). For the duration of the trial (light and dark periods), the mean movement was highest in the water control, and progressively decrease with increasing dosage of PEITC. However, only the water control was significantly different to other treatments (p < 0.001). The activity timeline for trial five at 2pdh (Figure 72) shows slightly less clear changes in activity across all treatments following stimulus than the older alevins in trial four at 4dph (Figure 71).

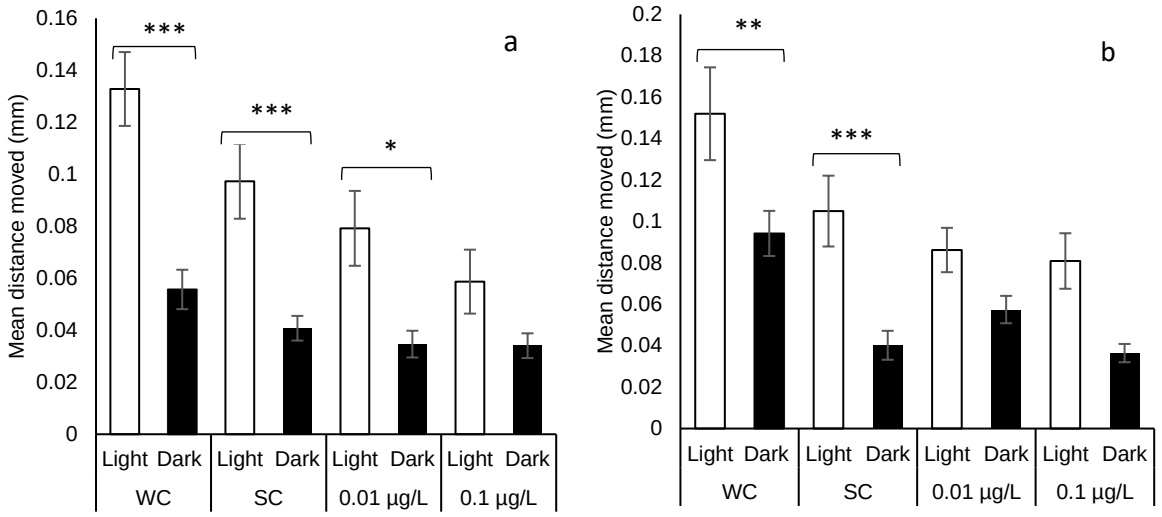

Figure 70 Brown trout (*Salmo trutta*) alevin mean (±SE) distance moved in light and dark periods following embryonic exposure of embryos to 0.1 and 0.01 µg/L PEITC; WC, water control; SC, DMSO solvent control. *N* = 24 alevins were used for each treatment, for trial four at 4dph (a) and trial five at 2 dph (b). Means generated from 10 s time bins during light and dark periods. Results of Repeated Measures ANOVA; *: p <0.05, **: p <0.01, ***: p < 0.001

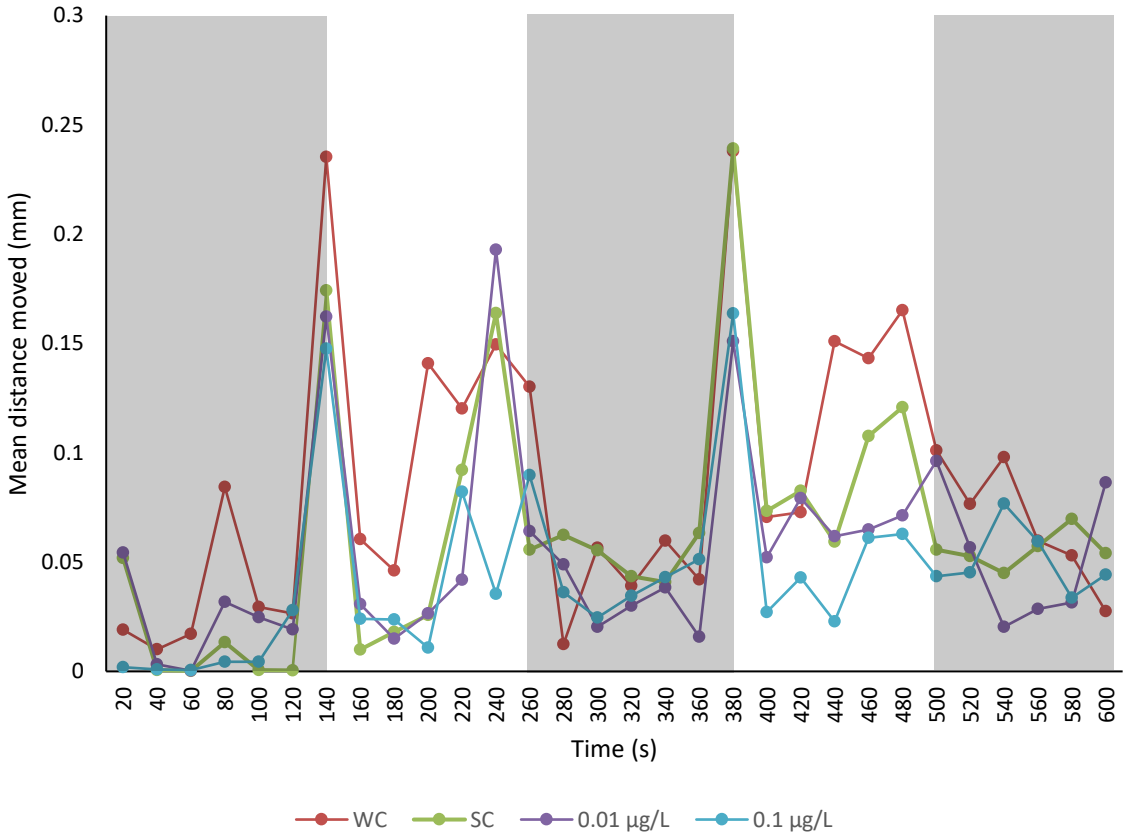

Figure 71 Brown trout (*Salmo trutta*) mean movement over time in trial four at 4dph of *n* = 24 alevins of each treatment; WC, water control; SC, solvent control and two PEITC treatments of 0.01 and 0.1 µg/. Alevins were tracked using a DanioVision™ behaviour chamber, which was illuminated during non-shaded timespans and dark during shaded timespans

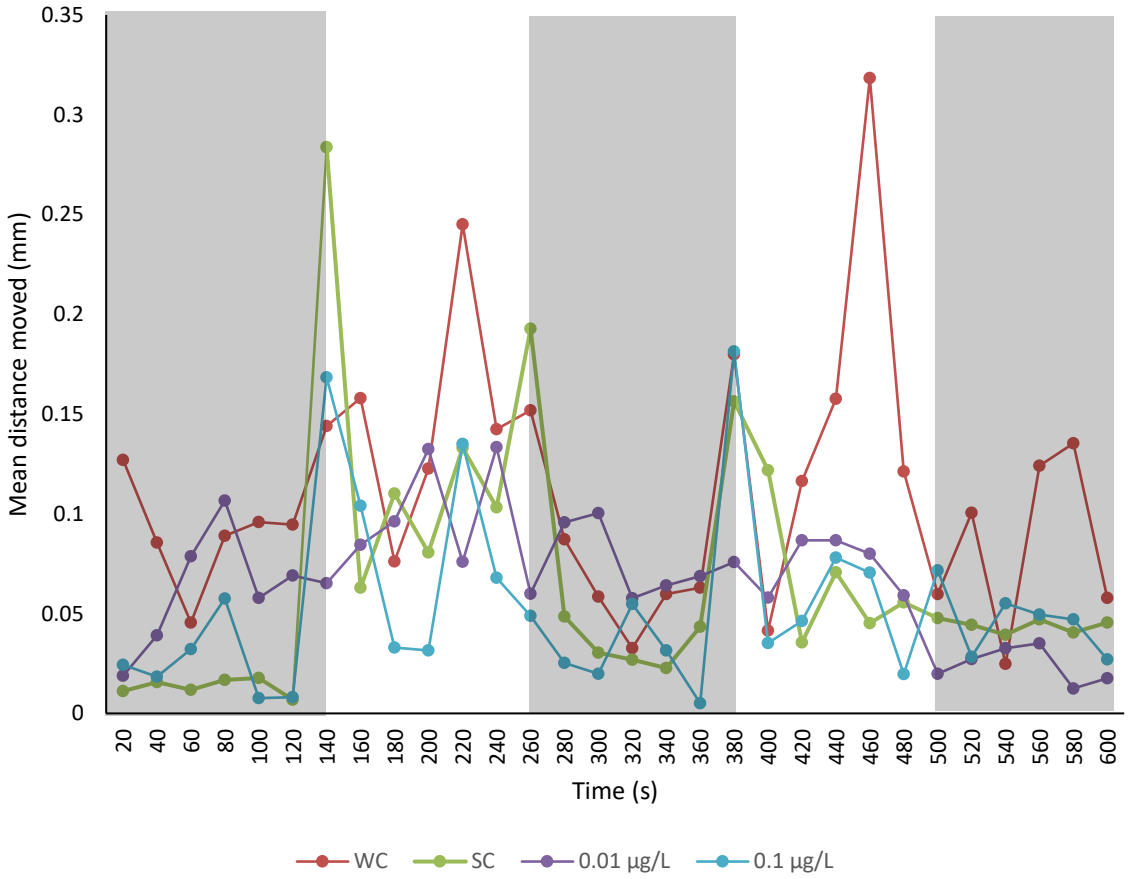

Figure 72 Brown trout (*Salmo trutta*) mean movement over time in trial five at 2dph of *n* = 24 alevins of each treatment; WC, water control; SC, DMSO solvent control and two PEITC treatments of 0.01 and 0.1 µg/L. Alevins were tracked using a DanioVision™ behaviour chamber, which was illuminated during non-shaded timespans and dark during shaded timespans

5.4.1.4 Spinal malformations: *S. trutta*

Exposure to 0.1 µg/L PEITC during embryonic development produced the highest percentage incidence of VCD in all trials with the exception of trials two and three which did not show any elevated incidences of VCD following PEITC exposure (Table 43). Moreover, no incidence of stump body was seen in trials two and three.

The percentage of all hatches sampled with VCD following exposure to 0.1 µg/L PEITC was 75% in trial one, 34.6% in trial four and 53.7% in trial five compared to the control range of 4.2% to 11.9%.

Exposure to 0.01 µg/L PEITC produced incidents of deformity within the range of the controls in trials five (8.5%) and six (9.9%), while in trial one it was 25% higher than the controls at 16.7%.

Incidents of VCD were higher in alevins that emerged later than the median hatch time (Table 44). In the 0.1 µg/L PEITC treatment, 52.6% (*n*=19) in trial four of those hatching after 41dpf, and in trial five 82.9% (*n*=41) of those hatching after 42dpf showing VCD. The condition 'stump body' was only observed in alevins exposed to 0.1 µg/L PEITC, accounting for 58.3% (*n*=24) of fish in trial one, 19.8% (*n*=81) of fish in trial four and 41.1% (*n*=95) of fish in trial five (Table 38). Similar to VCD, later hatches had a higher incidence of stump body than earlier hatches, accounting for 42.1% (*n*=19) and 69.5% (*n*=41) in trials five and six respectively after the median hatch date.

Table 43 Percentage incidence of spinal abnormalities in brown trout (*Salmo trutta*) alevins following embryonic exposure to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control. Included are the spinal deformities scoliosis, lordosis, kyphosis and stump body. The percentage of alevins exhibiting one or more of the aforementioned malformations summarised as total vertebrate column disorder (VCD)

Trial	*Treatment*	*n*	*Scoliosis*	*Lordosis*	*Kyphosis*	*Stump body*	*Total VCD*
Trial one	WC	24	0.0	4.2	0.0	0.0	4.2
	SC	24	0.0	4.2	0.0	0.0	4.2
	0.01 µg/L	24	0.0	12.5	4.2	0.0	16.7
	0.1 µg/L	24	12.5	33.3	4.2	58.3	75.0
Trial two (photoperiod embryos)	WC	24	0	4.2	0.0	0.0	4.20
	SC	24	0	0	0.0	0.0	0.00
	0.01 µg/L	24	4.2	4.2	0.0	0.0	4.20
	0.1 µg/L	24	4.2	0	0.0	0.0	4.20
Trial three (photoperiod embryos)	WC	24	4.2	4.2	0.0	0.0	4.20
	SC	24	4.2	4.2	0.0	0.0	4.20
	0.01 µg/L	24	4.2	8.3	0.0	0.0	8.30
	0.1 µg/L	24	0	4.2	0.0	0.0	4.20
Trial four	WC	*73*	6.8	0.0	0.0	0.0	6.8
	SC	*59*	10.2	1.7	1.7	0.0	11.9
	0.01µL L	*59*	3.4	5.1	0.0	0.0	8.5
	0.1µL L	*81*	16.0	6.2	3.7	19.8	36.4
Trial five	WC	*69*	1.4	4.3	0.0	0.0	4.3
	SC	*70*	1.4	4.3	0.0	0.0	5.7
	0.01µL L	*71*	8.5	2.8	0.0	0.0	9.9
	0.1µL L	*95*	15.8	11.6	6.3	44.2	53.7

Table 44 Percentage of brown trout (*Salmo trutta*) alevins displaying vertebral column disorder (VCD) and stump body after exposure to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control during embryonic development during trials four and five. The percentage of sampled alevins displaying spinal deformities at time of hatching in days post fertilisation (dpf) is shown, together with the percentage of all hatches over the duration of the study displaying spinal deformities

VCD	*Trial four*								*Trial five*							
	n	*40 dpf*	*n*	*41 dpf*	*n*	*> 41 dpf*	*n*	*all hatches*	*n*	*41 dpf*	*n*	*42 dpf*	*n*	*> 42 dpf*	*n*	*all hatches*
water	24	0.0	30	16.7	14	0.0	73	6.8	24	0.0	30	10.0	12	0.0	69	4.3
dmso	24	4.2	31	19.4	2	0.0	59	11.9	24	0.0	30	6.7	12	16.7	70	5.7
0.01 µg/L	24	8.3	30	10.0	0	n/a	59	8.5	24	16.6	30	6.7	15	6.7	71	9.9
0.1 µg/L	24	25.0	31	35.5	19	52.6	81	34.6	24	29.2	30	33.3	41	82.9	95	53.7

Stump body	*Trial four*								*Trial five*							
	n	*40 dpf*	*n*	*41 dpf*	*n*	*> 41 dpf*	*n*	*all hatches*	*n*	*41 dpf*	*n*	*42 dpf*	*n*	*> 42 dpf*	*n*	*all hatches*
water	24	0.0	30	0.0	14	0.0	73	0.0	24	0.0	30	0.0	12	0.0	69	0.0
dmso	24	0.0	31	0.0	2	0.0	59	0.0	24	0.0	30	0.0	12	0.0	70	0.0
0.01 µg/L	24	0.0	30	0.0	0	n/a	59	0.0	24	0.0	30	0.0	15	0.0	71	0.0
0.1 µg/L	24	12.5	31	22.6	19	42.1	81	19.8	24	16.6	30	23.7	41	65.9	95	41.1

5.4.1.5 Morphometrics

The morphometric endpoints total length, total weight, yolk weight, body weight and yolk:body ratio were examined for *S. trutta* alevins from trials four and five. Trial one results have not been described due to the age post hatch of alevins being unknown.

5.4.1.5.1 Total length

There was a significant difference in total length of alevins between treatments in both trials four and five and at all ages investigated (Table 45).

In trial four, at 1dph there were significant differences between treatments (H = 43.96, df = 2, p < 0.001). The 0.1 µg/L PEITC dosed alevins were significantly shorter than the solvent control (W =516.0, p < 0.001) and the 0.01 µg/L PEITC treatment (W =544.0, p < 0.001) (Figure 73). Alevins exposed to 0.01 µg/L PEITC during embryonic development did not exhibit any significant shortening relative to the control (W =856.0, p = 0.3871). At 4dph there were also significant differences between treatments (H = 45.88, df =2, p <0.001). Here again the 0.1 µg/L PEITC treatment was significantly shorter than the pooled control (W = 2278, p < 0.001) and the 0.01 µg/L treatment (W = 82800, p < 0.001). The 0.01 µg/L treated embryos were also significantly shorter than the pooled controls (W = 672, p = 0.015).

In trial five at 1dph, there were significant differences between treatments *(H* = 53.78, df = 2, p < 0.001), with the 0.1 µg/L PEITC treated alevins significantly shorter than the pooled control (W = 546, p < 0.001) and the 0.01 µg/L PEITC treatment (W = 1282.5, p < 0.001) (Figure 73). Once again, there was no significant difference in total length between the 0.01 µg/L PEITC and the pooled control (W =1169.0, p = 0.0943). At 2dph, there were significant differences (H = 28.72, df = 2, p < 0.001) with the 0.1 µg/L significantly shorter than the solvent control (W = 356.50, p <0.001) and the 0.01 µg/L treatment (W = 391.50, p <0.001). The 0.01 µg/L treatment was also significantly shorter than the solvent control (W = 687.00, p = 0.04).

Table 45. Brown trout (*Salmo trutta*) alevin morphometrics following embryonic exposure to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control, showing morphometry at 1, 2 and 4 days post hatch (dph)

	Trial four				*Trial five*			
	WC	*SC*	*0.01µg L*	*0.1gL L*	*WC*	*SC*	*0.01µg L*	*0.1µg L*
Morphometry at 1 dph								
n	*30*	*30*	*30*	*30*	*30*	*30*	*30*	*30*
Total length (mm)	14.80±0.44‡	15.55±0.41‡^{a}	15.41±0.47^{a}	13.76±1.56^{b***}	15.67±0.38§^{a}	15.50±0.49§^{a}	15.42±0.54^{a}	13.74±1.78^{b***}
Total weight (µg)	92.13±3.85§^{a}	93.81±3.14§^{a}	91.74±3.43^{a}	90.68±5.17^{b*}	72.91±3.05§	74.10±2.95§	74.16±3.22	73.30±3.91
Body weight (µg)	22.18±1.88‡	23.35±1.70‡^{a***}	25.42±1.62^{b***}	22.04±1.74^{c***}	22.18±1.19§^{a}	22.86±1.49§^{a}	21.73±1.82^{ab*}	21.50±1.97^{b***}
Yolk weight (µg)	69.95±3.37§^{a}	70.46±3.58§^{a}	66.32±3.35^{b***}	68.64±4.84^{ab*}	50.73±2.66§^{a}	51.27±2.84§^{a}	52.43±2.27^{b*}	51.80±2.81^{ab}
Yolk:body ratio	3.17±0.30§^{a}	3.04±0.33§^{a}	2.62±0.24^{b***}	3.12±0.34^{a}	2.30±0.16§^{a}	2.25±0.21§^{a}	2.43±0.21^{b***}	2.43±0.23^{b***}
Morphometry at 2dph								
n	-	-	-	-	*24*	*24*	*24*	*24*
Total length (mm)	-	-	-	-	15.73±0.31‡	16.07±0.42^{a}	15.86±0.33^{b*}	14.72±1.61^{c***}
Total weight (µg)	-	-	-	-	73.99±3.37§	73.90±3.40§	73.71±2.44	73.81±4.04
Body weight (µg)	-	-	-	-	23.28±1.78‡	24.75±1.60‡	23.66±1.36	24.00±2.35
Yolk weight (µg)	-	-	-	-	50.71±2.97§	49.15±2.63§	50.05±1.67	49.81±2.87
Yolk:body ratio	-	-	-	-	2.19±0.21‡	1.99±0.15‡	2.12±0.12	2.09±0.22
Morphometry at 4dph								
n	*24*	*24*	*24*	*24*	-	-	-	-
Total length (mm)	17.12±0.42§^{a}	16.93±0.47§^{a}	16.73±0.49^{b*}	15.54±0.99^{c***}	-	-	-	-
Total weight (µg)	93.61±4.21§	94.02±3.45§	94.26±3.77	93.23±3.49	-	-	-	-
Body weight (µg)	33.13±2.81§	32.30±1.98§	32.46±1.57^{a}	29.49±2.36^{b**}	-	-	-	-
Yolk weight (µg)	60.48±3.45§^{a}	61.72±3.61§^{a}	61.73±3.22^{ab}	63.74±2.63^{b**}	-	-	-	-
Yolk:body ratio	1.84±0.21§^{a}	1.92±0.20§^{a}	1.91±0.13^{a}	2.18±0.21^{b***}	-	-	-	-

Different letters refer to significant differences between treatments (mean ± SD, ANOVA or Mann-Whitney with 95% CI).
Asterisks indicate significant differences; *: p ≤ 0.05, **: p ≤0.01, ***: p ≤ 0.001
‡ WC was significantly different to SC, so only SC was used in statistical analysis
§ SC and WC were not significantly different so controls combined for statistical analysis

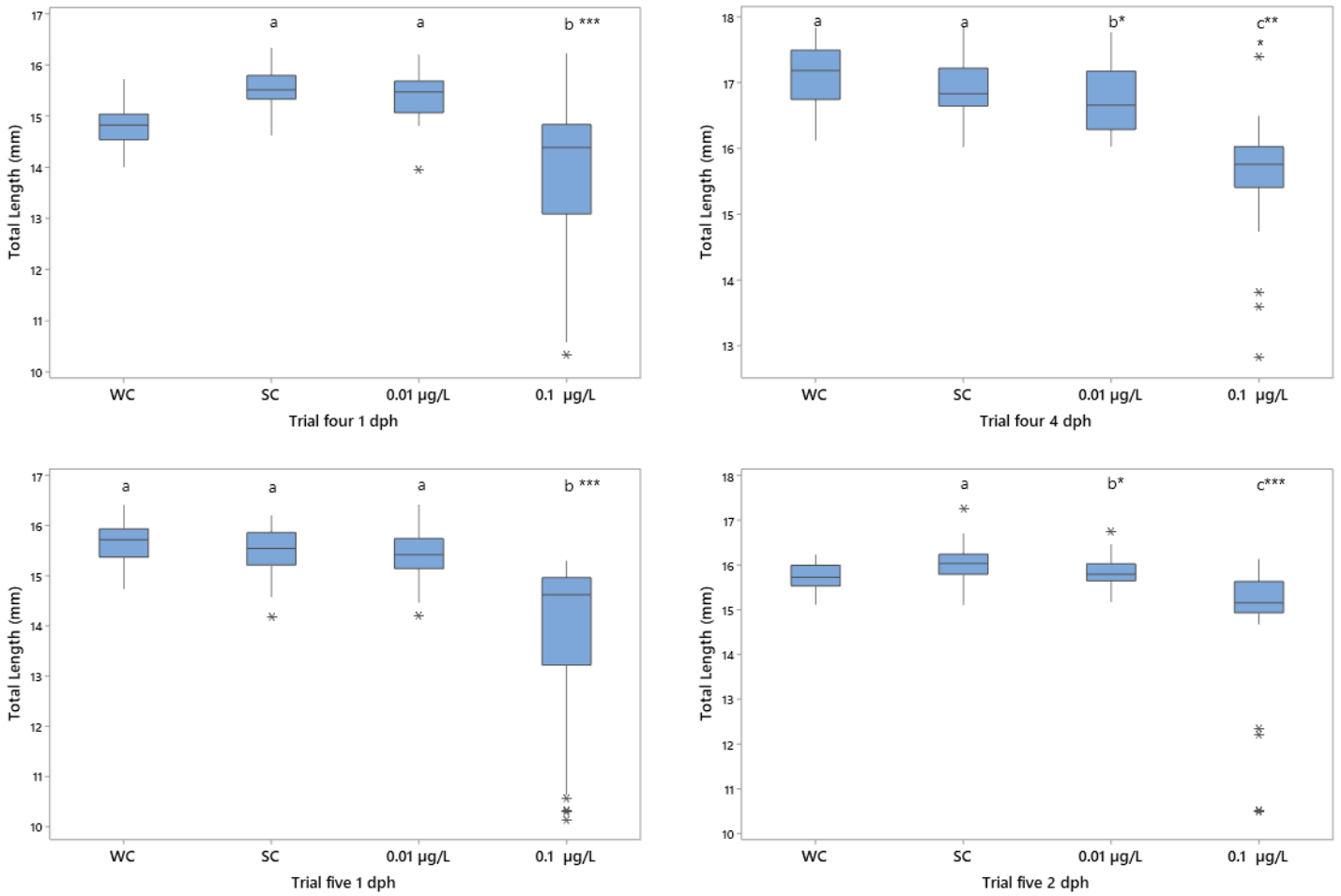

Figure 73 Total length of brown trout (*Salmo trutta*) alevins following exposure to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control, throughout embryogenesis in trial four at one and four days post hatch (dph) and in trial five at one and two dph (n = 24 per treatment). Median, interquartile range box (Q3-Q1), whiskers (25% of data) and outliers in asterisks. Different letters indicate significant differences in median values using Mann-Whitney tests (95% CI) A letter absent from the WC indicates that the WC was significantly different to the SC and so the SC was used for subsequent analysis. Where WC and SC share letters, the values of each were pooled for prior to statistical analysis. Asterisks indicate significant differences; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

5.4.1.5.2 Yolk and Body Weights

Trial four

The total weight of alevins collected at 1dph were significantly different between treatments ($F_{2,117}$ = 3.41, p = 0.036), with the 0.1 µg/L PEITC treatment significantly lighter than the pooled control ($F_{1,88}$ = 6.00, p = 0.016), but not the 0.01 µg/L PEITC treatment ($F_{1,58}$ = 0.88 , p = 0.352) (Table 45). The 0.01 µg/L PEITC treatment did not differ from the pooled control ($F_{1,88}$ = 2.40, p = 0.125). There were significant differences in both body ($F_{2,117}$ = 31.50, p < 0.001) and yolk ($F_{2,117}$ = 10.36, p < 0.001) weights across all treatments. This was driven by the 0.01 µg/L PEITC treatment having significantly heavier body weight and lighter yolk weights than the controls, resulting in a lower yolk:body ratio which differed significantly from the pooled control ($F_{1,88}$ = 53.38, p < 0.001), and the 0.1 µg/L PEITC ($F_{1,88}$ = 45.63, p < 0.001) treatment. The 0.1 µg/L PEITC treatment did not differ significantly from the pooled control ($F_{1,88}$ = 0.14, p = 0.712).

At 4dph, the total weight of alevins was not significantly different between treatments ($F_{2,93}$ = 0.46, p = 0.634). However, there were significant differences in body weight ($F_{2,93}$ = 17.82, p < 0.001) and yolk weight ($F_{2,93}$ = 5.28, p = 0.007) leading to significant differences in yolk:body ratios ($F_{2,93}$ = 20.68, p < 0.001). The yolk:body ratio of alevins exposed to 0.1 µg/L PEITC was significantly higher than the control ($F_{1,70}$ = 33.03, p <0.001) and the 0.01 µg/L PEITC treatment ($F_{1,46}$ = 28.43, p < 0.001), as a consequence of this treatment having a significantly lower body weight ($F_{1,70}$ = 28.46, p < 0.001) and higher yolk weight ($F_{1,70}$ = 10.41 , p = 0.002). In contrast to the alevins at 1dph, at 4dph the yolk:body ratio of the 0.01 µg/L PEITC treatment was not significantly different to the control ($F_{1,70}$ = 0.35, p = 0.556), nor was the yolk weight ($F_{1,70}$ = 0.65, p = 0.422) or the body weight ($F_{1,70}$ = 0.21 , p = 0.647).

Trial five

At 1dph the total body weight of alevins did not vary significantly across treatments ($F_{2,117}$ = 0.57, p = 0.564) (Table 45). However, there were significant differences in body weight ($F_{3,116}$ = 3.94, p = 0.010), yolk weight ($F_{2,117}$ = 3.11, p = 0.048) and yolk:body ratios ($F_{2,117}$ = 8.28, p < 0.001). The 0.1 µg/L PEITC treated fish were significantly lighter than the pooled controls ($F_{1,88}$ = 8.09, p = 0.006) but not significantly different to the 0.01 µg/L PEITC treatment ($F_{1,58}$ = 0.23, p = 0.636). The body weight of the 0.01 µg/L PEITC treatment was between the pooled controls and the 0.1 µg/L PEITC treatment and was significantly different to the pooled controls ($F_{1,88}$ = 5.16, P = 0.026) but not the 0.1 µg/L PEITC treatment ($F_{1,58}$ = 0.23, p = 0.636). Yolk weight of the 0.1 µg/L PEITC treatment was neither significantly different than the control ($F_{1,88}$ = 1.70, p = 0.195), nor the 0.01 µg/L PEITC treatment ($F_{1,58}$ = 0.91, p = 0.344), while the 0.01 µg/L PEITC treatment had significantly heavier

yolk weights than the control group ($F_{1,88}$ = 6.14, p = 0.015). The yolk:body ratio of the control was significantly lower than both the 0.1 µg/L PEITC treatment ($F_{1,88}$ = 11.07, p = 0.001) and the 0.01 µg/L PEITC treatment ($F_{1,88}$ = 12.15, p = 0.001), while the two PIETC treatments did not differ significantly ($F_{1,58}$ = 0.00, p = 0.993).

At 2dph in trial five, there were no significant differences in total weight ($F_{2,93}$ = 0.4, p = 0.959), body weight ($F_{2,69}$ = 1.25, p = 0.293) or yolk weight ($F_{2,93}$ = 0.05, p = 0.949).

5.4.2 Cyprinus carpio

5.4.2.1 Embryo mortality following embryonic exposure to PEITC

All embryos exposed to the highest PEITC concentration of 1 µg/L had died by 4 dpf, or three daily PEITC exposures (Table 46). There was an increase in percentage mortality and DMR with increasing PEITC concentrations, with the 0.01 µg/L treatment resulting in 28% mortality and a DMR of 2, and the 0.1 µg/L treatment having a 46% mortality and a DMR of 3.19. All PEITC treatments had higher percentage mortality and DMR than the controls, which were 8% and 0.615 for the WC and 11.33% and 0.872 for the SC respectively.

5.4.2.2 Hatch timing following embryonic exposure to PEITC

There were significant differences in hatch timing between treatments (df = 3, h-value 17.49, p = 0.001) with increasingly delayed hatch timing with increasing PEITC concentration (Table 46). There were significant differences between WC and SC (W-value 20485, p = 0.001) and between SC and 0.01 (W-value = 15211.5 p <0.001) and SC and 0.1 (W-value - 8931.5 p = 0.015). The 0.1 µg/L had a later median hatch than 0.01 µg/L, but not significantly so (W-value = 9845.5, p = 0.674).

Table 46 Mortality and hatch summary data of common carp (*Cyprinus carpio*) exposed during embryonic development to 0.01, 0.1 and 1 µg/L PEITC; WC, water control; SC, solvent control. Showing duration to hatch in days post fertilisation (Dn), actual mortality (Mort), percentage mortality (% Mort), daily mortality rate (DMR) and mean hatch (Mn) and median hatch (Md). Different letters in hatch groups refer to significant differences in hatch timing between treatments (Mann-Whitney with 95% CI). Asterisks indicate significant differences; *: *p < 0.05, **: p <0.01, ***: p <0.001*

Treatment	*n*	*Dn*	*Mort*	*% Mort*	*DMR*	*Mn*	*SD*	*Md*	*Hatch group*
WC	150	13	12	8.0	0.62	11.04	1.12	11	a***
SC	150	12	17	11.3	0.87	10.59	1.09	11	b***
0.01µg L	150	14	42	28.0	2.00	11.16	1.11	11	c***
0.1µg L	150	14	67	44.7	3.19	11.20	1.89	12	c
1µg L	150	4	150	100	25	n/a	n/a	n/a	n/a

5.4.2.3 Motor activity assay: *C. carpio*

In contrast to *S. trutta*, activity levels for *C. carpio* were greater during the dark phase than the light phase (Figure 74). Repeated measures ANOVA showed significant differences in the movement of *C. carpio* between treatments ($F_{1,152}$ = 21.36, *P* = <0.001), condition (light and dark) ($F_{1,152}$ = 97.85, *p* = <0.001), and the interaction between treatment and condition ($F_{3,152}$ = 15.72, *p* = <0.001). Across all treatments, activity levels were significantly higher in the dark phase than the light phase ($F_{1,158}$ = 65.80, *p* = <0.001). There were significant differences in activity levels between light and dark periods in the water control ($F_{1,38}$ = 36.38, *p* = <0.001), the DMSO control ($F_{1,38}$ = 48.82, *p* = <0.001) and the 0.01 µg/L treatment ($F_{1,38}$ = 37.29, *p* = <0.001), but not the 0.1µg/L treatment ($F_{1,38}$ = 1.92, *p* = 0.174).

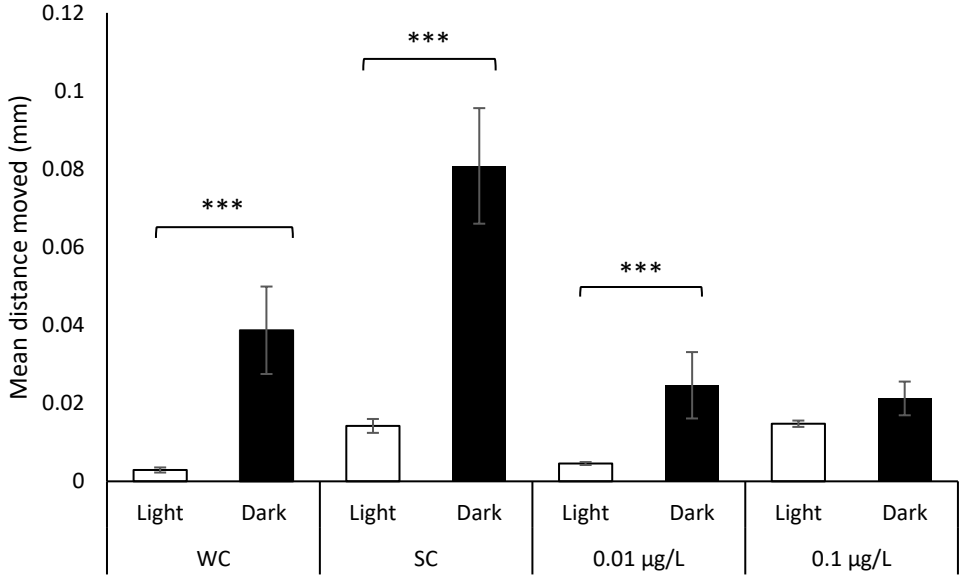

Figure 74 Common carp (*Cyprinus carpio*) mean (±SE) distance moved of fry in light and dark conditions following embryonic exposure of *n* = 36 fry per treatment to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control. Means generated from 10 s time bins during light and dark periods. Results of Repeated Measures ANOVA; *: *p* <0.05, **: *p* <0.01, ***: *p* < 0.001

Activity levels in *C. carpio* increased rapidly during the start of the dark phase at 380 s (Figure 75). Activity in the light phase was relatively high in the 0.1µg/L PEITC treatment, but the response to the dark phase was much weaker than all other treatments. The response to stimulus was greatest in the DMSO solvent control, while the water control and 0.01µg/L PEITC treatments were similar.

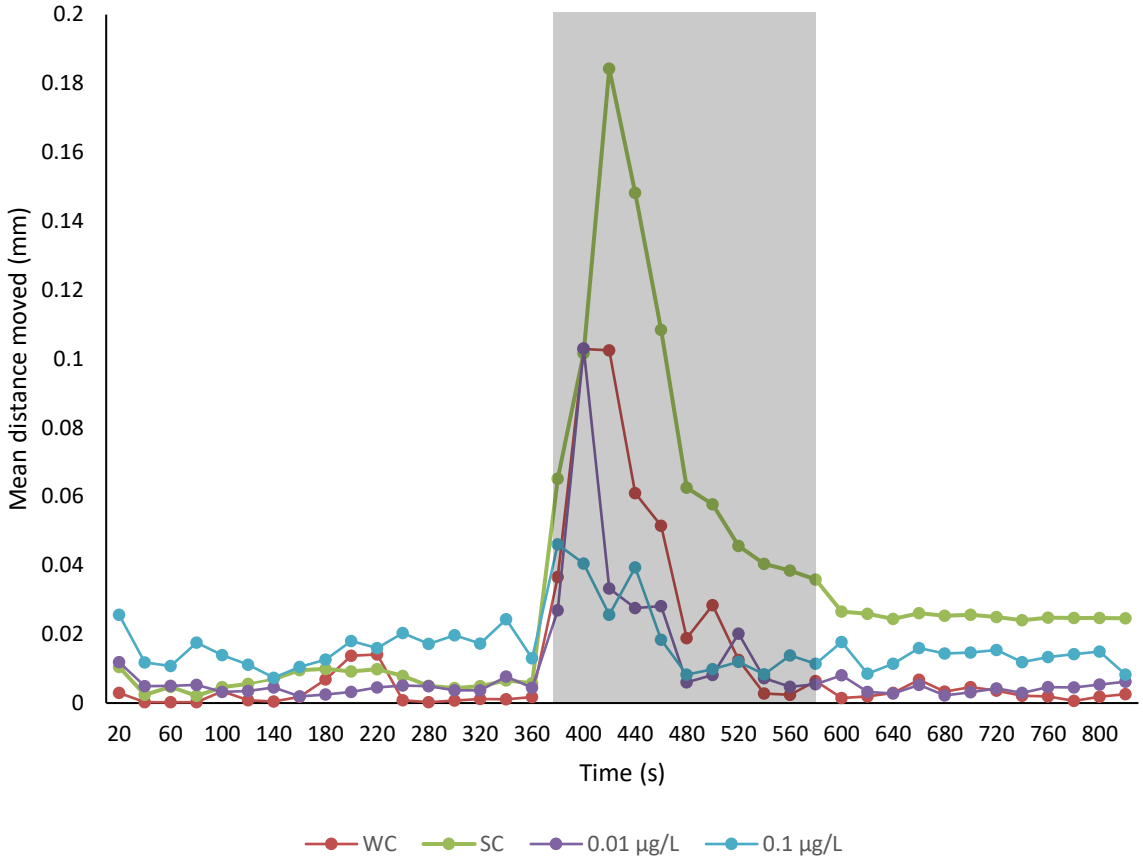

Figure 75 Common carp (*Cyprinus carpio*) mean distance moved of *n* = 36 larvae in 10 s time bins following exposure during embryonic development to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control. Movement recorded in an illuminated DanioVision™ behaviour chamber with shaded zone indicating timespan when light was switched off

5.4.2.4 Spinal malformations: *C. carpio*

Total incidents of VCD were greatest in the *C. carpio* larvae exposed to 0.1 µg/L PEITC during embryonic development, with nearly half (46.9%) of those exposed to 0.1 µg/L PEITC displaying some form of spinal malformation (Table 47). Incidences of VCD were closer to the controls for the 0.01 µg/L exposed animals, being 13.9% compared to 8.3% for the SC and 2.8% for the WC. Incidents of stump body were extremely low, with just one individual from the 0.1 µg/L PEITC treatment displaying the condition, giving a 3.1% incidence rate compared to 0% for all other treatments.

Table 47 Percentage incidence of spinal abnormalities in common carp (*Cyprinus carpio*) fry following embryonic exposure to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control. Included are the spinal deformities scoliosis, lordosis, kyphosis and stump body. Total vertebrate column disorder (VCD) is the percentage of fry exhibiting one or more of the aforementioned malformations

Treatment	*Embryo no*	*Scoliosis*	*Lordosis*	*Kyphosis*	*Stump body*	*Total VCD*
WC	36	2.8	0.0	0.0	0.0	2.8
SC	36	2.8	2.8	5.6	0.0	8.3
0.01 µg/L	36	8.3	5.6	2.8	0.0	13.9
0.1 µg/L	32	21.9	18.8	21.9	3.1	46.9

5.4.2.5 Morphometrics: *C. carpio*

Across all treatments, there were significant differences in mean standard length for *C. carpio*larvae ($F_{3,131}$ = 44.09, p = <0.001) (Figure 76). Standard length was significantly shorter for larvae exposed to 0.1 µg/L of PEITC during embryogenesis compared to the water control ($F_{1,61}$ = 77.46, p = <0.001), the DMSO control ($F_{1,63}$ = 81.16, p = <0.001) and the 0.01 µg/L PEITC treatment ($F_{1,66}$ = 47.71, p = <0.001). There were no other significant differences in pairwise comparisons of treatments.

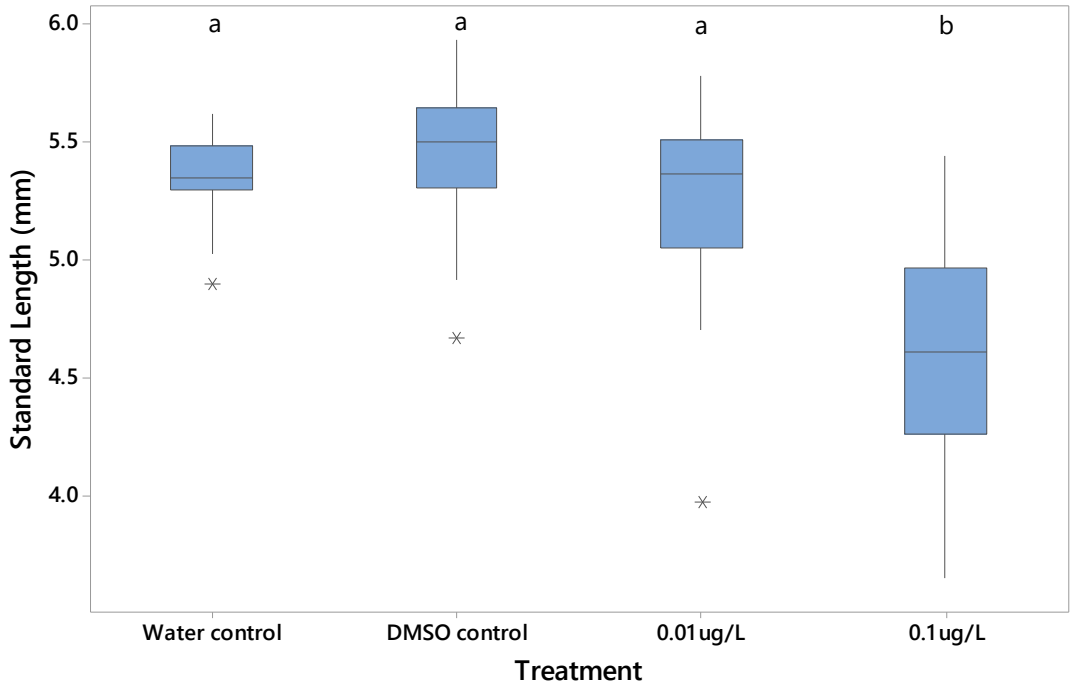

Figure 76. Standard length of common carp (*Cyprinus carpio*) larvae exposed to 0.01 and 0.1 µg/L PEITC; WC, water control; SC, solvent control throughout embryogenesis (n = 36 per treatment). Median, interquartile range box (Q3-Q1), whiskers (25% of data) and outliers in asterisks. Different letters indicate significant differences in means using Tukey's HSD (95% CI).

5.4.3 Danio rerio

5.4.3.1 Embryo mortality following embryonic exposure to PEITC and watercress assay

Embryo mortality was identical for the WC and SC, having a percentage mortality of 2.7% and a DMR of 0.333 (Table 48). Both mortality metrics increased with increasing concentrations of PEITC, being 8% with a DMR of 1.0 for the 0.01 µg/L exposed embryos and 9.3% with a DMR of 1.167 for the 0.1 µg/L exposed embryos. All Danio embryos exposed to 1 µg/L PEITC died within 24hrs, giving a 100% DMR.

The full-strength watercress assay (WX1) had the highest percentage mortality rate and DMR of all the watercress assays at 8.7% and 1.083 respectively. Both serial dilutions (WX 0.01 and WX 0.1) produced identical percentage mortality and DMR rates of 4.7 and 0.583 respectively. The deactivated myrosinase-deactivated treatment (WXD) produced the lowest percentage mortality and DMR of all treatments, including the controls (1.3% and 0.267 respectively).

5.4.3.2 Hatch timing following embryonic exposure to PEITC and watercress assay

There were significant differences in median hatch timing between treatments (*df* = 7, *H-value* = 212.11, *p* <0.001). The SC bought forward mean hatch timing (Table 42) and significantly bought forward median hatch timing relative to the WC (Z = -3.857, *p* = 0.001). The WC had significantly later hatches than all treatments other than WX 0.01. The SC was not significantly different to the PEITC treatments 0.01 and 0.1 ug/L (*p* = 1) (Table 49)

The full strength WX1 treatments hatched significantly earlier than the WC, and increasing dilutions produced hatch timings that were progressively later, with the lowest dilution WX 0.01 not being significantly different to the WC (*p* = 1). The WXD treatment produced the earliest hatch timings of all, being significantly different to all other treatments, including the second earliest-hatching treatment WX1 (*p* = 0.001)

Table 48 Mortality and hatch summary data of zebrafish (*Danio rerio*) exposed during embryonic development to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Showing duration to hatch in days post fertilisation (Dn), actual mortality (Mort), percentage mortality (% Mort), daily mortality rate (DMR), mean hatch (Mn) and median hatch (Md).

Treatment	*Embryo no*	*Dn*	*Mort*	*% Mort*	*DMR*	*Mn*	*SD*	*Md*
WC	150	8	4	2.70	0.333	5.145	0.717	5
SC	150	8	4	2.70	0.333	4.774	1.075	5
0.01 µg/L	150	8	12	8.00	1.000	4.839	0.964	5
0.1 µg/L	150	8	14	9.30	1.167	4.640	1.001	5
1 µg/L	150	1	150	100	100			
WX 0.01	150	8	7	4.70	0.583	5.441	1.320	5
WX 0.1	150	8	7	4.70	0.583	4.699	1.210	5
WX 1	150	8	13	8.70	1.083	4.102	1.107	4
WXD	150	5	2	1.30	0.267	3.662	1.187	4

Table 49 Dunn test results for pairwise comparisons of zebrafish (*Danio rerio*) median hatch rate following embryonic exposure to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control

	SC	0.01µg L	0.1µg L	WX 1	WX 0.1	WX 0.01	WXD
WC	**0.001**	**0.002**	**<0.001**	**<0.001**	**0.001**	1	**<0.001**
SC		1	1	**<0.001**	0.948	**<0.001**	**<0.001**
0.01µg L			1	**<0.001**	1	**<0.001**	**<0.001**
0.1µg L				**<0.001**	1	**<0.001**	**<0.001**
WX 1					**<0.001**	**<0.001**	**0.001**
WX 0.1						**<0.001**	**<0.001**
WX 0.01							**<0.001**

5.4.3.3 Motor activity assay

Repeated measures ANOVA showed no significant differences between treatments ($F_{7,352}$ = 1.2, p = 0.407), but significant differences in condition (light and dark) ($F_{1,352}$ = 100.65, p < 0.001) and the interaction between the both factors ($F_{7,352}$ = 92.49, p = < 0.001). Unlike the *S. trutta* and *C. carpio* trials, the mean movement during periods of darkness was significantly greater than for periods of illumination for all treatments (Figure 77). As it was evident that total movement in both light and dark conditions was reduced in the some of the treatments, the total movement over the duration of the *D. rerio* trial was explored for significant differences (Figure 78). This showed a step-wise decrease in movement with increasing PEITC concentration, with larvae exposed to 0.1 µg/L showing significantly less activity than 0.1 µg/L, which was significantly less than the controls. The water and solvent controls did not differ significantly. The full-strength watercress assay (WX 1), the deactivated WX and the lowest WX concentration (WX 0.01) were broadly similar and not significantly different from each other (p = 1) but had significantly activity than the controls. The intermediate watercress wash (WX 0.1) was significantly higher than all other WX treatments and was not significantly different to the controls.

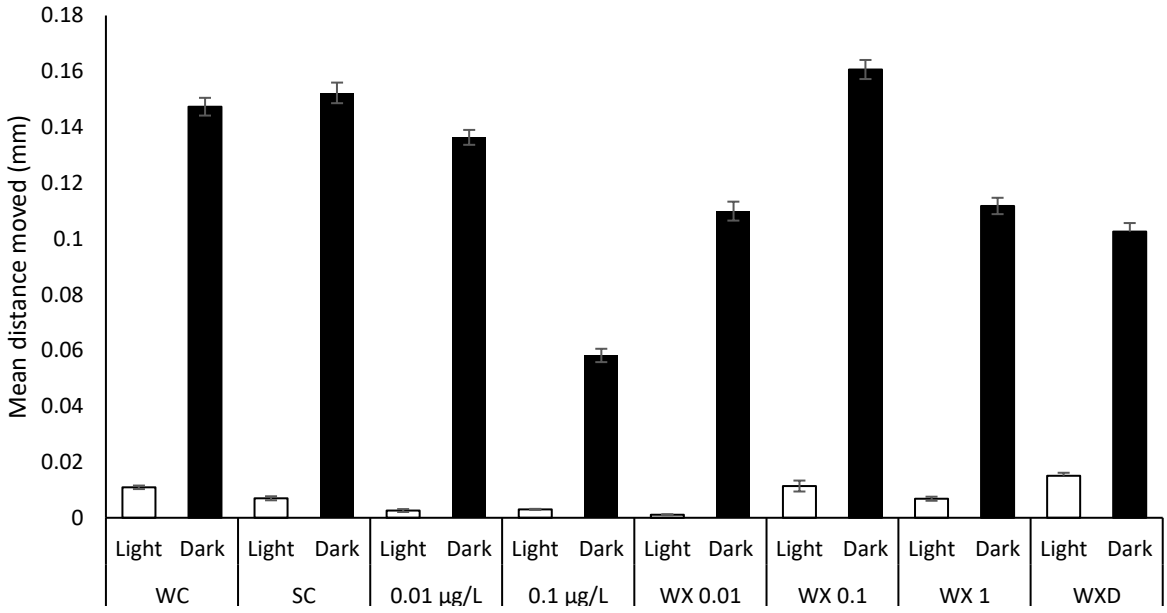

Figure 77. Mean distance moved (±SE) of zebrafish (*Danio rerio*) larvae in light and dark conditions following embryonic exposure of n = 36 larvae to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Means generated from 10 s time bins during light and dark periods. Differences between light and dark periods were significantly different for all treatments in repeated measures ANOVA/Bonferroni post-hoc comparisons (α 0.05)

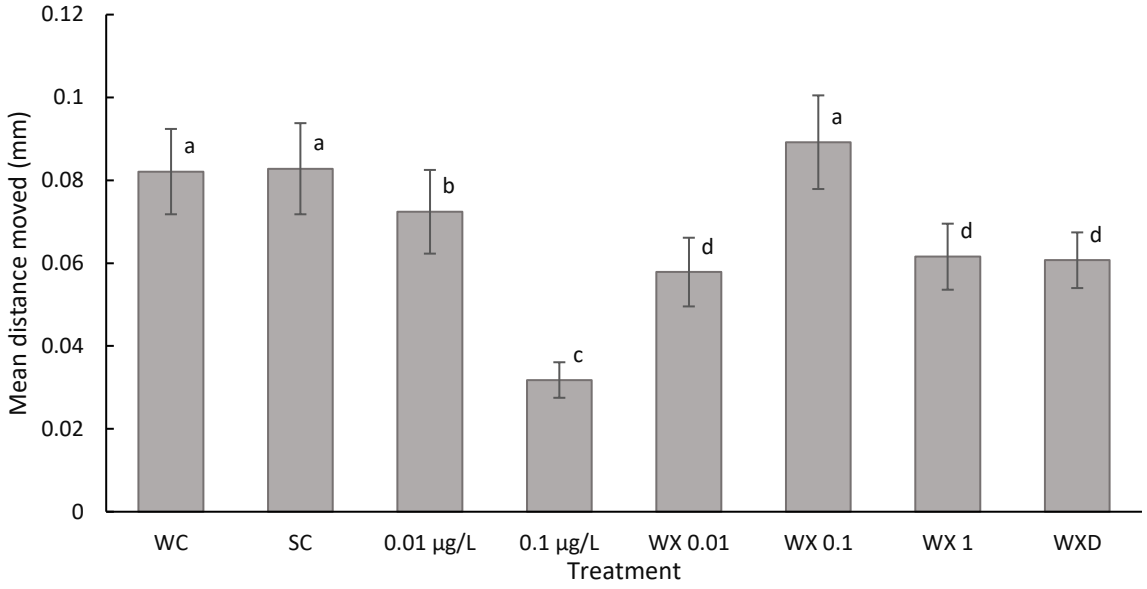

Figure 78. Mean movement (±SE) over duration of trial of zebrafish (*Danio rerio*) larvae (*n* = 36 per treatment) following embryonic exposure to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Different letters indicate significant differences in means using repeated measures ANOVA with Bonferroni post-hoc comparisons (α 0.05)

The activity levels of all but the 0.1 µg/L treated *D. rerio* larvae increased markedly when the chamber was darkened, then activity declined steeply over the next 40 s until activity levels became more stable (Figure 79). Activity dropped once more when the chamber was re-illuminated. The second darkness phase elicited an increase in activity but without the initial strong spike observed following the first dark phase. The 0.1 µg/L treated larvae displayed a reduced response to stimulus relative to all other treatments.

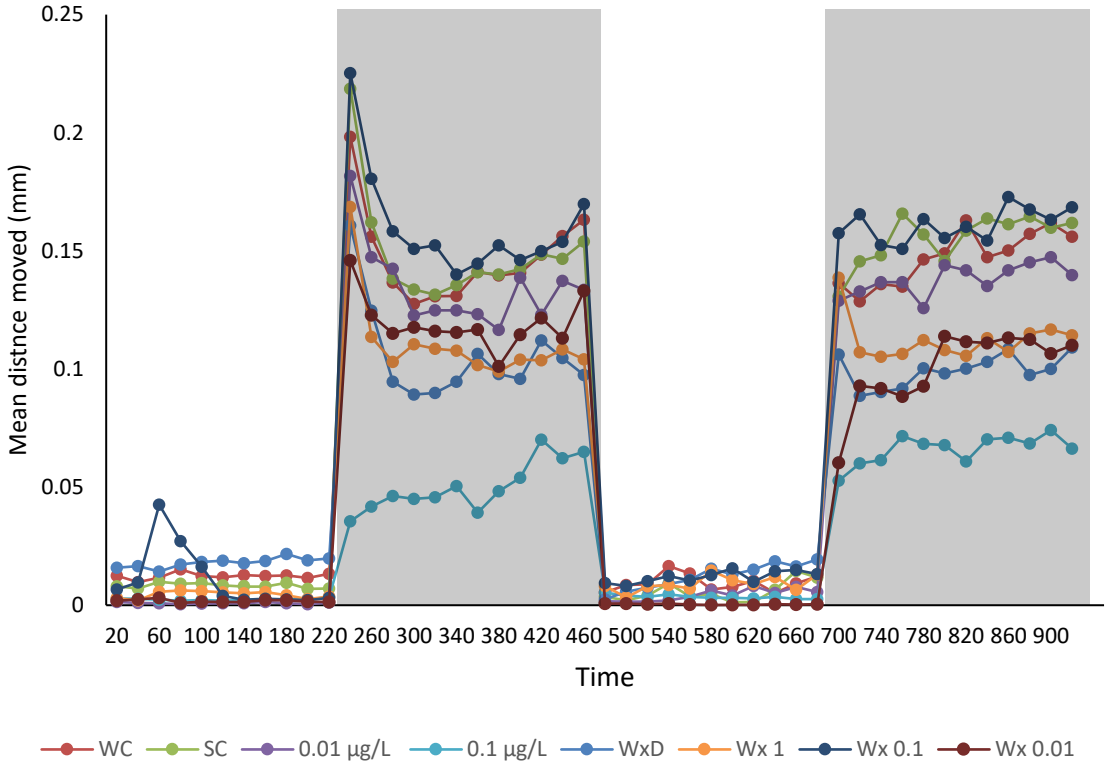

Figure 79 Zebrafish (*Danio rerio*) movement following embryonic exposure of 0.01 and 0.1 µg/L of phenethyl isothiocyanate, watercress assay to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Mean distance moved of *n* = 36 larvae for each treatment in 20 s time bins. Movement recorded in an illuminated DanioVision™ behaviour chamber with shaded zone indicating timespan when light was switched off

5.4.3.4 Spinal malformations

Incidents of spinal malformations in *D. rerio* were low in comparison to *S. trutta* and *C. carpio*, but similarly they increased in prevalence with increasing concentrations of PEITC (Table 50). The controls displayed no spinal malformations and the highest incidence of VCD (5.9%) was for the highest concentration of PEITC (0.1 µg/L). The myrosinase-deactivated WXD exposed embryos resulted in no malformations, while the undiluted WX1 resulted in 2.6%. The highest WX dilution had a VCD of 5.7% and the intermediate concentration WX 0.1 resulted in no malformations.

Table 50. Percentage incidence of spinal abnormalities in zebrafish (*Danio rerio*) larvae following embryonic exposure to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Included are spinal deformities scoliosis, lordosis, kyphosis and stump body. Total vertebrate column disorder (VCD) is the percentage of alevins exhibiting one or more of the aforementioned malformations

Treatment	*n*	*Scoliosis*	*Lordosis*	*Kyphosis*	*Stump body*	*Total VCD*
WC	29	0.0	0.0	0.0	0.0	0.0
SC	37	0.0	0.0	0.0	0.0	0.0
0.01 µg/L	30	0.0	0.0	3.3	0.0	3.3
0.1 µg/L	34	2.9	2.9	0.0	0.0	5.9
WX 0.01	35	5.7	0.0	0.0	0.0	5.7
WX 0.1	36	0.0	0.0	0.0	0.0	0.0
WX 1	35	2.6	0.0	0.0	0.0	2.6
WXD	33	0.0	0.0	0.0	0.0	0.0

5.4.3.5 Morphometrics

Significant differences in *D. rerio* larvae standard length were found between treatments ($F_{7,261}$ = 14.42, p = < 0.001). Tukey's HSD post-hoc analysis showed that of the PEITC treatments, the animals exposed to the highest concentration 0.1 µg/L had a significantly shortened standard length over the controls and all other treatments (Figure 80). Exposure to increasing concentrations of watercress assays resulted in step-wise decreased in total length, with the only significantly reduced total length relative to the controls caused by exposure to the highest concentration (WX1). The WXD watercress assay did not reduce standard length significantly relative to the controls.

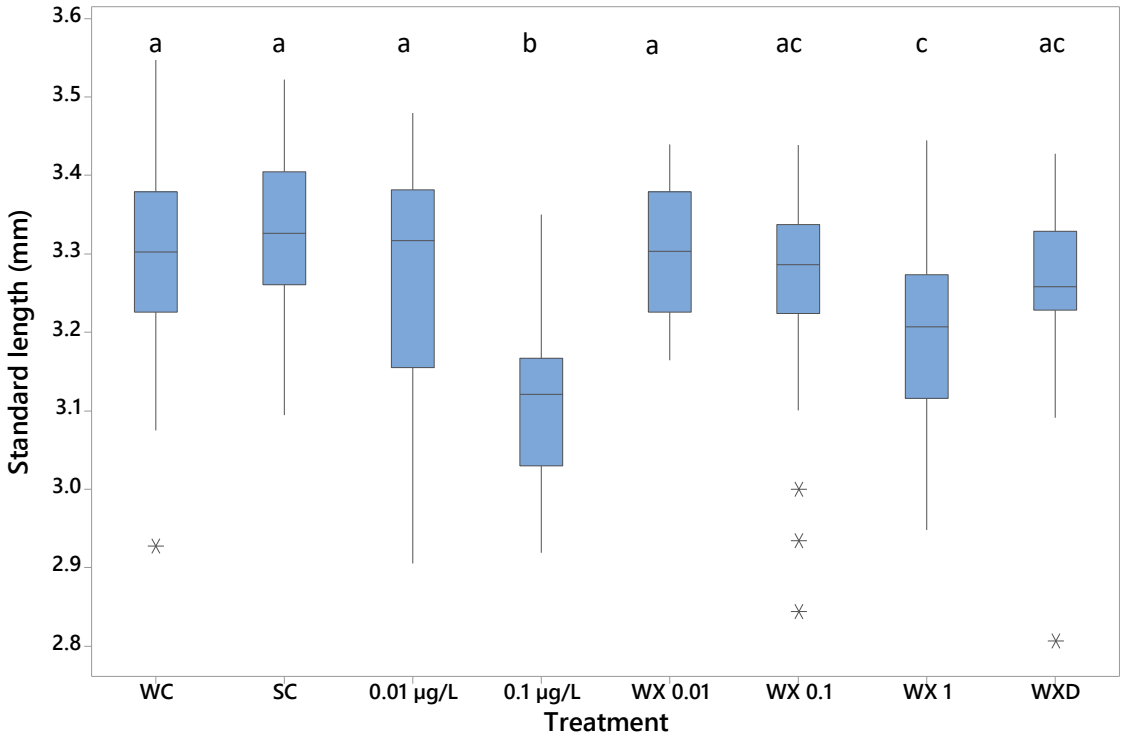

Figure 80 Standard length of *Danio rerio* larvae (*n* = 36 per treatment) at 1 dpf following embryonic exposure to 0.01 and 0.1 µg/L of PEITC; WX1, watercress assay at 1 g wet weight of watercress tissue per L; WX 0.1, serial dilutions by 10/1; WX 0.01, serial dilution 100/1; WXD, boiled watercress assay of 1g wet weight of watercress tissue per L; WC, water control; SC, solvent control. Median interquartile range box (Q3-Q1), whiskers (25% of data) and outliers in asterisks. Means that share letters are not significantly different (Tukey's post hoc HSD α 0.05)

5.5 Summary of key results

Exposure to 1 µg/L PEITC resulted in total mortality for all three species. For *S. trutta* and *C. carpio* this occurred within 4 dosing days, while for *D. rerio* it occurred more rapidly before two doses. Exposure to 0.1 µg/L PEITC raised embryo mortality rates above the controls and the 0.01 µg/L PEITC treatment for all species (Table 51). *S. trutta* and *C. carpio* exposed to 0.1 µg/L PEITC had non-significant responses to stimulus in a motor activity assay in contrast to the significant responses to stimulus seen in the controls. Though responses to stimulus were significant for all *D. rerio* treatments, the total movement during the trial was significantly reduced following exposure to 0.1 µg/L compared to the controls and the 0.01 µg/L PEITC treatment. For all species, incidences of vertebral column disorder (VCD) were highest in the 0.1 µg/L PEITC treatment, and total length was significantly reduced for all species relative to their respective controls. Exposures to 0.01 µg/L had a much less pronounced effect, though mortality rates were still in excess of the controls for all species. Sub-lethal impacts for 0.01 µg/L treatments were not consistent, with delayed hatching

evident in *S. trutta* and *C. carpio* but not *D. rerio* and a shorter total length in *S. trutta* but not the other two species. Motor activity was reduced for all species, but not as pronounced as the 0.1 µg/L PEITC treatment. Only *D. rerio* was exposed to the watercress assay, and unlike PEITC, this resulted in earlier hatching both in the full-strength assay and the PEITC deactivated assay. The full-strength assay resulted in the same mortality rate as the 0.1 µg/L PEITC treatment along with higher VCD incidence and shorter total length relative to the controls. The PEITC deactivated watercress assay resulted in lower mortality and no difference in total length relative to the controls along with zero incidents of VCD.

Table 51 Summary of key ecotoxicology results on *Salmo trutta*, *Cyprinus carpio* and *Danio rerio* trials using embryonic phenethyl isothiocyanate (PEITC) exposures of 1, 0.1 and 0.01 µg/L and for *Danio rerio* including a watercress assay (WX1) and a PEITC deactivated assay (WXD) at 1 g macerated watercress per L water. Percentage mortality of embryos for the duration of trial (% mort), the hatch timing relative to controls. Motor activity assay for S. trutta and C. carpio shows significance of difference between activity in light and dark conditions and for D. rerio the total activity relative to controls. The percentage of spinal deformities, vertebral column disorder (% VCD) and the length and weight relative to controls. Values are summarised as means for *S. trutta* as multiple trials were conducted, significant results where different in more than one trial separated by /. NS, not significant; nd, no difference; results of significance testing *: p <0.05, **: p <0.01, ***: p < 0.001

Species	*Treatment*	*% Mort*	*Hatch timing relative to control*	*Motor activity*	*% VCD*	*Length*	*Weight*
S. trutta	1 µg/L	100	-	-	-	-	-
	0.1 µg/L	29	later ***	NS	44	shorter***	lighter***
	0.01 µg/L	12	later	*/NS	12	shorter	lighter
	Controls	6	-	***	6	-	-
C. carpio	1 µg/L	100	-	-	-	-	-
	0.1 µg/L	45	later ***	NS	47	shorter***	-
	0.01 µg/L	28	later ***	***	14	nd	-
	Controls	10	-	***	11	-	-
D. rerio	1 µg/L	100	-	-	-	-	-
	0.1 µg/L	9	later	reduced ***	6	shorter	-
	0.01 µg/L	8	earlier	reduced **	3	nd	-
	Controls	3	-	-	0	-	-
	WX1	9	earlier ***	reduced ***	3	shorter	-
	WXD	1	earlier ***	reduced ***	0	nd	-

5.6 Discussion

The effect of embryonic exposure of PEITC was broadly similar for *S. trutta, C. carpio* and *D. rerio*, causing increased mortality, significantly delayed hatching, shorter body lengths, increased spinal malformations and reduced activity levels. Trials two and three using photoperiod manipulated *S. trutta* failed to replicate the sublethal and teratogenic impacts seen in all other trials. Photoperiod

manipulated rainbow trout (*Oncorhynchus mykiss*) have been shown to have higher levels of alevin malformations and lower embryo survival rates than natural photoperiod produced fish (Bonnet et al. 2007). The present study did not find such effects on the control photoperiod *S. trutta*, whose controls had similar mortality and malformation levels to *S. trutta* controls in the other trials. Aside from the use of photoperiod manipulated fish, trials two and three differed in that the PEITC used was from a bottle that had been repeatedly opened during use in a trial three months prior, with the PEITC/DMSO stock frozen in pre-weighed centrifuge tubes to be thawed before each dose. Kosson and Horbowicz (2009) investigated the decline in PEITC content of horseradish cream stored at 2, 8 and 18°C over nine months starting from two weeks after its production date. The maceration of horseradish produced concentrations of PEITC which declined from c.250 mg/kg at the start of the experiment to c.25 mg/kg at four months at 2 °C. The rate of degradation was higher still at 8 and 18 °C. The PEITC used in the present study was kept chilled at 4°C, but as the results of Kosson and Horbowicz (2009) suggest, the longer storage period in the *S. trutta* trials two and three may explain the failure of them to replicate prior and subsequent results. PEITC is volatile (Ji et al. 2005; Doheny-Adams et al. 2018) and the opening of the bottle is likely lead to increased degradation over time. Subsequent trials utilised newly opened bottles of PEITC and the results of earlier trials were replicated. As the photoperiod *S. trutta* trials two and three utilised degraded PEITC, they have been omitted from further discussion.

5.6.1 Embryotoxicity of PEITC

Fish embryos are sensitive to acute toxicity from a wide range of pollutants (McKim 1977; Belanger et al. 2010), and even small variations in mortality rates in early life stages can cause large fluctuations in fish recruitment (Houde 1987). It is therefore important to understand the levels of potential toxicants that cause mortality to early life stages fish where exposure is likely to occur. The potential for PEITC release from salad washing to cause embryotoxicity was confirmed in the watercress assay trials using *D. rerio*, which is discussed in section 5.6.6. Using analytic grade PEITC under experimental conditions, embryonic exposure led to concentration-related increases in percentage mortality and daily mortality rates of *S. trutta*, *C. carpio* and *D. rerio*. It was notable that exposure to 1 µg/L PEITC caused 100% embryo mortality by dose day four in both *S. trutta* trials and the *C. carpio* trials. It appeared that *D. rerio* embryos were more sensitive to PEITC, with complete mortality of all embryos occurring after just one dose at 1 µg/L. The DMSO solvent control did not elicit an acute toxicological response as mortality rates between the solvent and water controls were identical for *D. rerio*, similar for *C. carpio* and varied within the natural range of expected mortality for *S. trutta* embryos (Klemetsen et al. 2003; Ojanguren and Braña 2003; Réalis-Doyelle et al. 2016).

5.6.2 Delayed hatching following PEITC exposure

Delayed hatching in fish is a common response to toxicants such as heavy metals (Jezierska et al. 2009), microcystins (Malbrouck and Kestemont 2006), xenoestrogens (Schubert et al. 2014), pesticides (Lower and Moore 2003) and environmental stress (Wu et al. 2003). Across species, delayed hatching was observed to be more prevalent and for a longer duration following exposure to 0.1 µg/L compared to 0.01 µg/L PEITC indicating a concentration-related response. Species with longer incubation periods exhibited greater delayed hatching response to PEITC exposure. Of the three species studied, *D. rerio* had the shortest incubation period of up to 8 days, and there were no significant differences between the solvent control and the PEITC treatments. However, the solvent control and all PEITC treatments – which contain DMSO - hatched significantly sooner than the water controls. This agrees with a study by Hallare et al. (2006) which found accelerated hatching in *D. rerio* following embryonic exposure to DMSO. *C. carpio* had an incubation period of up to 14 days, and hatching was significantly delayed in both PEITC treatments relative to both solvent and water controls. Similar to *D. rerio*, the *C. carpio* embryos exposed to DMSO hatched significantly earlier. Early hatching under DMSO exposure may be a result of stimulatory effects on the hatching enzyme (chorionase) or the promotion of active uptake of water by the embryo (Denuce 1985; Hallare et al. 2006). The results from *D. rerio* and *C. carpio* studies suggest that the DMSO solvent may have acted antagonistically in the PEITC treatments in foreshortening hatch timings, so that PEITC may delay hatching to a greater degree in its absence. The DMSO treatment did not appear to hasten hatching in *S. trutta*, as there were no significant differences between the solvent control and the water control. Salmonid embryos have thick chorions compared to most teleosts, that may be 33-43 µm thick (Songe et al. 2015), while the thickness of *D. rerio* chorion is typically much thinner at 1.5-2.0 µm (Kunz 2004). The chorion is widely thought to be an uptake barrier for many toxicants, and although no chemically comprehensive survey of chorion permeability has been reported (Mandrell et al. 2012) the thicker chorion of *S. trutta* may be more effective at excluding DMSO than both D. *rerio* and *C. carpio*.

S. trutta saw the greatest degree of delayed hatching, where in both trials four and five the 0.1 µg/L treatment was significantly delayed relative to the controls and the 0.01 µg/L treatment. The more pronounced delay in hatch timing of *S. trutta* relative to the other species under study may have been a function of the longer incubation period and slower development of *S. trutta* embryos, where a PEITC-mediated inhibition of developmental processes over the longer incubation period resulted in a greater hatch delay. However, all three species were raised at different temperatures, and the PEITC-mediated delay in hatching tracks the differences in temperature at which the fish were raised. PEITC is degraded faster at higher temperatures (Ji et al. 2005; Kosson and Horbowicz 2009), so the more pronounced hatch delays in *S. trutta* compared to *C. carpio* and *D. rerio* may be

a function of the fish raised in warmer water having a reduced exposure due to higher breakdown rates in the PEITC treatments.

5.6.3 Length and weight

In all three species studied, embryonic exposure to 0.1 µg/L PEITC resulted in significantly reduced body length compared to all other treatments, while exposure to 0.01 µg/L PEITC did not significantly reduce body length for any species. This suggests a dose dependent impact on body length, which mirrors the findings of Njiwa et al. (2004) who studied the effects of the insecticide DDT exposure on *D. rerio*. Further, in addition to embryonic DDT exposure producing shorter larvae, they discovered toxicant-induced reduced growth may persist into later life stages as the cohort exposed to the highest DDT concentration maintained slower growth through the entire lifespan.

S. trutta alevins with small body length and delayed emergence from gravels are at higher risk of mortality than larger bodied individuals that appear later from gravels (Wankowski 1979; Einum et al. 2014). Smaller emergent salmonids produce weaker burst speeds than their larger conspecifics (Taylor and McPhail 1985) which make them more susceptible to predation (Rice et al. 1993). On emergence, *S. trutta* must establish and defend feeding territories against conspecifics, and these territories must be acquired before the fry can locate and catch prey items effectively (Skoglund and Barlaup 2006). Aggressive competition over territory from established resident fry often displaces smaller and later emerging fry downstream (Chapman 1962; Rhodes and Quinn 1998). Fry displaced on emergence experiencing higher levels of mortality through predation than residents with established territories (Elliott 1986; Einum et al. 2014). In addition, smaller fry are not equipped to survive as long without food (Bagenal 1969), and can experience higher winter mortality due to energy depletion than larger conspecifics (Hunt 1969; Cargnelli and Gross 1996; Schultz et al. 1998).

Despite the shorter total length seen in the highest PEITC exposed *S. trutta* alevins, total weights remained consistent across treatments, with just the 0.1 µg/L PEITC treatment in trial five being significantly lower. While there were no consistent trends in yolk:body ratios between treatments at 1dph, in the oldest cohort examined (4 dph) the 0.1 µg/L PEITC exposed animals showed signs of reduced yolk absorption having significantly reduced yolk:body weight ratio with significantly heavier yolk and lighter bodies. This suggests that exposure to PEITC at 0.1 µg/L reduced the efficiency of yolk to body tissue conversion in developing alevins. Similar reduced yolk-to-embryo gross conversion has been observed in *Salmo salar* following embryonic exposure to 2 parts per billion (ppb) of cadmium in trials by Peterson et al. (1983). In a natural scenario, this may delay emergence from the redd, compounding the effects of delayed hatching and small body size, putting the emergent fry at increased risk of predation.

5.6.4 Spinal malformations

Sfakianakis et al. (2015) state that in toxicant stressed teleost species, the most frequent expressed developmental anomalies are skeletal deformities in the vertebral column or its predecessor, the notochord. The notochord is the axial structure on which many other tissues depend for differentiation and proper formation. Toxicants that disrupt notochord development result in permanent skeletal deformities, muscle abnormalities and neurological dysfunction. The present study found that exposure to 0.1 µg/L PEITC was shown to significantly decrease total length in all three species and led to increased percentage of spinal malformations. The percentage incidence of spinal malformations was greatest in *S. trutta*, the species with the longest incubation period and lowest in *D. rerio*, the species with the shortest incubation period. It was intermediate in the species with intermediate incubation period, *C. carpio*, suggesting that length of exposure to PEITC during embryonic development may play a key role in the prevalence of spinal malformation in exposed fish. However, there are likely to be species-specific sensitives to PEITC which may account for the trends seen. In general, the 0.01 µg/L treatment either had no effect relative to the controls, or an intermediate effect.

Spinal deformities are found in wild fish populations from polluted waters, but are rarer in undisturbed waters (Dahlberg 1970; Boglione et al. 2001; Antunes and Lopes Da Cunha 2002; Messaoudi et al. 2009). This would indicate that afflicted individuals may persist in populations. However, a number of studies of wild fish populations indicate that the lifespan of fish displaying spinal deformities is likely to be reduced. Messaoudi et al. (2009) surveyed wild populations of grass goby *Zosterisessor ophiocphalus* in two locations in the Gulf of Gabès (Tunisia). One population was native in relatively pristine water, while the other in water polluted by heavy metals. The rates of spinal deformities in the unpolluted water were 4.58% compared to 17.67% from polluted water. The rates in polluted water were high in young fish and became significantly lower with increasing age. Similarly, Tutman et al. (2000) looked at wild populations of sandsmelt (*Atherina boyeri*) in the Neretva river estuary, middle eastern Adriatic, and found spinal deformities in ages classes 0+, 1+, 2+, but none in 3+ fish. With both studies, the reduction of spinal deformities in older fish was speculated to be a result of higher mortality rates of deformed fish, with spinal deformities impairing swimming performance (Weis and Weis 1976; Powel et al. 2009), and decreasing an individual's ability to escape predation and forage (Kroger and Guthrie 1971).

Stump body is frequently observed in cultured fish where it can render them poor swimmers, less able to compete for food and more susceptible to stress (Kvellestad et al. 2000). In this study the condition only occurred in 0.1 µg/L PEITC exposed fish. The condition did not occur at all in *D. rerio* and occurred in just one individual of *C. carpio*, while it occurred in 20-58% of *S. trutta* exposed to

0.1 µg/L PEITC. The higher incidences in *S. trutta* may be a function of the longer incubation period and/or lower incubation temperature relative to *D. rerio* and *C. carpio*. The condition was found in particularly frequently in later hatching *S. trutta* individuals, and it seems plausible that the condition impaired emergence from the egg and may be a driver of delayed hatching. Stump body has been observed in the sheepshead minnow *Cyprinodon variegatus* as a result of experimental exposure to trifluralin (Couch et al. 1979) and due to infestation of *Myxobolus sandrae* in wild *Perca fluviatalis* (Lom et al. 1991). Despite the *S. trutta* broodstock being obtained from a cultured fish population, the occurrence of stump body reflects a teratogenic effect of PEITC exposure, since the condition was only observed in alevins following exposure to the highest concentration of PEITC.

The present study found increased spinal malformations in all species following exposure to 0.1 µg/L of PEITC as embryos. Should environmental PEITC concentrations reach this level during embryo incubation, it could have a deleterious impact on the lifespans affected individuals and result in population-level impacts. As the teratogenic effect was found in distantly related species, the concentration-related effect is likely to be applicable to a wide range of teleost species. Species with long incubation periods, such as *S. trutta* and other salmonids, and those that incubate eggs at low temperatures are likely to be at particular risk.

5.6.5 Locomotory response to stimulus

Alevins of salmonids display photonegative behaviour, with their ability to orientate away from light increasing concurrently with morphological development (Woodhead 1957). Photonegative behaviour increases up until the final stages of yolk absorption when the behaviour is reversed and they become photopositive (Woodhead 1957; Noakes et al. 1981; Fast and Stober 1984). Bams (1969) suggested that photonegative behaviour is an adaptation to keep alevins in the safety of the gravel to avoid predation. The *S. trutta* alevins in the present study were in the photonegative stage, so the significantly higher mean movement during periods of light is likely to be a result the alevins attempting to burrow down to elude the light. Across all treatments, *S. trutta* alevins in both the 2 dph and 4 dph trials increased activity during exposure to light, indicating that they were photonegative. In both trials the solvent control groups exhibited decreased total activity compared to the water controls, and in trial five significantly so. The 0.01% v/v DMSO used to dose treatments was below that found to cause teratogenic effects in fish in previous studies (Hallare et al. 2006; Hutchinson et al. 2006; Maes et al. 2012). However, in *D. rerio*, Chen et al. (2011) found developmental exposure of DMSO as low as 0.01% can affect locomotor activity without causing any other observable developmental defects. Contrary to the *S. trutta* results, Chen et al. (2011) found embryonic exposure to 0.01, 0.1 and 1% v/v significantly *increased* distance moved by *D. rerio* relative to a water control, indicating that there may be species-specific impacts on behaviour

following DMSO exposure. However, the present study found no significant differences in activity between the water and solvent control in the *D. rerio* trial. While the solvent control caused a reduction in overall activity levels in *S. trutta*, and the solvent may have contributed to reduced activity in the PEITC treatments, only alevins exposed to 0.1 µg/L in trial both trials and to 0.01 µg/L PEITC in trial five failed to significantly increase activity levels in light, suggesting that PEITC exposure had diminished the response.

In response to light, Carey and Noakes (1981) found rainbow trout (*Oncorhynchus mykiss*) alevins migrate downwards into gravels. In situations where alevins become displaced from gravel, such as scouring events or superimposition of redds by later spawning salmonids, a photonegative behaviour would effectively help orientate them to the safety of the gravels (Bams 1969; Fast and Stober 1984). Should a wild population of salmonids be exposed to PEITC during embryonic development, a weakened photonegative response, as was observed in this study, may leave alevins above gravel for longer following disturbance. Such exposure may render them at greater risk of predation following displacement by water currents (Elliott 1986; Einum et al. 2014).

In contrast to *S. trutta* alevins, *C. carpio* larvae exhibited increased movement during darkness, and reduced activity during light. The relevance of this response is difficult to ascertain, but the sudden darkening of the chamber could have triggered a predator avoidance response in the larvae by approximately mimicking the dimming light caused by an approaching predator (Easter and Nicola 1996). Larvae incubated in water and solvent controls both showed a significant increase in activity when the light was turned off. Neither of the PEITC exposed larvae responded with a significant increase in activity, and the response was concentration dependent, with larvae incubated as embryos in 0.1 µg/L PEITC exhibiting a weaker response to darkness than those incubated at 0.01 µg/L. The reduced movement in PEITC exposed larvae in response to dark stimulus may translate to a reduced ability of PEITC exposed larvae in a wild population to elude predators.

Overall activity levels of *D. rerio* declined significantly with increasing concentrations of PEITC, indicating depressed activity or swimming performance. Activity levels in *D. rerio* were significantly higher during darkened periods for all treatments, and the reasons for increased activity may be predator-avoidance as speculated for *C. carpio*. Relative to *S. trutta* and *C. carpio*, the increase in activity following stimulus in *D. rerio* was much greater. Activity levels in the dark phase following PEITC exposure to 0.1 µg/L PEITC were less than half that of other treatments. However, despite this reduced activity, in contrast to *S. trutta* and *C. carpio,* the difference between stimulus and 'rest' was still significant in the 0.1 µg/L PEITC treatment, which may be attributable to the greater differences in activity levels between the two conditions in *D. rerio*.

Deviations in normal swimming behaviours may increase conspicuousness to predators and inhibit flight responses when confronted with a predator (Weis and Weis 1995). Studies on rainbow trout showed significantly inhibited foraging efficiency after sub-lethal exposure to six common agricultural chemicals (Little and Finge 1990). It was beyond the scope of the present study to look at specific foraging and flight responses in the fish studied. However, the results show a significant impairment of locomotory responses in eleutheroembryos exposed to sub-lethal concentrations of PEITC during incubation. If such levels were encountered in aquatic habitats during spawning, it would likely result in fry with impaired behavioural responses that would be deleterious to survival. The physiological mechanisms impacted by toxicants that can elicit behavioural changes in fish may be sensory, hormonal, neurological or metabolic in nature, and will usually be an integration of many physiological systems (Scott and Sloman 2004). The present study has for the first time identified altered behavioural responses in eleutheroembryos of *S. trutta* and *C. carpio* following exposure to PEITC though embryonic development. The precise physiological mechanisms by which PEITC caused degraded responses to stimulus has yet to be investigated.

5.6.6 Relating in-vitro PEITC effects to salad washing

The impact of PEITC exposure during embryonic development on *S. trutta, C. carpio* and *D. rerio* has been established. In addition to using analytical grade PEITC, the *D. rerio* trial utilised an approximation of salad wash effluent from the watercress farm situated on the Bourne Rivulet. This trial was undertaken to ascertain if the impacts of PEITC exposure are replicated by a simulated salad wash effluent. The full-strength watercress assay (WX1) exposed *D. rerio* had higher mortality rates, increased spinal malformations and significantly reduced total length relative to the controls, replicating the results of PEITC exposure seen in *D. rerio* and in previous trials using *S. trutta* and *C. carpio*. This suggests that PEITC produced and released by abrasion of watercress tissue in aqueous solution replicates the impacts on mortality, spinal malformations and body length recorded using analytical grade PEITC in vitro. In contrast, thermally deactivated myrosinase watercress assay (WXD) exhibited the lowest mortality rate, produced no spinal malformations and did not significantly reduce total length. As the thermally deactivated myrosinase watercress tissue was unable to synthesise PEITC on maceration (Newman et al. 1996; Ghawi et al. 2012), it confirms the aetiological role of PEITC in producing the teratogenic impacts seen in the WX1 assay. In contrast to delayed hatching following PEITC exposure in all other trials, exposure to the watercress assay had the reverse effect. Hatch timing occurred earliest at the highest watercress assay concentration and became progressively normalised to where it was not significantly different to the controls at the lowest concentration. A possible explanation may be that a compound other than PEITC released by macerated watercress tissue may hasten hatching and reduce mortality and spinal malformation rates. Such compounds may include carotenoids such as β-carotene, lutein and

zeaxanthin which are in high concentrations in watercress and can enhance immune system functions along with promoting a suite of other health benefits in mammals (Kopsell et al. 2007). Unlike PEITC, carotenoid concentrations are not diminished by boiling watercress tissues (Giallourou et al. 2016). However, whether carotenoids were available for utilisation by the embryos and eleutheroembryos is not known. While there is nothing in the existing literature examining watercress extract or PEITC exposure on fish, work has been conducted feeding sub-adult rainbow trout (*Oncorhynchus mykiss*) with a diet containing 1% watercress extract. The oral administration was found to enhance a number of immunological parameters such as lysozyme activity and compliment proteins (Asadi et al. 2012). Although it was not established which compounds in the watercress extract caused the positive effects seen in Asadi et al.'s (2012) study, It is possible in the present study that compounds in the watercress assay other than PEITC enhanced embryo immunity leading to the lessened mortality rates in the thermally-deactivated treatment.

The results of the motor activity assay following exposure to the watercress assay were less conclusive than the other toxicological endpoints measured. Activity levels following exposure to WX1 and WXD were not significantly different, but both were significantly reduced relative to the controls. However, unlike the PEITC treatments which displayed a concentration-related decline in activity with increasing PEITC concentration, the diluted watercress assays did not produce a comparative trend. As such, it is not possible to relate decreased motor activity following exposure to the watercress assay to PEITC exposure.

The similarity between the results on mortality, spinal malformations and total length in the watercress assay and PEITC exposures, in concert with the similarity of PEITC deactivated watercress assay to the controls indicate that PEITC derived from watercress salad washing processes is capable of producing teratogenic effects observed in previous PEITC trials. However, patterns in altered behaviour following exposure to the watercress assay did not clearly follow the patterns observed following PEITC exposure. As the watercress assay was used in only a single trial on a single species, replicate and further trials are recommended.

5.6.7 Potential PEITC impacts on fish recruitment

The results of this study indicate that PEITC exposure poses a potential risk to early life stages of wild populations of *S. trutta* and *C. carpio,* and potentially other species that are subjected to embryonic exposure. The concentrations of PEITC used were up to three orders of magnitude lower than estimates of leachate following biofumigation (Laegdsmand et al. 2007), in discharge water during harvesting of watercress (Worgan and Tyrell 2005), and from its salad wash effluent (Dixon 2010). However, the figures are estimates for discharge waters rather than estimates of

concentrations encountered in salmonid redds or receiving waterbodies, where the local hydrology would act to dilute PEITC to varying degrees. Exposure regimes experienced in fluvial environments are difficult to determine, with variable rates of biodegradation through temporal and spatial changes in physicochemical properties and rates of dilution (Hamilton et al. 2016). While dilution and biodegradation will almost certainly mean lower PEITC concentrations encountered by embryos in the environment that the estimates given, the relatively low concentrations that elicited significant toxicological effects remain a cause for concern. To retain enough eleutheroembryos for behavioural studies and to avoid the risk of experimenting on exogenously feeding fish for which the research was not licenced, the dosing of PEITC was halted when the first embryos hatched. However, it is well-established that the eleutheroembryo phase between hatching and exogenous feeding is considered the most vulnerable ELS to toxicants (Woltering 1984; Van Leeuwen et al. 1985; Von Westernhagen 1988; Farag et al. 1993; Witeska et al. 1995; Finn 2007; Sloman and Mcneil 2012), when the chorion protecting the embryo is lost and the gills are directly exposed to waterborne contaminants (Von Westernhagen 1988). Hence, it is probable that mortality and teratogenicity is likely to occur in this sensitive life stage at PEITC concentrations below those found to cause embryotoxic responses.

The suite of teratogenic impacts on development of embryos at PEITC concentrations of 0.01 µg/L were less marked or absent compared to exposure to 0.1 µg/L PEITC. These results indicate that for developing embryos, environmental concentrations of PEITC below 0.01 µg/L are unlikely to produce acute toxicity or teratogenicity. However, concentrations as low as 0.01 µg/L may result in higher predation through altered behaviours. Moreover, adverse effects of early life exposure to toxicants may not be manifest until adulthood in both reduced reproductive success (Coe et al. 2010) and altered behaviours (Brown et al. 2016). Fjeld et al. (1998) exposed developing embryos of grayling (*Thymallus thymallus*) to different concentrations of methylmercury (0.16, 0.8, 4.0 and 20 µg Hg/L). When tested three years after embryonic exposure, the fish exposed as embryos to 0.8 µg Hg/L or above had feeding efficiencies on *Daphnia magma* reduced by 15-24% relative to a control group. Moreover, in a competitive arena, control *T. thymallus* were able to capture two to six times more prey items than exposed groups.

5.7 Conclusions

These laboratory ecotoxicology trials suggest that ongoing exposure to environmental levels of 0.1 µg/L PEITC and above are liable to increase embryo mortality, delay hatching, and increase predation risk due to increased rates of morphological abnormalities and a reduced response to stimuli. Significant mortality of embryos is likely to occur if exposed to concentrations of PEITC at 1 µg/L or greater.

These findings highlight an urgent need to quantify PEITC levels in the aquatic environment emanating from watercress farms and biofumigation. It is hoped that these findings of acute toxicity and teratogenic impacts at low levels of PEITC will spur the development of a reliable standard methodology to test and monitor environmental levels of PEITC in the aquatic environment. Further, should environmental concentrations of PEITC be found to exceed the levels which cause acute toxicity and teratogenic impacts to embryos, then mitigation strategies for the treatment of effluents should be sought.

5.8 Further research and limitations

PEITC dosing in the present study was halted at hatch to avoid experimentation on exogenous feeding fry. As newly hatched fry are the most sensitive early life stage to toxicants, PEITC dosing of this stage under Home Office licence is recommended to gain greater insight into mortality levels following exposure.

The experimental set up used in the present study did not allow for the calculation of LC50 values for PEITC. Future studies could employ a continual dosing system to calculate LC50 values.

It has been recommended in ecotoxicological research that analytical methods are used to quantify actual concentrations of test compounds in the media to which organisms are exposed (Harris et al. 2014). Due to the absence of facilities to undertake analysis of concentrations of PEITC in the test beakers, the present study relied on concentrations derived by serial dilution.

The present study used farm and laboratory reared fish, which is common in ecotoxicology studies. Such stocks may have lower levels of genetic diversity than wild populations, which can have a significant effect on behaviour, fitness and response to toxins, which should be given consideration for studies that attempt to extrapolate results of ecotoxicological laboratory tests to wild populations (Coe et al. 2009).

FINAL DISCUSSION

6.1.1 Synthesis

This research was undertaken in response to the absence of published research investigating fish population changes in rivers below watercress farm outfalls. A small number of privately commissioned reports on fish populations have been conducted (Gent 2005; Longley 2006) but they were limited to a single watercress farm only and their findings were often contradictory, possibly as a result of different surveying and analytical methodologies employed by each. Moreover, there has been concern among fishery owners that altered macroinvertebrate abundances below watercress farms were having a negative impact on *S. trutta* stocks in their waters, but this until now has not been investigated.

It had been speculated that salad washing would lead to a greater release of PEITC into receiving chalk streams than the irrigation and harvesting of watercress crop alone (Worgan and Tyrell 2005; Dixon 2010; Cotter 2012). Previous studies of ecological impacts on a range of rivers and watercress farm discharges did not differentiate between discharges of watercress bed irrigation effluent and salad wash effluent. Studies such as Smith (1992) and Roddie et al. (1992) had largely described impacts on macroinvertebrates as a result of the discharge of fine sediment and zinc. However, such studies were conducted prior to the implementation of tighter controls to limit and monitor fine sediment release as well as the cessation of zinc applications and reductions in pesticide and fertilizer use.

To explore the possibility that watercress farm discharges were impacting fish populations and to examine potential drivers, fieldwork and laboratory ecotoxicology studies were carried out. Fieldwork was conducted in sites upstream and downstream of three watercress farms to quantify changes in habitat and macroinvertebrate assemblages resulting from discharges. These were then related to results of electric fishing surveys to assess if changes in habitat or macroinvertebrate prey availability wrought by discharges were having indirect impacts on fish population structures. The possibility that PEITC released from watercress farming and salad washing may have a direct impact on fish recruitment, and by extension, populations, was examined with ecotoxicology trials. These trials were the first of their kind to expose developing embryos of three fish species to PEITC to ascertain the concentration during embryonic development that may result in mortality, teratogenic impacts or altered behaviours that may be deleterious to survival.

6.1.2 Summary of key findings

6.1.2.1 Macroinvertebrate assemblages altered by salad wash effluent discharges

The first aim of the study was to investigate whether watercress farms were impacting on habitat and macroinvertebrate assemblages. It has been previously speculated that the discharge of salad wash effluent is likely to have a more adverse impact on macroinvertebrates than watercress bed irrigation effluent alone (Worgan and Tyrell 2005; Dixon 2010; Cotter 2012) and this was demonstrated in chapter three. The macroinvertebrate assemblage at each site, summarised using the Walley Hawkes Paisley Trigg (WHPT) biotic index (Paisley et al. 2014), demonstrated that sites downstream of salad wash effluent indicated the presence of organic pollution, while conversely those that received watercress irrigation effluent only had higher than predicted WHPT scores and higher abundances of pollution-sensitive macroinvertebrates. It is likely that little to no PEITC is discharged from watercress beds via irrigation discharges, as in contrast to the significantly increased gammarid mortalities seen in caged animals in salad wash effluent discharge channels (Dixon 2010), watercress beds are often densely populated by gammarids (Cox 2009).

Gammarids contributed the greatest between-site differences in macroinvertebrate assemblage. Moreover, their key role in chalk stream ecosystems (Mann and Orr 1969; Wright and Symes 1999; Macneil et al. 1999; Woodward et al. 2008; French et al. 2016) and the interest generated by their historically low abundances below watercress farm discharges (Smith 1992; Roddie et al. 1992; Cox 2009) singles them out for discussion. The abundance of gammarids were lower immediately downstream of discharges than above on all rivers. However, abundances have increased over historical records which is likely due recent changes in watercress farming practices such as the cessation of zinc and pesticide applications (Cox 2009; AFS 2016). All but the Bourne rivulet east channel which receives the greatest volume of salad wash effluent had mean abundances of over 500 per kick sample, the threshold that the Salmon and Trout Conservation charity and Environment Agency agree to indicate a healthy population (S&TC 2019). They were once absent on the east channel (Medgett and Court 2008), so finding them at this site is an improvement. However, the low abundances remain a concern. In contrast to the WHPT score, which was of intermediate value at the confluence of the east and west channels, gammarid abundances were at their highest at the confluence, which suggests that longitudinal recovery downstream of the east channel was relatively rapid.

On the Bourne, the impact on Ephemeroptera, Plecoptera and Trichoptera (EPT) was striking, with many taxa absent in the east channel below the salad wash effluent. Percentage abundance and family richness of EPT in samples improved with increasing distance from the east channel but did not return to values seen in the west channel even at the furthest downstream sampling point at

1.2 km from salad wash discharge point. This is of concern, as there has been a global decline in EPT orders since in the 1950s (Wagner 2020). Though much focus of previous work on macroinvertebrates and watercress farming has been on gammarids, these findings suggest that renewed focus should be directed towards the EPT taxa which play important roles in nutrient cycling and aquatic/terrestrial energy subsidies (Wallace and Webster 1996; Marcarelli et al. 2011) and which this study has shown to be impacted to a greater extent.

6.1.2.2 Watercress farm discharges and fish populations

The second thesis aim was to determine if watercress farm discharges were having a population impact on fish populations. In general, non-salmonids were at higher density below salad wash discharges, while *S. trutta* were at lower densities. This may have occurred for several reasons. It may be in part be a function of the relatively high sensitivity of *S. trutta* to toxins. For example, Sanchez-Galan et al. (1999) showed that *S. trutta* to be more sensitive to heavy metal pollution than the minnow (*Phoxinus phoxinus*). However, the lower densities of *S. trutta* may have led to less predation pressure on, and competitive exclusion of, smaller non-salmonids. The concept that top-down pressure by *S. trutta* may have excluded conspecifics is supported by a study of fish assemblages in two southern chalk streams England by Prenda et al. (1997). Their study examined patterns of habitat use of twelve species, finding that low densities of adult *S. trutta* were associated with higher densities of other chalk stream species. The increased abundance of non-salmonids may also be a function of improved prey abundances. For example, prey items favoured by *C. gobio* and *A. anguilla* were at greater abundance downstream of the discharges, particularly Asellidae and Molluscs and Chironomidae favoured by *C. gobio* (Mills and Mann 1983; Welton et al. 1983) and Ephemeroptera, Chironomidae, Simuliidae and Asellidae favoured by *A. anguilla* (Mann and Blackburn 1991). The lower abundances of gammarids did not appear to have a negative impact on adult *S. trutta* as had been speculated by fishery owners. Despite lower gammarid abundances downstream of discharges, total macroinvertebrate abundances were higher and there existed a significant positive correlation between macroinvertebrate abundance and relative weight of *S. trutta* suggesting well-fed and healthy individual adults and sub-adults below discharges.

6.1.2.3 Embryotoxicity of PEITC

The third aim of this research was to investigate the embryotoxicity of PEITC to assess its potential to impact fish recruitment when discharged from watercress farms into chalk streams. A concentration of just 1µg/L was found to result in total mortality of *S. trutta*, *C. carpio* and *D. rerio* embryos. In the case of *S. trutta* and *C. carpio*, total mortality of all embryos occurred by dose day four in all trials, while *D. rerio* embryos appeared more sensitive, occurring after a single dose. It

was discovered that exposure to concentrations as low as 0.1µg/L resulted in significantly increased embryo mortality, and those that survived to hatch had an increase in the percentage of incidents of spinal malformations for all species, particularly *S. trutta*, perhaps owing to the longer incubation period and lower temperatures increasing exposure duration and intensity. Moreover, a motor assay showed that concentrations as low as 0.1µg/L can reduce motor reactions in *S. trutta* to a sudden illumination from a significant startle response in the controls to a non-significant one at 0.1µg/L. The startle response to a sudden dark period was similarly compromised by exposure to 0.1µg/L in carp, while total activity levels in *D. rerio* were significantly reduced by embryonic exposure to 0.1µg/L PEITC. Such teratogenic and behavioural impacts were generally not apparent following exposure to 0.01µg/L PEITC, giving an indication of the concentrations of PEITC to result in acute toxicity and sublethal effects.

6.1.3 Salad wash effluent discharge and PEITC: impacts on macroinvertebrates and young-of-year fish

Several studies have highlighted the value in examining both fish and macroinvertebrate assemblages in bioassessment programmes in rivers (Freund and Petty 2007; Flinders et al. 2008; Johnson and Ringler 2014). Examining both assemblages is considered beneficial as both groups provide valuable information on ecological condition and may respond to differently to a variety of stressors (Johnson and Ringler 2014). It was therefore notable that downstream of salad wash effluent, reductions in pollution-sensitive macroinvertebrates occurred concurrently with lower densities of young-of-year *S. trutta*. In contrast, relative abundances of both appeared to be improved where watercress bed irrigation water only was discharged.

Impacts to macroinvertebrates from salad wash effluent linked to PEITC discharge had been previously reported (Dixon 2010) and so it was not unexpected that macroinvertebrate impacts were observed in this study. However, the high embryotoxicity of PEITC discovered in the laboratory trials and low densities of young-of-year *S. trutta* suggest that PEITC in salad wash effluent may be acting as a stressor for both macroinvertebrates and impinging on *S. trutta* recruitment. The ecotoxicology trials saw total mortality of embryos at PEITC concentrations up to three orders of magnitude lower than the estimated 600-1040 µg/L PEITC in salad wash effluent (Dixon 2010). Concentrations of PEITC occurring in receiving rivers and gravel redds will naturally be lower than these estimates, being be subject to variable rates of dilution and biodegradation (Hamilton et al. 2016). However, even with high levels of dilution and biodegradation, the relatively very low levels of PEITC found to cause adverse impacts on fish embryos in the laboratory trials suggests that *S. trutta* embryos and early life stages may be exposed to concentrations high enough to impact recruitment.

6.1.4 Linking low densities of young-of-year *Salmo trutta* to habitat and laboratory findings

As salmonids defend territories close to the redds from which they emerge, the lower than expected densities of this age class below salad wash discharges may indicate reduced spawning success, or higher mortality rates of embryos or fry (Crisp 1995; Klemetsen et al. 2003). Mortality rates of *S. trutta* embryos subjected to WWTP discharges in the River Wyna in Switzerland were found to be as high as 95% (Kobler 2004). This impacted on recruitment, leading to an altered *S. trutta* population structure, which was depauperate in young-of-year fish and characterised by lower densities of adult fish in higher condition than unimpacted sites (Kobler 2004). This altered population structure is strikingly similar to that found in sites downstream of salad wash discharges in the present study, suggesting that there may have been embryo mortality.

As gravel beds with low fines are required for successful incubation of salmonid embryos (Turnpenny and Williams 1980; Soulsby et al. 2001; Heywood and Walling 2007) and emergence of alevins (Rubin and Glimsäter 1996; Rubin 1998), the higher fine sediment loads may have created suboptimal conditions for spawning success in sites downstream of discharges. However, fine sediments were also increased below the irrigation water discharge on the Frome, which saw above predicted densities of the young-of-year class, suggesting that fine sediment may not be the primary cause. As levels of interstitial fine sediment were not quantified, It is possible that they were higher below salad wash effluent discharges, which may or may not be related to salad washing activity. Kondolf and Wolman (1993) make the point that it is important to distinguish between the presence of redds and the perceived quality of spawning substrates as the presence of fine sediment does not necessarily impinge on spawning. Salmonids clean gravels when cutting redds, which displaces fine sediments and increases porosity (Crisp and Carling 1989; Peterson and Quinn 1996; Armstrong et al. 2003). The amount of displaced sediment can be large, for example, redd cutting by Chum salmon (*Oncorhynchus keta*) can purge approximately 75% of fines from the streambed sediment (Peterson and Quinn 1996). The small increase in observed surface fine sediment downstream of watercress farm discharges, which was largely limited to channel margins, is perhaps unlikely to have resulted in decreased spawning success and emergence of fry. However, it is not possible to definitively rule out the possibility that fine sediment accumulation in gravels rendered them suboptimal for embryo incubation. This possibility requires further investigation with the use of techniques such as freeze core sampling as suggested in 6.1.6.

Perhaps the most parsimonious explanation for low densities of young-of-year *S. trutta* may be that there were fewer redds constructed at sites where salad wash effluent was discharged. If so, this may be linked to a factor in discharges, a factor independent of discharges or may simply be a

random effect. To identify potential causative factors would require data on spawning activity at each site, but this was unknown in the present study. Indeed, Armstrong et al. (2003) argue that a weakness in the predictive power of the HABSCORE model is that it does not take into account spawning levels. Even so, of all size classes of *S. trutta* and *S. salar*, young-of-year *S. trutta* are one of the categories which have been shown to be most accurately predicted by the HABSCORE model (Armstrong et al. 2003). A methodlogy for future studies which would circumvent the unknown extent of redd formation is proposed in section 6.1.6. Young-of-year *S. trutta* densities are limited by habitat availability (Borsuk et al. 2006), so should spawning and emergence have been successful, if habitat was suboptimal for emergent fry and they may have migrated to more favourable habitat. However, HABSCORE predictions are based on variables known to fulfil habitat requirements for each of the size classes of *S. trutta*. This suggests that in terms of physical habitat structure such as channel topography, substrate, site location and available instream and overhead cover, sites downstream of the salad wash effluent should have supported greater densities of young-of-year than were observed.

6.1.5 Fish exposed to PEITC from salad wash: confounding variables, potential genetic adaptations and long-term consequences of exposure

Most fish are exposed to complex mixtures of chemicals in natural populations, and so identifying health outcomes of specific chemicals is extremely difficult (Hamilton et al. 2016). The waters in the chalk river sites in the present study are likely to contain a suite of potentially harmful compounds emanating from catchment sources such as septic tanks, agricultural run-off and for all sites except those on Bourne, from sewerage treatment plant discharges which may contain a myriad of low concentration xenobiotics (Luo et al. 2014; Margot et al. 2015; Berger et al. 2017). With the short spatial distances between upstream and downstream sites, those downstream of salad wash effluent will contain a similar if not the same suite of potential harmful compounds as upstream with the addition of any compounds released from salad wash. Therefore, comparison between upstream and downstream sites should allow effects of salad wash to be determined. In terms of the physicochemical determinands recorded in the present study, sites downstream of salad wash discharge were not altered such that they would be deleterious to salmonid embryo incubation. However, antagonistic or additive effects of xenobiotics from the catchment and PEITC cannot be ruled out. Relating laboratory ecotoxicology experiments conducted in controlled conditions to natural field settings, where there are a multiplicity of potentially confounding factors has limits (Vignati et al. 2007). An experimental strategy which seeks to address such issues is proposed in section 6.1.6.

While lower than predicted densities of young-of-year *S. trutta* suggest that recruitment may have been negatively affected, populations of *S. trutta* persisted. This may be explained by older individuals which are naturally more tolerant to toxicants than early life stages (Belanger et al. 2010; Sloman and Mcneil 2012) migrating into reaches below discharges and taking advantage of reduced intraspecific competition. A further explanation may be cohort variation in sensitivity to PEITC, as the laboratory trials showed individual variability in PEITC sensitivity. If susceptibility and resistance to PEITC has a genetic basis this could provide the potential for evolutionary adaptation via selection of less susceptible individuals. Local adaptations to pollutants have been reported for mosquitofish (*Gambusia affinis*) to insecticides (Culley and Ferguson 1969; Andreasen 1985), and killifish (*Fundulus heteroclitus*) and Atlantic tomcod (*Microgadus tomcod*) to PCBs (Wirgin and Waldman 2004). These adaptations to pollutants may be genetic. For example, Lind and Grahn (2011) applied amplified fragment length polymorphism analysis to identify adaptive genetic variation in the three-spined stickleback associated with pollution tolerance to kraft mill effluents in the Baltic Sea. Populations at polluted sites were genetically distinct, indicating that they were under divergent selection. In southern chalk streams, significant genetic differences in *S. trutta* populations have been discovered between populations separated by little over 1 km in response to heavy metal contamination (Durrant et al. 2011). If *S. trutta* are subjected to chronic PEITC exposure downstream of salad wash effluent, selection pressure on resident populations may explain their persistence.

In the present study, embryonic PEITC exposure caused delayed hatching and resulted in smaller eleutheroembryos. Small sizes at hatching following embryonic exposures to xenobiotics have been linked to slower growth rates through life history (Njiwa et al. 2004). Moreover, exposure to xenobiotics in early life stages may manifest in impacts in later life stages. Heintz et al. (2000) incubated embryos of pink salmon (*Oncorhynchus gorbuscha*) under varying concentrations of polynuclear aromatic hydrocarbons (PAH). Surviving fish that appeared healthy were tagged and released into the marine environment in Alaska, USA. Compared to control fish, those exposed to 5.4 ppb PAH experienced a 15% reduction in return rates. A cohort maintained at a hatchery experienced reduced growth rates following PAH exposure, leading to Heintz et al. (2000) to conclude that reduced growth rates impacted marine survival. It is therefore possible that embryonic PEITC exposure could lead to reduced growth rates and survival of anadromous *S. trutta*, *S. salar* and other species. Further ecotoxicology trials are recommended to quantify growth rates post-hatch following embryonic exposure to PEITC to assess if growth is compromised.

PEITC exposure from salad wash effluent may have resulted in lower than expected densities of young-of-year *S. trutta*. Moreover, low densities of young-of-year *S. trutta* may have arisen through a combination of stressors, such as lower rates of spawning activity, fine sediment ingress into

spawning gravels or other environmental stressors not recorded. Potential strategies for future research which may help address such confounding effects will be discussed.

6.1.6 Potential directions for future research

To identify environmentally relevant concentrations of PEITC will require a methodology to quantify PEITC concentrations in river water, something outside of the scope of the study. Analytical methods such as liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) are available that are capable of measuring concentrations of many chemical contaminants in water, including pharmaceuticals in sewage effluents in the ng/L range (Corcoran et al. 2010). PEITC has been quantified in solution by GC-MS (Palaniswamy et al. 2003; Dixon 2010) and by LC-MS (Ji et al. 2005). However, the high volatility of PEITC (Chen and Ho 1998; Doheny-Adams et al. 2018) poses challenges when attempting to accurately quantify PEITC concentrations in sampled river water (Dixon 2010). Problems may arise due to the rapid breakdown of the compound, which is likely to occur during transport from site to laboratory. Portable testing equipment able to take measurements more-or-less in situ would be desirable. Passive samplers, or 'chemical catchers' that bind chemicals from the aqueous phase have been applied to study chemical exposures over time. PEITC is a hydrophobic organic compound, a type of compound polyethylene chemical catchers can be used to sample (Adams et al. 2007). However, the problems associated with degradation may still persist with PEITC captured on such a device. A method to accurately quantify environmental concentrations of PEITC is clearly needed.

There are, however, avenues for further research into potential impacts of PEITC on *S. trutta* embryos in chalk streams that may be conducted without access to analytic methods. For example, in vivo exposures using caged embryos, or artificial redds *(sensu*. Malcolm et al. 2003) set into the gravel beds of chalk streams. Positioning caged embryos in discharge channels and in sites upstream and downstream of discharges, mortality rates and the hatch timing and morphology of alevins could be compared between sites. A benefit of this approach is that exposures would account for any synergistic/antagonistic effects of PEITC and other compounds found in the hyporheos. In addition, it would take into account the often highly dynamic levels of chemicals fluctuating in river habitats resulting from fluctuating rates of biodegradation and physiochemical conditions, water flow rates and levels of dilution (Hamilton et al. 2016)

Future in vitro testing may be conducted with water collected directly from salad wash lines from a range of salad washing facilities, which would provide a direct link to salad washing and its effects on embryonic development. Such studies if conducted during the natural embryo incubation period, where the crops grown and the attendant PEITC content of the salad wash effluent would be an accurate representation of the discharge potentially encountered in chalk stream

headwaters. However, this approach would not account for dilution of effluent water in the receiving water, nor the extent to which PEITC would ingress into gravels to reach the embryos. Therefore, the previously described in vivo methodology may be a preferable future approach.

The present study strongly suggests that salad wash effluent discharges rather than watercress bed irrigation water is impacting on biota in chalk streams. Future studies may aim to survey upstream and downstream of at least three salad wash effluent discharges. Such an approach would enable data to be aggregated for statistical analysis, such as the use of mixed effect models. Doing so while holding site as a random factor would circumvent pseudoreplicaton which constrained the statistical comparison of upstream and downstream sites in the present study.

Future studies may aim to more accurately quantify fine sediments, particularly interstitial quantities to assess if substrates below watercress farm discharges are rendering habitat suboptimal for sediment-sensitive macroinvertebrates, *Ranunculus* growth and salmonid spawning. Future studies could explore the interstitial fine sediment loading using freeze core sampling (eg. Rood and Church 1994).

The orders EPT appeared to be strongly impacted by salad wash discharges, particularly Trichoptera, which were much reduced in family richness and abundance in sites receiving salad wash effluent. Future ecotoxicology trials using EPT taxa and PEITC may be performed to isolate the action of PEITC in the observed declines.

6.1.7 Future challenges

Future challenges associated with global warming may exacerbate some of the issues raised in this study. At present, chalk aquifers provide 70% of public drinking water in south east England (Visser et al. 2019). The projected increases in temperatures under future global warming trends may place ever greater demands on chalk aquifers for abstraction (Collet et al. 2018). Increases in water abstraction allied to more frequent drought events would invariably result in low river levels being more commonplace. In chapter four the negative effects of a low flow year on *S. trutta* recruitment was observed, with young-of-year densities being significantly below expected following a dry winter and spring. Should such events become more frequent due to global warming, this may have profound impacts on salmonid recruitment. Moreover, a reduction in river flow reduces the extent a river can dilute pollutants from both point and diffuse sources (Bowes et al. 2008). Low flow events may therefore lead to higher concentrations of PEITC and other potential toxicants, such as micropollutants (Margot et al. 2015) and microplastics (Mintenig et al. 2017; O'Connor et al. 2020) from WWPTs, and nutrient and pesticide run-off from agriculture (Hendry et al. 2003). It is thought that over 60,000 anthropogenic compounds are released from wastewater discharges (Hamilton et

al. 2016) any number of which may interact with PEITC in ways not yet understood. Under increased concentrations, mixing of xenobiotics may impact further on pollution-sensitive macroinvertebrates and *S. trutta* recruitment. Moreover, in chalk streams reduced flows can increase fine sediment deposition (Wood and Petts 1999) which may degrade salmonid spawning habitat.

S. trutta populations in Europe have declined dramatically in recent years, particularly in Switzerland and France, largely due to the stress of increasing water temperatures (Hari et al. 2006; Poulet et al. 2011). Freshwater fish are particularly vulnerable to increasing water temperatures as they lack mechanisms to regulate body temperature (Angilletta et al. 2002) and have limited opportunity to disperse to escape temperature shifts (Comte et al. 2013). In terms of recruitment success, non-salmonids such as cyprinids may benefit from increased river water temperatures (Nunn et al. 2003) while increases in incubation temperatures to just 12°C can be detrimental to salmonid recruitment (Johnson and Sumpter 2014; Réalis-Doyelle et al. 2016). A small rise in winter chalk stream temperatures may therefore exacerbate the observed low abundances of salmonids and high abundances of non-salmonids below salad wash discharges. Should *S. trutta* populations decline as they have in much of mainland Europe, this would not only be damaging in economic terms, but would be leave the UK's chalk streams denuded of its most iconic species (Lobón-Cerviá and Sanz 2017).

6.1.8 Potential mitigation strategies

The results of this study indicate that salad wash effluent is negatively impacting macroinvertebrate assemblages and may be leading to mortality of *S. trutta* early life stages. Further research as discussed previously is required to conclusively link lower densities of young-of-year *S. trutta* to salad wash effluent discharges. However, to improve abundances of sensitive macroinvertebrates and to mitigate against potential impacts on *S. trutta* recruitment, there are some steps that may be taken to minimise PEITC release into chalk streams from salad wash facilities.

There is a large body of research into the use of artificially constructed wetlands to improve water quality (Mander et al. 2017). Passing discharges from agriculture, aquaculture and wastewater treatment through macrophyte beds has been shown to successfully reduce nutrients (Land et al. 2016) and heavy metals (Mays and Edwards 2001). It is intuitive that nutrient uptake by macrophytes would reduce nutrient loads of water passed through growing macrophyte beds, and the uptake and assimilation of heavy metals into plant tissues is well understood (Rezania et al. 2016). However, it is not known if an artificial wetland will reduce PEITC concentrations. Some encouragement may be drawn from the improvement in Gammarid abundances in the Bourne Rivulet east channel post 2005 following the rerouting of salad wash effluent through watercress

beds prior to discharge (Medgett and Court 2008; Cotter 2012). The increased residence time in the watercress beds increases potential for phytodegradation, phytolysis, adsorption, desorption and degradation of volatile PEITC (McEldowney et al. 1993; Cotter 2012). Such processes in artificial wetlands have been shown to significantly reduce concentrations of a range of pesticides in agricultural run-off (Elsaesser et al. 2011; Tournebize et al. 2013) which are almost certainly more stable in the environment than PEITC. As PEITC is volatile (Chen and Ho 1998; Doheny-Adams et al. 2018) it would be expected that degradation would occur more rapidly than many pesticides, so the use of artificial wetlands to reduce PEITC concentrations suggests much promise. Moreover, the use of an appropriately designed artificial wetland could reduce the input of particulate matter into the receiving watercourse (Zachritz and Fuller 1993) which may reduce fine sediment loading in the receiving chalk stream.

The watercress producers on the Bourne Rivulet have recently drawn up plans for the construction of artificial wetlands (VCT Chalk Stream Headwater Forum, pers. comms. 2019). The proposed plan will pump salad wash effluent to an artificially created wetland at a slightly higher altitude than the watercress farm and river. The pumped salad wash effluent will pass through a series of five interlinked planted ponds and lagoons under gravity before finally entering the Bourne Rivulet (Blaxland 2019). Once constructed and operational, monitoring of the biota in the Bourne Rivulet below the discharge will reveal the efficacy of the artificial wetland approach, which, if effective, may be implemented more widely among watercress growers.

As activated carbon can adsorb Isothiocyanates (Konieczka et al. 1992), filtering salad wash effluent through a bed of activated carbon may be a viable mechanism by which to strip PEITC from effluent prior to discharge. Efficient removal of micropollutants using powdered activated carbon has already been established in WWTP processes (Margot et al. 2013; Thellmann et al. 2014). As the construction of a wetland would be constrained by available land, and an activated carbon filter may require little space, it may prove to be a solution for a greater number of salad-washing facilities.

The use of UV light has been established as an effective means to degrade organic micropollutants in waste water treatment, including phenols, insecticides, pharmaceuticals, estrogens and cyanotoxins (Matafonova and Batoev 2018). The use of UV to increase PEITC breakdown rates shows promise due to its high volatility. Moreover, recent advances in LED UV lamp technology results in lamps of smaller size, longer lifespan and much lower power consumption compared to conventional mercury lamps (Chen et al. 2017). Such units could be powered by solar arrays (Chen et al. 2017) making the application of UV treatment appealingly low-cost to operators of salad washing facilities.

6.1.9 Final remarks

The results of electric fishing surveys suggest that rivers with discharges from watercress farms support higher densities of non-salmonid species and lower densities of *Salmo trutta*. However, populations of *S. trutta* below discharges were in better condition than upstream sites, likely as a result of increased macroinvertebrate food availability. A distinction was observed between discharges from salad wash facilities, which resulted in macroinvertebrate biotic index scores below expected values indicating organic pollution, and watercress bed irrigation, which appeared to have no negative effect on biotic indices. This suggests that a compound such as PEITC released from salad wash may be exerting a toxic effect on sensitive macroinvertebrate taxa in chalk streams. Furthermore, the embryotoxic effect of PEITC may be responsible for the lower than expected young-of-year *S. trutta* below salad wash discharges. This research for the first time highlights the negative impact that PEITC release from salad wash effluent may be having on *S. trutta* in chalk streams and proposes potential mitigation strategies to improve chalk stream habitat for macroinvertebrates and salmonids.

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HABSCORE for Windows v1.1 : HAB*form*
Site habitat record

NB - this form is double sided

Site identification

Site code		Catchment	
Site name		NGR	
River name		Survey date	

Riparian shading of the site

What percentage of the water surface of the site is overhung by riparian vegetation? Estimate this percentage, for the three vegetation classes indicated, to the nearest 5%.

Deciduous trees & shrubs ☐ Coniferous trees ☐ Herbaceous vegetation ☐

Migratory access

What is the accessibility of the site ?

	Salmon	Sea trout
Always accessible		
Sometimes accessible		
Never accessible		

Substrate embededness

What is the degree of substrate embededness throughout the site? Tick one box.

High ☐ Medium ☐ Low ☐

Flow conditions

Briefly describe the prevailing flow conditions (as observed at the time of the HABSCORE survey) in the space provided below.

.....

Upstream land-use considerations

What is the principal land-use immediately upstream of the site? Tick appropriate box(es).

Moor / heathland	☐	Coniferous woodland	☐	Deciduous woodland	☐
Rough pasture	☐	Urban development	☐	Other	☐
Improved pasture	☐	Industrial land	☐	.....	
Arable land	☐	Tips / waste	☐	.....	

Potential impacts

Are there likely to be any impacts at the site from the following sources? Tick appropriate box(es).

pH effects	☐	Stocking	☐	Other	☐
Migration barriers	☐	Habitat modification	☐	.....	
River engineering	☐	Low flows	☐	.....	
Pollution	☐	Flow regulation	☐		

Width and depth profile at bottom stop net

Record widths to the nearest 0.1m and depths to the nearest 1.0cm.

Channel width	
Depth at ¼ channel width	
Depth at ½ channel width	
Depth at ¾ channel width	

Section dimensions

Record section lengths and widths to the nearest 0.1m and depths to the nearest 1.0cm.

Section length										
Section width										
Depth at ¼ channel width										
Depth at ½ channel width										
Depth at ¾ channel width										

Substrate

Absent	Scarce	Common	Frequent	Dominant
0%	>0% & <5%	≥5% & <20%	≥20% & <50%	≥50%
A	***S***	***C***	***F***	***D***

What percentage of the stream bed area in each section is composed of the following substrate types? Enter *A*, *S*, *C*, *F* or *D* as appropriate (see above table).

Substrate category										
Bedrock / artificial										
Boulders ***>25.6 cm***										
Cobbles ***6.4-25.6 cm***										
Gravel / coarse sand ***0.2-6.4 cm***										
Fine sand / silt ***<0.2 cm***										
Compacted clay										

Flow

What percentage of the water surface area in each section is composed of the following flow types? Enter *A*, *S*, *C*, *F* or *D* as appropriate.

Flow category										
Cascade / torrential										
Turbulent / broken ***deep***										
Turbulent / broken ***shallow***										
Glide / run ***deep***										
Glide / run ***shallow***										
Slack ***deep***										
Slack ***shallow***										

Sources of cover for >10cm trout

What percentage of the stream bed area in each section could provide cover (for a >10cm trout) in the form of *submerged overhang*, or *overhang within 0.5m of the water surface*?

Indicate the abundance of cover within the various categories which are listed below. For 'submerged vegetation' include all macrophytes, mosses and algae which are providing cover. Estimate as 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, ... 100%.

Source of cover										
Submerged vegetation										
Boulders, cobbles, etc.										
Tree root systems										
Branches and logs										
Undercut banks										
Other submerged cover										
Overhang ***within 0.5m***										
Area of deep water										

Appendix 2a. List of macroinvertebrate families captured on Bourne Rivulet sites BRWC, BREC, BRDS1 and BRDS2 including mean abundance ±SD from *n* = 5 kick samples

MAJOR GROUP	FAMILY	SITE							
		BRWC	SD	BREC	SD	BRDS1	SD	BRDS2	SD
EPHEMEROPTERA	Caenidae	405.6	449.7	4.8	10.7	117.4	190.1	221.2	297.4
	Heptageniidae	0.8	0.8	-	-	-	-	5.6	7.2
	Baetidae	137.0	137.2	584.6	434.9	408.2	269.0	386.8	280.2
	Ephemeridae	1.2	1.6	-	-	1.4	1.5	4.4	6.1
	Leptophlebiidae	-	-	-	-	-	-	0.6	1.3
	Ephemerellidae	972.4	1620.7	470.2	450.0	1383.0	1329.0	666.4	710.4
TRICHOPTERA	Hydropsychidae	14.0	7.2	-	-	4.8	5.3	32.8	46.7
	Limnephilidae	13.4	11.9	1.4	2.6	4.4	7.1	7.6	14.8
	Leptoceridae	4.0	8.9	-	-	2.6	5.8	-	-
	Glossosomatidae	139.8	81.1	-	-	0.6	1.3	25.6	23.9
	Rhyacophilidae	4.4	1.5	0.6	1.3	3.2	3.1	1.8	2.0
	Sericostomatidae	2.2	3.2	3.8	7.4	6.6	6.8	4.8	3.3
	Psychomyiidae	0.8	1.8	-	-	-	-	-	-
	Brachycentridae	0.2	0.4	-	-	-	-	-	-
	Lepidostomatidae	0.2	0.4	-	-	-	-	0.2	0.4
	Goeridae	0.6	0.9	-	-	5.0	8.0	3.2	5.1
	Beraeidae	7.2	13.9	-	-	1.0	2.2	0.2	0.4
	Odontoceridae	0.4	0.5	0.2	0.4	3.8	2.6	8.2	4.9
	Hydroptilidae	0.4	0.5	-	-	0.2	0.4	-	-
PLECOPTERA	Perlodidae	1.6	2.3	-	-	-	-	0.2	0.4
	Nemouridae	-	-	-	-	0.2	0.4	-	-
	Leuctridae	3.0	4.1	-	-	-	-	1.2	1.3
CRUSTACEA	Gammaridae	520.2	458.5	228.2	222.9	1609.0	899.1	995.4	395.2
	Asellidae	1.6	1.7	709.4	745.3	14.0	11.5	0.8	0.8
MOLLUSCA	Planorbidae (excluding Ancylus)	4.2	6.3	16.4	18.0	3.2	4.4	1.4	1.7
	Physidae	2.8	2.6	11.4	16.2	1.6	2.1	-	-
	Ancylidae	9.8	11.5	0.4	0.9	58.8	91.1	5.2	7.8
	Lymnaeidae	1.2	2.2	0.4	0.5	1.6	3.6	1.4	3.1
	Sphaeriidae	1.2	1.3	15.2	16.3	-	-	5.0	7.1
HEMIPTERA	Coroxidae	-	-	0.2	0.4	-	-	-	-
	Veliidae	0.2	0.4	-	-	-	-	-	-
COLEOPTERA	Gyrinidae	-	-	-	-	0.2	0.4	-	-
	Dryopidae	3.6	8.0	0.4	0.9	0.2	0.4	0.4	0.9
	Helophoridae	0.2	0.4	0.2	0.4	-	-	-	-
	Circulionidae	0.4	0.9	-	-	-	-	-	-
	Elmidae	94.2	53.1	-	-	16.6	13.4	7.8	6.1
	Dytiscidae	23.6	14.1	3.0	2.9	77.4	49.4	18.4	22.3
DIPTERA	Chironomidae	137.8	131.4	1228.6	2322.1	132.8	209.4	57.0	74.2
	Thaumaleidae (chironimid)	-	-	0.6	1.3	-	-	-	-
	Simuliidae	74.4	56.6	234.6	236.3	118.2	210.0	126.4	118.4
	Stratiomyidae	-	-	0.2	0.4	-	-	-	-
	Tipulidae	-	-	-	-	-	-	0.2	0.4
	Dixidae	0.2	0.4	0.2	0.4	0.2	0.4	-	-
	Rhagionidae	-	-	0.2	0.4	-	-	-	-
	Pedicidae	0.2	0.4	-	-	0.2	0.4	2.2	3.2
	Ceratopogonidae	11.4	16.8	17.4	22.1	16.0	18.1	1.4	2.6
	Psychodidae	-	-	3.4	5.1	-	-	-	-
	Limoniidae	-	-	3.0	6.2	-	-	-	-
	Ephydridae	-	-	2.0	3.5	-	-	-	-
	Empididae	0.8	1.1	1.4	2.6	0.8	1.8	0.2	0.4
OLIGOCHAETA		33.6	61.8	255.4	230.7	290.4	171.3	115.6	86.2
HIRUDINEA	Erpobdellidae	3.6	3.4	12.8	13.7	21.6	11.4	7.0	1.9
	Piscicolidae	0.6	0.9	2.0	2.8	0.4	0.5	-	-
	Hirudinidae	-	-	2.0	4.5	0.8	1.8	0.6	1.3
	Glossiphoniidae	4.4	3.6	43.8	37.7	10.2	8.4	5.0	3.4
ARACHNIDEA	Arachnida (acarii)	16.0	20.5	5.4	8.8	15.6	20.7	6.6	12.5
TRICLADIDA	Planariidae	4.6	5.1	175.6	161.0	6.6	7.6	2.0	2.3
	Dendrocolidae	-	-	9.0	6.3	0.6	0.9	1.4	0.9

Appendix 2b Similarity Percentage (SIMPER) analysis of macroinvertebrates on the Bourne Rivulet, listed in order of contribution to dissimilarity between sites (CONT. %) and the cumulative percentage (CUM. %) and for illustration the mean abundances (standard errors and *n* in appendix 2a)

TAXON	SITE				DISSIMILARITY	CONT. %	CUM. %
	BRWC	BREC	BRDS1	BRDS2			
Ephemerellidae	972.4	470.2	1383.0	666.4	15.6	23.49	23.49
Gammaridae	520.2	228.2	1609.0	995.4	13.92	20.95	44.44
Chironomidae	137.8	1228.6	132.8	57.0	7.273	10.95	55.39
Asellidae	1.6	709.4	14.0	0.8	5.913	8.901	64.29
Baetidae	137.0	584.6	408.2	386.8	5.526	8.318	72.6
Caenidae	405.6	4.8	117.4	221.2	4.127	6.213	78.82
Oligochaeta	33.6	255.4	290.4	115.6	3.418	5.145	83.96
Simuliidae	74.4	234.6	118.2	126.4	2.723	4.1	88.06
Planariidae	4.6	175.6	6.6	2.0	1.51	2.273	90.34
Glossosomatidae	139.8	-	0.6	25.6	1.344	2.023	92.36
Elmidae	94.2	-	16.6	7.8	0.808	1.216	93.57
Dytiscidae	23.6	3.0	77.4	18.4	0.7001	1.054	94.63
Ancylidae	9.8	0.4	58.8	5.2	0.5704	0.8587	95.49
Hydropsychidae	14.0	-	4.8	32.8	0.3996	0.6015	96.09
Glossiphoniidae	4.4	43.8	10.2	5.0	0.3443	0.5183	96.61
Ceratopogonidae	11.4	17.4	16.0	1.4	0.2927	0.4407	97.05
Arachnida (acarii)	16.0	5.4	15.6	6.6	0.2242	0.3375	97.38
Erpobdellidae	3.6	12.8	21.6	7.0	0.2065	0.3109	97.7
Limnephilidae	13.4	1.4	4.4	7.6	0.1591	0.2395	97.94
Planorbidae (ex. Ancylus)	4.2	16.4	3.2	1.4	0.1559	0.2346	98.17
Sphaeriidae	1.2	15.2	-	5.0	0.1248	0.1879	98.36
Physidae	2.8	11.4	1.6	-	0.118	0.1776	98.54
Odontoceridae	0.4	0.2	3.8	8.2	0.09473	0.1426	98.68
Sericostomatidae	2.2	3.8	6.6	4.8	0.09426	0.1419	98.82
Beraeidae	137.0	584.6	408.2	386.8	0.08763	0.1319	98.95
Dendrocoelidae	-	9.0	0.6	1.4	0.0818	0.1231	99.08
Leptoceridae	4.0	-	2.6	-	0.0679	0.1022	99.18
Goeridae	0.6	-	5.0	3.2	0.06614	0.09957	99.28
Heptageniidae	0.8	-	-	5.6	0.05678	0.08548	99.36
Ephemeridae	1.2	-	1.4	4.4	0.05528	0.08322	99.45
Rhyacophilidae	4.4	0.6	3.2	1.8	0.05451	0.08205	99.53
Lymnaeidae	1.2	0.4	1.6	1.4	0.03977	0.05987	99.59
Hirudinidae	-	2.0	0.8	0.6	0.03034	0.04568	99.63
Leuctridae	3.0	-	-	1.2	0.02798	0.04212	99.68
Tipulidae	-	-	-	0.2	0.02526	0.03803	99.71
Piscicolidae	0.6	2.0	0.4	-	0.0247	0.03718	99.75
Pedicidae	0.2	-	0.2	2.2	0.02323	0.03497	99.79
Empididae	0.8	1.4	0.8	0.2	0.02266	0.03412	99.82
Dryopidae	3.6	0.4	0.2	0.4	0.02224	0.03348	99.85
Psychodidae	-	3.4	-	-	0.01887	0.02841	99.88
Limoniidae	-	3.0	-	-	0.01325	0.01995	99.9
Perlidae	1.6	-	-	0.2	0.01083	0.01631	99.92
Ephydridae	-	2.0	-	-	0.009862	0.01485	99.93
Psychomyiidae	0.8	-	-	-	0.008985	0.01353	99.95
Leptophlebiidae	-	-	-	0.6	0.006929	0.01043	99.96
Hydroptilidae	0.4	-	0.2	-	0.005648	0.008502	99.97
Dixidae	0.2	0.2	0.2	-	0.00514	0.007738	99.97
Helophoridae	0.2	0.2	-	-	0.00377	0.005675	99.98
Stratiomyidae	-	0.2	-	-	0.002437	0.003669	99.98
Circulionidae	0.4	-	-	-	0.001953	0.00294	99.99
Gyrinidae	-	-	0.2	-	0.001719	0.002588	99.99
Brachycentridae	0.2	-	-	-	0.001636	0.002463	99.99
Corixidae	-	0.2	-	-	0.001611	0.002426	99.99
Rhagionidae	-	0.2	-	-	0.001602	0.002412	99.99
Nemouridae	-	-	0.2	-	0.001381	0.002079	100
Lepidostomatidae	0.2	-	-	0.2	0.0009766	0.00147	100
Veliidae	0.2	-	-	-	0.0009766	0.00147	100

Appendix 3a List of macroinvertebrate families captured on the River Crane sites CRUS, CRDS1, CRDS2 and CRDS3 including mean abundance ±SD from *n* = 5 kick samples for all sites except CRDS2 where *n* = 4

MAJOR GROUP	FAMILY	SITE							
		CRUS	SD	CRDS1	SD	CRDS2	SD	CRDS3	SD
EPHEMEROPTERA	Caenidae	-	-	-	-	0.8	1.5	0.4	0.9
	Heptageniidae	18.2	24.4	4.4	5.0	5.5	5.0	62.8	18.5
	Baetidae	165.8	245.8	312.0	232.2	60.0	64.2	99.2	67.1
	Ephemeridae	2.4	3.4	3.0	2.4	80.8	43.3	6.4	4.6
	Leptophlebiidae	-	-	0.6	0.9	8.8	10.7	2.2	2.3
	Ephemerellidae	241.8	334.6	112.0	112.6	291.0	534.8	144.0	134.5
TRICHOPTERA	Hydropsychidae	0.4	0.9	0.2	0.4	0.5	1.0	21.4	25.7
	Limnephilidae	27.6	32.8	13.8	12.9	21.0	5.8	3.6	3.8
	Leptoceridae	2.2	3.5	2.8	5.2	1.8	2.9	-	-
	Lepidostomatidae	-	-	-	-	-	-	0.2	0.4
	Polycentropodidae	-	-	0.2	0.4	7.8	9.0	2.6	4.3
	Philopotomidae	-	-	0.2	0.4	-	-	-	-
	Glossosomatidae	6.6	9.4	15.2	12.9	23.5	26.7	71.2	83.6
	Rhyacophilidae	4.0	4.8	2.4	2.9	1.3	1.9	3.4	3.0
	Sericostomatidae	7.2	6.8	2.4	2.1	9.8	4.8	1.8	3.0
	Lepidostomatidae	0.2	0.4	-	-	-	-	-	-
	Goeridae	16.6	18.1	5.4	9.4	2.8	2.8	14.8	16.1
	Beraeidae	-	-	0.2	0.4	-	-	-	-
	Odontoceridae	0.8	1.8	1.4	2.1	6.3	11.2	0.4	0.9
PLECOPTERA	Perlodidae	0.2	0.4	0.4	0.9	-	-	-	-
	Capniidae	4.2	8.3	0.4	0.9	1.0	2.0	-	-
	Leuctridae	5.8	6.6	0.8	1.1	20.3	27.1	7.6	7.5
CRUSTACEA	Gammaridae	967.0	822.5	693.2	556.1	360.0	279.8	403.2	211.1
	Asellidae	45.0	19.8	67.8	44.5	25.0	18.7	-	-
MOLLUSCA	Bithyniidae	-	-	-	-	-	-	0.2	0.4
	Planorbidae (excluding Ancylus)	3.0	2.8	9.2	14.7	1.0	2.0	-	-
	Valvatidae	1.6	2.1	84.0	166.1	0.5	1.0	-	-
	Hydrobiidae	-	-	-	-	20.8	41.5	49.8	33.9
	Physidae	-	-	0.8	1.3	-	-	-	-
	Ancylidae	38.0	44.7	4.0	5.2	1.0	1.2	15.8	12.3
	Lymnaeidae	0.8	1.1	0.6	1.3	-	-	-	-
	Sphaeriidae	5.8	9.7	121.4	76.5	121.8	74.1	11.4	8.4
HEMIPTERA	Coroxidae	0.2	0.4	0.6	0.9	-	-	-	-
COLEOPTERA	Gyrinidae	-	-	-	-	-	-	0.2	0.4
	Hydrophilidae	0.2	0.4	-	-	-	-	-	-
	Dryopidae	4.8	10.7	2.4	5.4	-	-	16.0	35.8
	Helophoridae	0.4	0.9	0.6	1.3	-	-	-	-
	Haliplidae	0.4	0.9	0.8	1.8	-	-	-	-
	Elmidae	132.4	79.4	57.0	25.2	43.8	25.1	93.6	52.4
	Dytiscidae	6.6	4.8	1.6	1.3	17.8	28.4	0.8	1.3
DIPTERA	Chironomidae	84.8	82.2	21.8	13.6	77.5	60.9	42.4	39.1
	Simuliidae	124.6	183.7	76.2	93.5	20.0	22.0	367.8	422.5
	Stratiomyidae	0.8	0.8	0.6	0.5	-	-	0.2	0.4
	Tipulidae	0.2	0.4	0.2	0.4	-	-	-	-
	Dixidae	-	-	-	-	2.3	3.9	0.6	1.3
	Rhagionidae	-	-	-	-	-	-	0.2	0.4
	Pedicidae	4.0	3.8	3.8	4.8	10.3	18.6	6.6	4.2
	Ceratopogonidae	3.4	4.9	1.2	1.1	1.3	1.5	-	-
	Psychodidae	0.2	0.4	0.2	0.4	-	-	-	-
	Limoniidae	-	-	0.2	0.4	-	-	-	-
	Ephydridae	-	-	0.8	1.8	-	-	0.6	1.3
	Tabanidae	-	-	-	-	0.3	0.5	-	-
	Empididae	0.2	0.4	-	-	-	-	-	-
OLIGOCHAETA		25.2	15.5	33.8	27.5	29.8	16.6	25.2	27.3
HIRUDINEA	Erpobdellidae	0.8	0.4	1.6	1.1	3.3	5.9	-	-
	Piscicolidae	2.0	2.7	0.8	0.4	-	-	0.4	0.5
	Hirudinidae	-	-	0.2	0.4	-	-	-	-
	Arhynchobdellida	-	-	-	-	0.3	0.5	-	-
	Glossiphoniidae	2.6	1.8	19.2	12.8	1.3	1.5	-	-
ARACHNIDEA	Arachnida (acarii)	5.2	6.4	6.0	5.8	5.0	4.2	8.6	8.0
MEGALOPTERA	Sialis	0.4	0.5	0.6	1.3	-	-	-	-
ODONATA	Cordulegastridae	0.4	0.5	0.2	0.4	0.3	0.5	0.2	0.4
	Coenagrionidae	-	-	-	-	-	-	-	-
	Calopterygidae	0.2	0.4	-	-	1.8	2.1	0.4	0.5
TRICLADIDA	Planariidae	1.4	2.1	5.4	2.4	0.5	1.0	-	-
	Dendrocolidae	0.4	0.9	2.0	2.4	0.5	1.0	-	-

Appendix 3b Similarity Percentage (SIMPER) analysis of macroinvertebrates on the River Crane, listed in order of contribution to dissimilarity between sites (CONT. %) and the cumulative percentage (CUM. %) and for illustration the mean abundances (standard errors and *n* in appendix 3a)

TAXON	SITE				DISSIMILARITY	CONT. %	CUM. %
	CRUS	CRDS1	CRDS2	CRDS3			
Gammaridae	967	693.2	360	403.2	15.09	25.33	25.33
Ephemerellidae	241.8	112	291	144	7.528	12.63	37.96
Simuliidae	124.6	76.2	20	367.8	6.995	11.74	49.7
Baetidae	165.8	312	60	99.2	6.567	11.02	60.72
Sphaeriidae	5.8	121.4	121.75	11.4	3.071	5.154	65.87
Chironomidae	84.8	21.8	77.5	42.4	2.195	3.684	69.56
Elmidae	132.4	57	43.75	93.6	1.933	3.245	72.8
Glossosomatidae	6.6	15.2	23.5	71.2	1.928	3.236	76.04
Asellidae	45	67.8	25	-	1.672	2.807	78.85
Ephemeridae	2.4	3	80.75	6.4	1.628	2.732	81.58
Valvatidae	1.6	84	0.5	-	1.028	1.725	83.3
Hydrobiidae	-	-	20.75	49.8	1.024	1.718	85.02
Heptageniidae	18.2	4.4	5.5	62.8	1.013	1.701	86.72
Oligochaeta	25.2	33.8	29.75	25.2	0.8487	1.424	88.15
Limnephilidae	27.6	13.8	21	3.6	0.8053	1.351	89.5
Ancylidae	38	4	1	15.8	0.7725	1.296	90.79
Goeridae	16.6	5.4	2.75	14.8	0.5744	0.964	91.76
Leuctridae	5.8	0.8	20.25	7.6	0.4644	0.7794	92.54
Dytiscidae	6.6	1.6	17.75	0.8	0.4195	0.704	93.24
Hydropsychidae	0.4	0.2	0.5	21.4	0.4194	0.7039	93.94
Glossiphoniidae	2.6	19.2	1.25	-	0.4048	0.6793	94.62
Dryopidae	4.8	2.4	-	16	0.3905	0.6553	95.28
Pedicidae	4	3.8	10.25	6.6	0.2565	0.4304	95.71
Sericostomatidae	7.2	2.4	9.75	1.8	0.2556	0.429	96.14
Planorbidae (ex. Ancylus)	3	9.2	1	-	0.2521	0.423	96.56
Arachnida (acarii)	5.2	6	5	8.6	0.2495	0.4188	96.98
Tipulidae	0.2	0.2	-	-	0.2338	0.3924	97.37
Polycentropodidae	-	0.2	7.75	2.6	0.2063	0.3461	97.72
Leptophlebiidae	-	0.6	8.75	2.2	0.1908	0.3202	98.04
Leptoceridae	2.2	2.8	1.75	-	0.1251	0.21	98.25
Planariidae	1.4	5.4	0.5	-	0.1206	0.2024	98.45
Rhyacophilidae	4	2.4	1.25	3.4	0.111	0.1864	98.64
Odontoceridae	0.8	1.4	6.25	0.4	0.1017	0.1706	98.81
Ceratopogonidae	3.4	1.2	1.25	-	0.09822	0.1648	98.97
Erpobdellidae	0.8	1.6	3.25	-	0.08883	0.1491	99.12
Capniidae	4.2	0.4	1	-	0.07292	0.1224	99.24
Piscicolidae	2	0.8	-	0.4	0.05862	0.09837	99.34
Dixidae	-	-	2.25	0.6	0.0516	0.0866	99.43
Calopterygidae	0.2	-	1.75	0.4	0.04532	0.07605	99.51
Dendrocoelidae	0.4	2	0.5	-	0.03424	0.05747	99.56
Ephydridae	-	0.8	-	0.6	0.02946	0.04945	99.61
Haliplidae	0.4	0.8	-	-	0.02789	0.04681	99.66
Stratiomyidae	0.8	0.6	-	0.2	0.02396	0.04021	99.7
Helophoridae	0.4	0.6	-	-	0.02318	0.0389	99.74
Lymnaeidae	0.8	0.6	-	-	0.01814	0.03044	99.77
Physidae	-	0.8	-	-	0.01678	0.02815	99.8
Cordulegasteridae	0.4	0.2	0.25	0.2	0.01533	0.02572	99.82
Sialidae	0.4	0.6	-	-	0.01455	0.02442	99.85
Perlidae	0.2	0.4	-	-	0.01395	0.02341	99.87
Caenidae	-	-	0.75	0.4	0.01364	0.0229	99.89
Corixidae	0.2	0.6	-	-	0.01293	0.0217	99.92
Psychodidae	0.2	0.2	-	-	0.008723	0.01464	99.93
Gyrinidae	-	-	-	0.2	0.006383	0.01071	99.94
Lepidostomatidae	0.2	-	-	0.2	0.005401	0.009063	99.95
Limoniidae	-	0.2	-	-	0.00485	0.00814	99.96
Hirudinidae	-	0.2	-	-	0.00485	0.00814	99.97
Philopotamidae	-	0.2	-	-	0.004715	0.007913	99.97
Bithyniidae	-	-	-	0.2	0.003111	0.005221	99.98
Hydrophilidae	0.2	-	-	-	0.002948	0.004947	99.98
Rhagionidae	-	-	-	0.2	0.00291	0.004884	99.99
Tabanidae	-	-	0.25	-	0.002686	0.004508	99.99
Beraeidae	-	0.2	-	-	0.002224	0.003733	100
Empididae	0.2	-	-	-	0.001751	0.002939	100

Appendix 4a. List of macroinvertebrate families captured on the River Frome sites FRUS, FRDS1, FRDS2 and FRDS3 including mean abundance ±SD from *n* = 3 kick samples

MAJOR GROUP	FAMILY	SITE							
		FRUS	SD	FRDS1	SD	FRDS2	SD	FRDS3	SD
EPHEMEROPTERA	Caenidae	6.3	11.0	6.3	11.0	1.7	2.9	14.7	25.4
	Heptageniidae	31.7	19.6	18.0	14.5	28.3	24.4	22.7	14.0
	Baetidae	211.3	176.3	203.0	252.9	210.3	174.0	274.3	241.5
	Ephemeridae	23.0	19.5	179.3	215.7	86.7	68.7	55.3	15.9
	Leptophlebiidae	0.3	0.6	0.3	0.6	0.3	0.6	3.0	4.4
	Ephemerellidae	0.7	0.6	2.3	3.2	4.0	2.6	2.7	1.5
TRICHOPTERA	Hydropsychidae	60.3	43.8	109.0	88.9	246.7	208.3	94.3	71.0
	Limnephilidae	3.3	3.5	18.0	27.8	8.3	8.0	6.7	5.5
	Leptoceridae	-	-	0.3	0.6	1.7	2.9	1.0	1.0
	Polycentropodidae	3.0	3.6	1.7	1.5	4.3	4.9	3.0	3.5
	Glossosomatidae	8.7	11.7	2.3	4.0	18.3	29.2	14.0	15.1
	Rhyacophilidae	9.0	5.3	3.3	2.1	9.0	9.2	5.0	4.0
	Sericostomatidae	4.3	6.7	9.3	5.5	1.0	1.0	3.7	2.3
	Psychomyiidae	-	-	-	-	0.3	0.6	-	-
	Brachycentridae	0.7	0.6	0.7	0.6	1.0	1.7	0.3	0.6
	Goeridae	3.0	3.5	4.7	4.5	6.3	1.2	58.0	78.9
	Beraeidae	-	-	2.0	2.0	-	-	-	-
	Odontoceridae	2.7	2.5	3.3	4.0	5.3	4.7	2.7	1.5
	Hydroptilidae	5.7	9.0	24.0	41.6	1.3	2.3	-	-
PLECOPTERA	Perlodidae	0.3	0.6	0.7	1.2	1.3	2.3	2.3	4.0
	Nemouridae	-	-	-	-	-	-	0.3	0.6
CRUSTACEA	Gammaridae	1787.7	1324.1	850.3	44.6	1507.0	1183.5	1445.3	590.1
	Asellidae	1.0	1.0	4.7	1.5	3.0	1.0	12.0	17.3
MOLLUSCA	Neritidae	97.7	54.3	19.0	11.3	17.3	11.6	5.0	5.3
	Bithyniidae	0.3	0.6	4.3	3.5	1.0	1.0	0.7	1.2
	Valvatidae	0.3	0.6	0.3	0.6	4.7	5.7	4.0	6.1
	Hydrobiidae	33.7	38.6	46.0	77.1	3.7	3.8	26.0	14.0
	Physidae	1.7	2.9	1.3	2.3	3.0	5.2	-	-
	Ancylidae	5.3	4.7	3.0	2.6	1.0	1.0	9.0	10.8
	Acroloxidae	-	-	0.3	0.6	0.3	0.6	-	-
	Lymnaeidae	14.3	12.4	23.7	21.5	43.0	51.1	13.3	12.9
	Sphaeriidae	0.7	1.2	1.0	1.7	0.3	0.6	8.0	11.4
HEMIPTERA	Pleidae	-	-	-	-	-	-	2.0	3.5
	Coroxidae	-	-	3.3	3.1	0.7	1.2	-	-
COLEOPTERA	Gyrinidae	1.3	2.3	1.0	1.7	5.7	4.5	2.3	2.3
	Elmidae	274.3	101.1	467.0	261.4	407.7	116.5	406.3	141.6
	Dytiscidae	-	-	0.3	0.6	1.0	1.0	0.7	0.6
DIPTERA	Athericidae	1.7	2.9	1.7	1.5	1.3	2.3	-	-
	Chironomidae	62.0	91.2	34.0	53.7	29.3	25.2	107.3	133.7
	Simuliidae	86.0	87.7	60.0	97.1	21.3	30.3	28.0	26.2
	Tipulidae	1.3	1.5	-	-	0.3	0.6	-	-
	Dixidae	0.3	0.6	-	-	-	-	0.3	0.6
	Pedicidae	1.7	2.9	5.0	5.0	20.0	6.2	3.7	4.7
	Ceratopogonidae	16.3	28.3	10.7	18.5	10.0	10.0	9.7	16.7
	Limoniidae	-	-	1.0	1.7	-	-	-	-
	Ephydridae	0.3	0.6	0.3	0.6	-	-	-	-
	Tabanidae	-	-	0.3	0.6	0.3	0.6	3.3	3.1
	Empididae	-	-	-	-	0.3	0.6	-	-
OLIGOCHAETA		71.0	83.0	37.0	14.0	15.3	7.6	65.3	14.6
HIRUDINEA	Erpobdellidae	7.0	7.5	2.0	2.6	4.3	2.1	9.7	8.1
	Piscicolidae	0.3	0.6	-	-	-	-	0.3	0.6
	Glossiphoniidae	0.7	0.6	5.0	4.4	4.7	1.5	7.7	5.5
ARACHNIDEA	Arachnida (acarii)	-	-	2.7	4.6	12.0	19.1	18.0	31.2
MEGALOPTERA	Sialis	-	-	-	-	-	-	2.3	4.0
ODONATA	Cordulegastridae	-	-	-	-	-	-	1.0	1.0
TRICLADIDA	Planariidae	-	-	1.0	1.7	0.7	1.2	0.7	1.2
	Dendrocolidae	-	-	-	-	0.3	0.6	-	-

Appendix 4b Similarity Percentage (SIMPER) analysis of macroinvertebrates on the River Frome, listed in order of contribution to dissimilarity between sites (CONT. %) and the cumulative percentage (CUM. %) and for illustration the mean abundances (standard errors and *n* in appendix 4a)

TAXON	SITE				DISSIMILARITY	CONT. %	CUM. %
	FRUS	FRDS1	FRDS2	FRDS3			
Gammaridae	1787.7	850.3	1507.0	1445.3	19.88	44.22	44.22
Baetidae	211.3	203.0	210.3	274.3	4.378	9.737	53.96
Elmidae	274.3	467.0	407.7	406.3	4.309	9.583	63.54
Hydropsychidae	60.3	109.0	246.7	94.3	2.826	6.286	69.83
Ephemeridae	23.0	179.3	86.7	55.3	2.091	4.651	74.48
Chironomidae	62.0	34.0	29.3	107.3	1.827	4.063	78.54
Simuliidae	86.0	60.0	21.3	28.0	1.443	3.211	81.75
Neritidae	97.7	19.0	17.3	5.0	0.9179	2.042	83.79
Oligochaeta	71.0	37.0	15.3	65.3	0.894	1.988	85.78
Hydrobiidae	33.7	46.0	3.7	26.0	0.8227	1.83	87.61
Lymnaeidae	14.3	23.7	43.0	13.3	0.5826	1.296	88.91
Goeridae	3.0	4.7	6.3	58.0	0.5355	1.191	90.1
Heptageniidae	31.7	18.0	28.3	22.7	0.4059	0.9027	91
Ceratopogonidae	16.3	10.7	10.0	9.7	0.3792	0.8433	91.85
Glossosomatidae	8.7	2.3	18.3	14.0	0.3376	0.7509	92.6
Arachnida (acarii)	-	2.7	12.0	18.0	0.3128	0.6956	93.29
Hydroptilidae	5.7	24.0	1.3	-	0.2967	0.6599	93.95
Caenidae	6.3	6.3	1.7	14.7	0.2555	0.5684	94.52
Limnephilidae	3.3	18.0	8.3	6.7	0.2487	0.5532	95.07
Tipulidae	1.3	-	0.3	-	0.2315	0.5149	95.59
Pedicidae	1.7	5.0	20.0	3.7	0.2276	0.5062	96.09
Asellidae	1.0	4.7	3.0	12.0	0.1537	0.342	96.44
Erpobdellidae	7.0	2.0	4.3	9.7	0.1309	0.2911	96.73
Rhyacophilidae	9.0	3.3	9.0	5.0	0.1291	0.2872	97.02
Ancylidae	5.3	3.0	1.0	9.0	0.1265	0.2814	97.3
Sericostomatidae	4.3	9.3	1.0	3.7	0.1255	0.2792	97.58
Glossiphoniidae	0.7	5.0	4.7	7.7	0.1054	0.2345	97.81
Sphaeriidae	0.7	1.0	0.3	8.0	0.09242	0.2056	98.02
Valvatidae	0.3	0.3	4.7	4.0	0.08539	0.1899	98.21
Odontoceridae	2.7	3.3	5.3	2.7	0.07836	0.1743	98.38
Gyrinidae	1.3	1.0	5.7	2.3	0.0765	0.1701	98.55
Polycentropodidae	3.0	1.7	4.3	3.0	0.07157	0.1592	98.71
Physidae	1.7	1.3	3.0	-	0.05947	0.1323	98.84
Bithyniidae	0.3	4.3	1.0	0.7	0.05551	0.1235	98.97
Ephemerellidae	0.7	2.3	4.0	2.7	0.05442	0.121	99.09
Leptophlebiidae	0.3	0.3	0.3	3.0	0.0497	0.1105	99.2
Corixidae	-	3.3	0.7	-	0.0435	0.09675	99.29
Perlidae	0.3	0.7	1.3	2.3	0.04129	0.09184	99.39
Athericidae	1.7	1.7	1.3	-	0.03898	0.08671	99.47
Leptoceridae	-	0.3	1.7	1.0	0.02896	0.06442	99.54
Sialidae	-	-	-	2.3	0.02758	0.06135	99.6
Tabanidae	-	0.3	0.3	3.3	0.02753	0.06123	99.66
Beraeidae	-	2.0	-	-	0.02172	0.0483	99.71
Pleidae	-	-	-	2.0	0.02066	0.04595	99.75
Planariidae	-	1.0	0.7	0.7	0.02022	0.04497	99.8
Brachycentridae	0.7	0.7	1.0	0.3	0.01867	0.04151	99.84
Cordulegasteridae	-	-	-	1.0	0.01132	0.02519	99.86
Dytiscidae	-	0.3	1.0	0.7	0.0106	0.02357	99.89
Limoniidae	-	1.0	-	-	0.00956	0.02126	99.91
Piscicolidae	0.3	-	-	0.3	0.00701	0.01559	99.93
Ephydridae	0.3	0.3	-	-	0.006787	0.01509	99.94
Acroloxidae	-	0.3	0.3	-	0.006636	0.01476	99.96
Dixidae	0.3	-	-	0.3	0.006254	0.01391	99.97
Psychomyiidae	-	-	0.3	-	0.003845	0.008552	99.98
Dendrocoelidae	-	-	0.3	-	0.003806	0.008465	99.99
Nemouridae	-	-	-	0.3	0.003443	0.007658	99.99
Empididae	-	-	0.3	-	0.002796	0.006219	100