Effects of agricultural intensification on the ecology of upland stream invertebrate communities.

Thesis submitted for the degree of Doctor of Philosophy
by

Caitlin Pearson MSc

School of Biosciences
Cardiff University
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Thesis summary

Agricultural land use is a leading cause of habitat degradation and biodiversity loss in streams**.** Understanding the mechanisms by which land use change affects stream ecosystems is essential for their effective management. Despite this, the consequences of agricultural intensification for community composition and ecosystem functioning in streams remain poorly resolved.

Using national-scale monitoring data and new field data from upland streams in South Wales, this study investigated the effects of pastoral intensification on the community composition, functional diversity and feeding interactions of stream macroinvertebrates. A combination of analytical tools were used, including propensity modelling, ecological traits, stable isotopes and Next Generation DNA sequencing to quantify diet.

Taxonomic and functional diversity had non-linear relationships with pastoral intensity, declining beyond a threshold of 4 mg L^{-1} nitrate and 8% fine sediment cover. This decline occurred as a non-random loss of species possessing specific traits, including large body size and lack of resistance forms. Although monitoring data showed that at a UK-wide scale pastoral agriculture (*cf*. other land uses) had a positive effect on richness and sensitive species representation, the threshold intensity at which effects become negative is exceeded in many locations within the U.K. and globally.

Invertebrates that feed by grazing algae were particularly vulnerable to agricultural stressors. Combined with changes in the availability and quality of basal resources with pastoral intensification, this decline in grazer representation resulted in invertebrate communities becoming increasingly reliant on detrital resources. Further, there was indication that methane-derived carbon contributed to the food web in high intensity sites, which has not previously been observed in upland streams. Although only relatively minor changes were observed in predator-prey interactions across the intensity gradient, there was a suggestion of simplification of the food web in high intensity sites. Together these changes could radically alter ecosystem properties such as secondary production, nutrient processing and resilience.

Overall, the results highlight the management priorities of reducing fine sediment and nutrient inputs to agricultural streams. The identification of a threshold at which agricultural effects become deleterious will assist in guiding mitigation efforts. Further work is required to determine the generality of this threshold across stream ecosystems.

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1. General Introduction

Freshwater ecosystems cover only 0.8% of the Earth's surface but contain 6% of the world's species and provide an estimated $4 trillion worth of ecosystem goods and services to society (Costanza *et al*., 2014*)*. Direct economic benefit is gained from freshwater ecosystems through fisheries, water supply, energy generation and recreation, with additional, but less tangible, value provided by their cultural importance and aesthetics. Further, freshwater ecosystems provide a range of functions such as carbon storage, biogeochemical cycling and local climate regulation for which there is insufficient knowledge to appreciate fully their value to society, economic or otherwise.

Despite recognition that freshwaters are essential to humankind, rivers are among the world's most threatened habitats, experiencing degradation and declines in biodiversity at a faster rate than most terrestrial ecosystems (Sala *et al.*, 2000; National Ecosystem Assessment, 2010). This is partially a result of overexploitation which has resulted in alteration of flow and sediment regimes, pollution, introduction of invasive species and channel modification (Dudgeon *et al.* 2006; Gurnell and Petts, 2010). The position of river systems, as receivers of all activities within the landscape makes them especially vulnerable to anthropogenic activities. Land use intensification, through its effects on fine sediment delivery, hydrology and inputs of nutrients, pesticides and heavy metals, is the greatest contributor to habitat change and biodiversity loss in river ecosystems (Allan and Flecker, 1993; Matson *et al.* 1997).

Globally, agriculture is the predominant anthropogenic land use, covering 38% of the Earth's ice-free land surface (Foley *et al.*, 2011). Growing global populations and increased demand for food have resulted in intensification of agricultural activities over recent decades. Meat production increased by 245% between 1961 and 2001 with only a 10% increase in the extent of grazing land area. Likewise, crop production increased by 47% between 1985 and 2005, with only a 2.4% increase in cropland area (Food and Agriculture Organisation, 2006). With this trend of intensification likely to continue, reconciliation of food production and environmental protection is a principal challenge for sustainable development and essential for freshwater conservation (Robertson and Swinton, 2005).

Pastoral agriculture, farming sheep and cattle, is the most widespread form of agriculture in the UK, covering over half of the UK land area (ca. 54%; Morton *et al*. 2011). Within this area there is a continuum of management intensity, from the extensively grazed uplands with little direct management, to the 25% of the UK classified as improved grassland, where management can be intensive with high stocking densities, re-seeding, fertilizing and draining of the land (Morton *et al*., 2011). The physico-chemical effects of pastoral intensification on rivers are well documented and include increases in fine sediment, elevation of dissolved nutrients, and alteration to riparian habitat, channel form and flow regime (eg. Belsky *et al.*, 1999; Allan, 2004). There is substantial evidence of the effects of each of these stressors on the biotic community, with the vast majority of studies concentrating on benthic macroinvertebrates. Stream macroinvertebrate taxa are highly variable in their sensitivity to stressors which, combined with their functional importance in stream ecosystems (Wallace and Webster, 1996), makes them an instructive model community for assessing anthropogenic effects. The stressors associated with pastoral agriculture can produce large changes in the composition of macroinvertebrate communities but consensus is lacking on the effects of multiple interacting stressors along a gradient of intensification. Responses to such complex stress gradients may be non-linear, with changes in community structure only occurring above critical thresholds of physico-chemical stress (Burdon *et al.*, 2013). The position of these thresholds is poorly understood, yet could be of great value in managing rivers and their catchments. Similarly, the underlying mechanisms that drive these changes in the invertebrate community are often neglected, but could include direct stress effects (eg. fine sediments clogging gills) or indirect mechanisms, such as the changes in terrestrial subsidies to the channel or other basal energy resources (Hladyz *et al.*, 2011).

Further, there remains considerable uncertainty about the consequences of stressors and the associated changes in community structure for ecosystem functioning. The complex interactions between organisms within a community can result in perturbations to one population producing unexpected changes in ecosystem properties such as productivity, resilience and community composition, but the link between changes in community structure and ecosystem functions remain poorly resolved (Gucker, 2009; Sandin and Solimini, 2009). Feeding interactions determine the pathways of energy and nutrient movement through ecosystems such that changes to feeding behaviour are likely to be a critical determinant of the ecosystem level response to stress (Woodward *et al.*, 2008; Woodward, 2009). Despite the potential for study of food web properties to help resolve the links between structure and function, very few studies have considered how food webs change across land use gradients (Wodward and Hildrew, 2002; Woodward, 2008).

In light of the preceding discussion, this study sought to evaluate the effects of intensifying pastoral agriculture on community composition, functional attributes and feeding interactions of benthic macroinvertebrates. Understanding the relationship between farming practices, biodiversity and ecosystem functioning is a priority for ecological research (Sutherland *et al.*, 2006) and the conclusions drawn from this work will aid the development of policy and practical mitigation measures to safeguard stream ecosystem services whilst maintaining agricultural productivity (Magbuna *et al.*, 2010).

1.1. Aims and hypotheses

Using catchments along gradients of increasing agricultural management intensity, this study aimed to test the overarching hypothesis that pastoral intensification would result in changes to both stream physico-chemical habitat and the food sources available to macroinvertebrates, leading to non-linear changes in macroinvertebrate taxonomic composition and functional diversity, and a simplification of feeding interactions.

The specific hypotheses tested were that increasing the intensity of pastoral agriculture would result in:

1) Changes in physical characteristics of streams across England and Wales, including increased nutrient and fine sediment concentrations;
2) A non-linear reduction in taxonomic and trait diversity in macroinvertebrate communities;
3) Macroinvertebrate primary consumers having an increased reliance on algal food resources, compared to detrital material;

4) Changes to the diet of invertebrate predators with a narrower diet breadth and stronger selection for fewer prey taxa in sites with high intensity agriculture.

Chapter 2 reviews the literature on the effects of agricultural land use on stream systems, focussing on macroinvertebrate communities. Specifically, the effects of agricultural intensification for taxonomic and trait composition are reviewed, and the current knowledge on the likely consequences for trophic interactions and ecosystem functioning are explored. Knowledge gaps are highlighted; these are then explored in the following experimental chapters.

In Chapter 3, Environment Agency monitoring data from over 3,000 sites in England and Wales are used to assess the effects of increasing agricultural land cover at the national scale. In addition to pastoral agriculture, the effects of arable farming are also investigated. Application of a statistical technique novel to land use studies, which can control for the effects of potentially confounding variables, allows the responses of stream habitat, water chemistry and invertebrate community composition to be investigated at the large spatial scale over which policy is implemented. A version of this chapter is in press for *Journal of Applied Ecology*.

Chapters 4-6 use field data collected from upland headwater streams in South Wales spanning a gradient of pastoral intensity. In Chapter 4 non-linear changes in invertebrate taxonomic composition and functional diversity in response to increasing land use intensity are appraised, with an assessment of the mechanisms driving changes in community structure and the potential effects on stream functioning. In Chapter 5, analysis of functional feeding guild representation and stable isotopes are combined to test the hypothesis that the macroinvertebrate community would become increasingly reliant on algal (*cf.* detrital) food sources with agricultural intensification. In Chapter 6, Next Generation Sequencing is used to analyse predator gut contents to determine how agricultural stressors affected the feeding interactions of two dominant predatory invertebrates. This is the first study to apply this technique to freshwater foodwebs.

Finally, Chapter 7 draws together the salient points from the evidence presented in the thesis and, whilst acknowledging some of the limitations in the study, presents the wider implications of the work for ecosystem and land use management. Remaining knowledge gaps are highlighted and further avenues of research suggested.

2. Literature review: The effects of pastoral intensification on stream ecosystems.

2.1. Introduction

With the global population expected to reach 7 billion, and the demand for food likely to at least double between 2000 and 2050 (Green *et al.*, 2005), food security is one of the greatest challenges of the 21st Century. Rising demand has encouraged increases in intensity of both arable (cultivation of crops) and pastoral (animal husbandry) agriculture (Matson *et al.*, 1997). Between 1961 and 2001, global meat production increased by 245% yet grazing land area increased by less than 10% (Food and Agriculture Organisation, 2006). Likewise, crop production increased by 47% between 1985 and 2005 with only a 2.4% increase in cropland area (Food and Agriculture Organisation, 2006). In order to achieve these higher yields per unit area, drainage, regular fertilisation, pesticide use, high stocking densities and multiple crop rotations per year have become routine agricultural practices. This trend of intensification is predicted to continue over the coming decades (Food and Agricultural Organisation, 2006). These practices have resulted in widespread declines in the extent and condition of both terrestrial and aquatic habitats, with diffuse water pollution, soil erosion, carbon loss and habitat fragmentation driving declines in biodiversity (eg. Tilman, 1999, Green *et al*., 2005, Piscart *et al*., 2009). Reconciling agricultural production and environmental protection, and ensuring that increased food production does not come at the expense of other ecosystem services, is one of the greatest challenges for sustainable development (Sutherland *et al.*, 2006).

The effects of agriculture on freshwaters are of particular interest due to the conservation, economic and cultural importance of these systems, and their vulnerability to land use change (Dudgeon *et al.*, 2006). Freshwaters provide drinking water, recreation opportunities, pollutant removal, fisheries and cultural value, while vectoring important biogeochemical links between land, water and marine systems (Dudgeon *et al.*, 2006; Maltby and Ormerod, 2011). Angling alone brings £2.4 billion to the U.K. economy each year (Lyons *et al.*, 2002) but non-market services are harder to assess and remain undervalued (Bateman, 2011). Upland streams are particularly important in ecosystem service provision, providing 70% of the U.K.'s drinking water for people and livestock (Heal, 2003), and acting as sources of water, organic material and biota to fuel downstream reaches.

The characteristics of river systems are determined by the landscape through which they flow, with hydrology, diffuse pollution, organic matter and nutrient loading determined by the land uses within the catchment (Hynes, 1975). Intensification of agricultural land use has contributed to the loss of stream biodiversity, which is occurring at a faster rate than in any other ecosystem (Strayer and Dudgeon, 2010). Few freshwaters now exhibit the biological and chemical attributes characteristic of a river in a pristine condition, which is considered to represent 'healthy' ecosystem functioning (Jax, 2005; Dudgeon *et al.*, 2006). A better understanding of the links between physico-chemical effects of agricultural change and ecosystem functioning is necessary to inform land use management and ensure sustained provision of the economic and societal benefits delivered by river ecosystems.

This literature review examines possible changes in agricultural practices in the UK and discusses the effects of changes in intensity on the physico-chemical characteristics of streams. The consequences of these changes for community composition, ecosystem functioning and trophic interactions are then explored, highlighting where knowledge gaps remain.

This study focusses on pastoral agriculture in upland catchments, reflecting the predominant agricultural land use in the U.K (DEFRA, 2015). Upland headwater streams constitute the majority of the length of the river network in the U.K. (Mainstone *et al.*, 2014) and are particularly vulnerable to land use change as their small size reduces their ability to buffer perturbations (Trimble and Mendel, 1995).

2.2. Pastoral farming in the U.K.

In the U.K., agriculture covers over 77% of the land area, of which 65% is pastoral farming (DEFRA, 2015). A diverse range of pastoral systems operates in the U.K., from extensive sheep grazing to intensive dairy and beef farming, with their distribution reflecting spatial patterns in climate and soils. On a national scale, pastoral agriculture intensified over the second half of the 20th Century, with increased chemical inputs, larger herd sizes and a switch from hay to silage systems (Stoate, 1996). This was a result of both technological advances and the adoption of the E.U. Common Agricultural Policy (CAP) in 1973 which gave financial incentives for high production. The CAP resulted in large areas of rough grazing land being drained, reseeded and fertilised to create enclosed 'improved' pastures on which high densities of livestock were grazed (Condliffe, 2009). This period of intensification coincided with a decline in the condition of neighbouring habitats, increased nutrient concentrations in stream waters (Croll and Hayes, 1988) and a reduction in farmland biodiversity (Chamberlain *et al.*, 2000).

Presently, an increasing proportion of financial subsidy for farmers is linked to environmental protection. Since 2003, CAP payments have been decoupled from production and attached to a baseline standard of 'Good Environmental and Agricultural Condition' and agri-environment payments now comprise a significant proportion of farming incomes in the U.K. (Whitfield, 2006). These schemes (Countryside Stewardship in England and GLASTIR in Wales) have resulted in reductions in stocking rates (Gardner *et al.*, 2008) and demonstrable benefits for water quality and biodiversity (Whittingham, 2011).

The future trajectory of pastoral intensity in the U.K., and hence its environmental impact, is likely to be governed by the response of the farming community to ongoing changes in the structure of financial subsidies. Increased uptake of agri-environment schemes may result in further declines in stocking density in upland areas and active management for ecosystem service provision (Dwyer *et al.*, 2010). If, however, these payments are insufficient to support farming activities in less productive areas, a reduction in subsidies linked to production could result in abandonment of upland areas and intensification of valley bottoms (Reed *et al.*, 2009; Termansen *et al.*, 2009). Alternatively, farmers may increase stocking levels to maintain their income, with the potential for environmental degradation from overgrazing (Davies-Jones, 2011). The reality is likely to be complex and spatially dependent, driven by the continued evolution of financial subsidy schemes, set against global market forces and climatic change.

In summary, pastoral farming is likely to experience substantial change over the coming decades (Davies-Jones, 2011). With such uncertainty surrounding the future of upland land management, understanding the consequences of changing agricultural intensity on neighbouring habitats, and the way they function to provide ecosystem services, is essential to inform land use policy.

2.3. Impacts of pastoral farming on the physico-chemical characteristics of streams

The physical impacts of pastoral farming on stream systems are well documented. Livestock compact soil and remove vegetation, reducing infiltration rates and leading to increased overland flow (Mullholland and Fullen, 1991; Daniel *et al.*, 2002). This change can alter hydrological response time to rainfall events, flow regime and, ultimately, channel morphology (Mueller *et al.*, 2010). Further, overland flow entrains surface sediment, increasing fine sediment delivery to the channel (Heathwaite and Burt, 1991; Owens *et al.*, 1996; Daniel *et al.*, 2002). This leads to increased sediment deposits and higher turbidity in the stream (eg. McDowell *et al.*, 2003). Further sediment influx can occur from direct bank erosion as livestock access the stream (Trimble and Mendel, 1995). Manure produced by livestock and inorganic fertilisers applied to improved pastures are rich in nitrates (Jarvie *et al.*, 2008), phosphates (Palmer-Felgate *et al.*, 2009), organic carbon (Owens *et al.*, 1989), potassium (McDowell *et al.*, 2003) and heavy metals (Clements *et al.*, 2000). These fertilisers leach into water courses where they can dramatically alter both the nutrient budget and water chemistry of the system.

These physicochemical changes have non-linear relationships with stocking density (Heathwaite and Burt, 1991; Smith *et al.*, 1993; Daniel *et al.*, 2002). Light grazing may have positive impacts on soil quality and nutrient retention by stimulating shoot and root growth (Haan *et al.*, 2007), and adding organic matter to the soil, which in turn improves water and nutrient retention (Hubbard *et al.*, 2004). Above a threshold stocking density, soil compaction does, however, result in greater overland flow and elevated sediment and nutrient inputs to river systems. For example, Gary *et al.* (1983) found no changes in water column nitrate and ammonia levels compared to ungrazed reaches when up to 60 cattle were grazed on pastures adjacent to a stream but significant increases when stocking densities were increased beyond this. Similarly, a review of U.S. studies found that “light” and “moderate” stocking densities had no detectable effects on infiltration rates whereas “heavy” grazing significantly reduced infiltration rates (Gifford and Hawkins, 1978).

2.4. Impacts of pastoral farming on stream communities

Physico-chemical changes are considered to be stressors when, as a result of human activity, they exceed their normal range of variation and impact the biological community (Townsend *et al.*, 2008; Wagenhoff *et al.*, 2011). Several of the physico-chemical changes associated with agriculture act as stressors in stream ecosystems, with nutrient enrichment and sedimentation being the most pervasive (Matthaei *et al.*, 2010; Wagenhoff *et al.*, 2011). Responses to agricultural stressors have been observed for a range of taxa but the majority of studies focus on macroinvertebrates due to their abundance and diversity, wide ranging sensitivity to stressors and functional importance in stream ecosystems making them instructive model organisms (Wallace and Webster, 1996).

The responses of community metrics to both sediment and nutrient inputs are non-linear. Small increases in fine sediment deposits can result in increased taxonomic richness due to increased habitat diversity providing substrata for macrophytes and refugia for burrowing invertebrates (Braccia and Voshell, 2007; Matthaei *et al.*, 2010). High sediment inputs, however, can reduce biomass and species richness of primary producers as turbidity reduces light penetration and fine sediment abrades cells and smothers growth (Wetzel and McGregor, 1968; Townsend and Riley, 1999; Parkhill, 2002; Barlocher and Corkum, 2003). In high concentrations sediment is also damaging for invertebrates and fish, smothering habitat (Wood and Armitage, 1997), clogging respiratory surfaces and impeding filter feeding (Lemly, 1982). This results in declines in richness (Lemly, 1982; Fairchild *et al.* 1987; Cuffney, 2000; Zweig and Rabeni, 2001; Niyogi *et al.*, 2003; Matthaei *et al.*, 2006; Larsen *et al.*, 2009; Herbst, 2012), abundance (Fairchild *et al.*, 1987; Quinn and Hickey, 1990; Zweig and Rabeni, 2001; Larsen and Ormerod, 2010b) and biomass (Matthaei *et al.*, 2010).

Similarly, nutrient enrichment stimulates macrophyte and algal growth as nitrate and phosphate are limiting nutrients (Quinn and Hickey, 1990; Cuffney *et al.*, 2000; Bernot *et al.*, 2006; Riddle, 2009). Algal growth from mild nutrient enrichment can stimulate secondary production of herbivorous invertebrates and their predators through an increase in available energy, and is associated with increased invertebrate richness (Riley *et al.*, 2003), biomass (Niyogi *et al.* 2003; Matthaei *et al.* 2006) and abundance (Riley *et al.*, 2003; Young and Collier, 2009). High nutrient concentrations, however, can alter the composition of algal communities, with taxa that are less palatable to invertebrates having a competitive advantage (Rosemond *et al.* 1993; Slavik *et al.* 2004, Riddle, 2009). Further, high nutrient concentrations have direct negative impacts on stream invertebrates and fish by modifying oxygen-carrying pigments to forms incapable of carrying oxygen (Grabda *et al.*, 1974).

Although the effects of these stressors acting in isolation are well documented, complex interactions between stressors mean that biological responses to the aggregate effects of intensification can be unpredictable from knowledge of single stressor effects (Townsend *et al.*, 2008; Matthaei *et al.*, 2010). For example, in the presence of low oxygen levels, the toxicity of trace metals to invertebrates increases due to enhanced gill movement increasing metal uptake (Van der Geest *et al*. 2002). Conversely, mild nutrient enrichment and sediment may interact antagonistically to produce less change in species richness than would be expected from their individual effects (Matthaei *et al.*, 2010).

Quinn (2000) captured these non-linear effects of multiple stressors by postulating that the overall effect of increasing pastoral intensity on biological metrics, such as invertebrate richness and abundance, would be a ‘subsidy-stress response’: mild increases in nutrients and fine sediment would supplement habitat and food supply, resulting in greater richness and abundance, but above a threshold concentration the negative effects of sedimentation, and other stressors associated with agriculture, would cause these metrics to decline. Determining the level of pastoral development at which overall effects become negative is critical for sustainable land management.

The predicted subsidy-stress response to increasing pastoral intensity has been observed for total invertebrate richness and number of sensitive taxa by Braccia and Voshell (2007), with these metrics peaking at stocking densities of 0.7 and 1 cattle per hectare, then declining with further increases in stocking density. This response pattern, however, is not ubiquitous. Pastoral intensification has been associated with both lower (Quinn and Hickey, 1990; Liess *et al.*, 2012) and higher invertebrate richness and sensitive species representation (Thompson and Townsend, 2004), with other studies finding no significant associations (Riley *et al.*, 2003). This lack of consensus on the effects of land use change may reflect variability in the relationship between stocking levels and in-channel impacts or could be an artefact of a limited range of in-stream conditions sampled within individual studies. Further work across a broader gradient of in-stream conditions will help to identify the level of intensity that acts as a stressor for macroinvertebrate communities (Townsend *et al.*, 2008).

2.5. Effects of pastoral intensification on ecosystem function

Metrics of community structure, such as richness, diversity or abundance, are the primary measures of ecosystem condition used in both scientific studies and environmental monitoring programmes. Due to their predictable responses to stress gradients, wide distribution, high abundance and relative ease of sampling and identification, macroinvertebrates are the most common focus of such metrics. Over 50 macroinvertebrate bio-monitoring metrics are currently in use (Friberg *et al.*, 2006) which, as discussed above, can provide an indication of the level at which stressors begin impacting the community (Magbuna *et al.*, 2010; Herbst, 2012). Being based on taxonomy, however, most metrics ignore the functional roles of species in ecosystems and provide little indication of the effects of stressors on ecosystem functions (Tilman and Downing, 1994; Díaz and Cabido, 2001), defined as the ‘properties of an ecosystem resulting from the collective activities and interactions of all its biota’ (Jax, 2005; Naeem *et al.*, 2002).

As the limitations of structural measures have been recognised, direct measures of ecosystem functioning have increasingly been used in research studies, although uptake into monitoring and assessment programmes has been low (Dolédec *et al.*, 2006). There is now substantial evidence that stressors associated with agriculture impact ecosystem functions, including leaf litter breakdown rate, primary productivity, ecosystem metabolism and rates of nutrient uptake (Hall and Tank, 2003; Sandin and Solimini, 2009). As with community structure, however, ecosystem functions respond to a multitude of interacting stressors, often resulting in non-linear responses to the combined effects of agricultural intensification (eg. Matthaei *et al.*, 2010).

Ecosystem metabolism, the ratio of carbon production by photosynthesis (gross primary productivity) and carbon use (respiration by autotrophs and heterotrophs) across the whole ecosystem, is widely used as a metric for ecosystem functioning (Young *et al.*, 2008; Bernot *et al.*, 2010). It provides an integrated measure of carbon cycling, which in turn determines secondary production and the cycling of other nutrients, making it a key indicator of ecosystem functioning (Bernot *et al.*, 2010). Agricultural stressors directly affect ecosystem metabolism through impacts on algal productivity and decay rates. Elevated nutrient concentrations result in increased stream productivity whereas sedimentation reduces the area of stream bed suitable for algal growth, reducing overall periphyton biomass (Hagen *et al.*, 2010) and can accelerate decay rates due to increased abrasion (Hagen *et al.*, 2006). A subsidy-stress response to agricultural intensity may therefore be expected for ecosystem metabolism (Young and Collier, 2009; Matthaei *et al.*, 2010). Such a relationship has been observed in response to the conversion of forest into pasture. Forested streams tend to be heterotrophic with production:respiration ratios < 1 (Bunn *et al.*, 1999; Hagen *et al.*, 2010) and as low as 0.2 (Hagen *et al.*, 2010). Forest clearance and an increase in agricultural intensity result in increased autotrophy, until a threshold level at which excessive sedimentation results in streams returning to being heterotrophic (Young and Collier, 2009; Bernot *et al.*, 2010; Hagen *et al.*, 2010). Few studies have addressed changes in ecosystem metabolism that are not accompanied by vegetation clearance within the catchment, making it difficult to resolve the effects of more intensive pastoral management. As for structural measures, continued investigation into the response of functional metrics to a broad gradient of agricultural intensity is required to improve understanding of the links between land use and stream ecosystem properties.

2.6. Linking structure and function

In addition to the direct effects of physico-chemical stressors, ecosystem functions are modulated by changes in the structure of biological communities along stress gradients (DÌaz *et al.*, 2007). Experimental and theoretical studies have demonstrated links between species richness and ecosystem functioning rates: communities with a greater number of species are likely to contain a greater diversity of traits, fulfilling a greater number of functional roles (Tilman *et al.*, 1997; Dukes, 2001). Taxonomic and trait diversity may, however, be weakly correlated if a pool of species displaying diverse traits is replaced with a community displaying similar traits and high redundancy, which would result in functional diversity (the range and relative abundance of functional traits present in the system) falling much more quickly than taxonomic diversity (Cornwell *et al.*, 2006). Therefore, trait-based metrics can provide greater insight into the links between structure and function than taxonomic measures, but the limitations of trait based approaches must be acknowledged. Firstly, trait based approaches do not reflect the interactions between traits. Due to ecological and evolutionary constraints, possession of a given trait may predispose a species to have certain other traits (Verberk *et al.*, 2013). Environmental filtering will act upon the whole subset of traits possessed by an organism such that interactions between traits may obscure the response of an individual trait to a stress gradient or change a trait's adaptive significance. Secondly, there is a lack of understanding of the functional importance, which will depend on both the environmental context and the combination of other traits with which it is manifest (Verberk *et al.*, 2013).

Despite these challenges to trait-based analyses, identifying changes in functional diversity along stress gradients has great potential to improve our mechanistic understanding of ecosystem responses to stressors. A wealth of theoretical studies have developed the functional diversity approach but empirical studies of changes in functional diversity along stress gradients have been limited (Pakeman, 2011).

Changes in trait representation in macroinvertebate communities along agricultural stress gradients are well documented but the overall changes in functional diversity are unresolved and remain a major source of uncertainty for predicting the effects of land use change on ecosystem function and service provision. The representation of behavioural and morphological traits responds to both nutrient and sediment pressures. Gill respiration, free swimming, crawling and clinging behaviour become less frequent in response to both these stressors (Rabeni and Zweig, 2005; Richards *et al.*, 1997; Wood and Armitage, 1997; Braccia and Voshell, 2006; Braccia and Voshell, 2007; Larsen and Ormerod, 2010; Magbuna *et al.*, 2010) with concomitant increases in representation of plastron, tegumental or aerial respiration, burrowing behaviour and streamline body shapes (Doledec *et al.*, 2006; Braccia and Voshell, 2007). Taxa with large body sizes tend to be the most susceptible to sedimentation pressures due to their requirement for large interstitial spaces and lack of resilience traits such as short life cycles and high reproductive rates (Quinn and Hickey, 1990; Richards *et al.*, 1997; Townsend *et al.*, 1997a; Larsen and Ormerod, 2010; Magbuna *et al.*, 2010).

The representation of different feeding traits is particularly relevant to ecosystem functioning, as the transfer of energy and nutrients through the system governs many ecosystem functions (Townsend and Riley, 1999; Benke *et al.*, 2001; Gessner *et al.*, 2010). The representation of taxa with specialised feeding traits can provide a measure of the reliance of the community on different basal resources (Huxel *et al.*, 2002; Hagen *et al.*, 2010). Stream food webs are fuelled by two distinct resource types, living ‘algal’ material from in-stream primary producers, and detrital material from both aquatic and terrestrial plants (Hall and Eggert, 2000). The relative utilisation of these alternative basal resources exerts a major influence on several ecosystem properties, including stability, secondary production and nutrient cycling rates (Polis *et al.*, 1997; Wallace *et al.*, 1997).

Representation of grazers, primary consumers adapted to eat algae, has been shown to increase with initial pastoral intensification as a result of increased primary production (Quinn and Hickey, 1990; Townsend *et al.*, 1997b; Harding *et al.*, 1999; Townsend and Riley, 1999) but to decline at high stocking densities due to the negative effects of sedimentation (Harding *et al.*, 1999; Braccia and Voshell; 2007; Wagenhoff *et al.*, 2011). Detrital feeders can be subdivided into shredders, which feed on coarse particulate organic matter, collector gatherers, which feed on fine particles and filterers which remove particles from suspension (Merrit and Cummins, 1978). Studies have generally found that shredders decline in livestock-grazed reaches compared to ungrazed ones, probably due to a reduction in allochthonous inputs from riparian vegetation (Townsend *et al.*, 1997b; Harding *et al.*, 1999; Kyriakeas and Watzin, 2006; Braccia and Voshell, 2007). Where riparian vegetation cover is unaltered, intensification of pasture can, however, increase shredders due to the higher nutritional quality of litter from nutrient enrichment (Hladyz *et al.*, 2009; 2011). Shredders are also negatively impacted by sediment levels and are therefore likely to show a subsidy-stress response along agricultural intensity gradients (Niyogi *et al.*, 2003). Food supply for collector-gatherers and filterers is increased by manure, increased fungal production and increased dead algal cells, such that their representation tends to increase along agricultural intensity gradients (Braccia and Voshell, 2007).

Although the relative representation of these feeding traits can provide valuable insight into energy transfers, a large proportion of invertebrates are generalist consumers, able to utilise both detrital and algal food sources (Chapman and Demory, 1963), with actual ingestion rates dependent on the elemental ratios of their food sources and hence variable between streams (Lauridsen *et al.*, 2014). Their ability to switch between basal resource pathways could act to stabilise the food web and increase the resilience of the ecosystem to perturbations (Hladyz *et al*., 2011), but site-specific ingestion rates cannot be ascertained from trait information. Stable isotope analysis is increasingly being used for this purpose (Woodward and Hildrew, 2002). The ratios of carbon isotopes (δ^{13}C) vary between primary producers and are enriched by one part per thousand with each trophic level (DeNiro and Epstein, 1981) such that the δ^{13}C of a consumer's tissues shows the relative contributions of different resources to its diet over the time period during which those tissues were formed (Fry and Sherr, 1984; Bearhop *et al.*, 2002). Employed together, trait information and isotopic data can provide valuable insight into the consequences of changes in the structure of biological communities for ecosystem functioning (Dìaz and Cabido, 2001).,

2.7. The importance of interactions

The importance of resolving the links between taxonomic composition, trait diversity and ecosystem functioning have received considerable attention in freshwater systems and in wider ecological literature (Loreau *et al.* 2001; Covich *et al.*, 2004). Several studies have demonstrated a positive relationship between functional diversity and ecosystem processing rates in terrestrial plant and soil communities (eg. Tilman and Downing, 1994; Tilman *et al.*, 1997; Dukes, 2001) but there remains considerable uncertainty as to the generality of this result across ecosystems. Complex patterns of interactions between individuals, including competition, mutualism and predation may result in unexpected changes to ecosystem functioning as a result of changes in community composition. For example, experimental removal of taxa with large body sizes from stream communities (two predatory perlid stoneflies, a detrivorous limnephilid caddisfly and a detrivorous stonefly) resulted in cascades in both algal and detrital food webs due to removal of feeding interactions associated with these taxa (Lecerf and Richardson, 2011). The importance of interactions may explain why taxonomic identity of shredding taxa, as well as relative trait representation, has been shown to be an important determinant of leaf litter breakdown rates (eg. Covich *et al.*, 1999; Huryn *et al.*, 2002). Understanding the patterns of interactions and their response to stressors will help to determine the links between physico-chemical stress, community composition and ecosystem functions, including system stability and resilience (Townsend and Riley, 1999; Woodward *et al.*, 2008; Woodward, 2009).

Recently, theoretical understanding of the consequences of altered predator-prey interactions for ecosystem properties has improved. The distribution and strength of interactions determines the stability of the food web (Montoya *et al.*, 2009) with complex food webs (those with a greater number of links per species) being the most stable, provided that most trophic interactions are weak (eg. McCann *et al*., 1998; Borrvall *et al.*, 2000). Despite this understanding of the importance of trophic structure, there has been relatively little attempt by ecologists to quantify changes along anthropogenic stress gradients. Study of food webs in agricultural streams has mainly been limited to connectance-based approaches, which assume that if a feeding link has been observed previously, in any system, then it will be present in the study system. Using this approach, both Riley (2003) and Thompson and Townsend (2005) found that measures of connectance and food chain length increased with agricultural intensity. Both studies covered only a small portion of an intensity gradient such that these results probably reflect the subsidy effect of mild nutrient enrichment. No studies have assessed changes in connectance over a broad range of agricultural intensities and no attempt has been made to assess whether agricultural stressors affect the feeding of invertebrate predators.

Recent advances in molecular technology may revolutionise the study of aquatic food webs over the next few years. The ability to screen the gut contents of invertebrates for the DNA of potential prey species could allow rapid identification of their diets at different locations so that site-specific feeding can be determined (*cf.* connectance webs). Traditionally, gut content analysis was done visually but this was time consuming, had high error rates and could not be used for prey items that did not have identifiable remains (Woodward and Hildrew, 2002; Thompson and Townsend, 2005). Genetic based techniques, using PCR amplification of mitrochondrial DNA, have largely replaced visual analyses in terrestrial ecology and offer great potential for resolving changes in feeding behaviour along anthropogenic stress gradients. This will afford greater insights into the mechanisms underlying anthropogenic stressors effects on ecosystem functioning (Gray *et al.*, 2014).

2.8. Temporal aspects

The majority of stream ecosystem studies are conducted within a single season (usually spring/early summer), reflecting the traditional focus of macroinvertebrate sampling and biological monitoring at this time of year (Clarke *et al.*, 2002). A consequence of this focus is that the potential temporal variability of ecosystem responses to anthropogenic stressors is often overlooked, despite strong seasonality in both abiotic and biotic characteristics (Ledger and Hildrew, 1998). The 'insurance hypothesis' predicts that depauperate communities, as expected in anthropogenically stressed systems, have reduced functional redundancy and therefore lower temporal stability (Yachi and Loreau, 1999). Under this hypothesis, structural and functional measures in streams draining more intensive pastures would vary more strongly in response to natural seasonal patterns such as river-fly emergence and changes in incident light, temperature, organic matter inputs and discharge (Thompson and Townsend, 1999). Empirical testing of this prediction could afford novel insights into the effects of anthropogenic stress on ecosystem stability and persistence (Cottingham *et al.*, 2001).

2.9. Conclusions

Intensification of agriculture has contributed to the loss of stream biodiversity and has impaired stream ecosystem functioning. With expected changes in the intensity of agriculture in upland areas, ensuring sustained delivery of the ecosystem services provided by stream ecosystems is a challenge that will require a robust understanding of the links between physico-chemical changes, community composition and ecosystem functioning. Although a growing number of studies have addressed these links, considerable knowledge gaps remain.

Firstly, most studies have assessed the effects of single stressors on biological responses but pastoral intensification results in a multitude of physico-chemical changes that can interact in complex ways. Further evidence is needed to determine the aggregate effects of land use change. Studies that have assessed the combined effects of agricultural intensification have found different and often contradictory results, which are likely to be attributable to differences in the range of intensities considered. Further studies across a broader gradient of in-stream conditions will help to identify the level of intensity that acts as a stressor for stream communities (Townsend *et al.*, 2008).

Secondly, most monitoring focuses on taxonomic measures and provides little indication of the effects of stressors on ecosystem functions. Measures of functional diversity provide useful metrics to elucidate these links but changes in functional diversity along stress gradients remain under explored. Thirdly, there has been little attention afforded to the response of feeding interactions, both basal resource use and transfers to higher trophic levels, to stress gradients despite their role in shaping ecosystem properties and stability (Woodward *et al.*, 2008). Finally, the interaction between the seasonal variation in stream communities and land use stressors remains unexplored, yet could deepen understanding of the mechanisms that generate stress responses and help determine the threshold intensity at which stream ecosystems are impaired. Addressing these gaps in current knowledge will increase the ability to identify impairment thresholds, and to predict and mitigate perturbations to stream ecosystems caused by agricultural land use.

3. Resolving large-scale agricultural effects on streams using propensity modelling

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3.1. Summary

Although agriculture is among the world's most widespread land uses, studies of its effects on stream ecosystems are often limited in spatial extent. National monitoring data could extend spatial coverage and increase statistical power, but analysis of such data is challenging where covarying environmental variables confound relationships of interest.

Propensity modelling is used widely outside ecology to control for confounding variables in observational data. Here, monitoring data from over 3,000 English and Welsh river reaches are used to assess the effects of intensive agricultural land cover (arable and pastoral) on stream habitat, water chemistry and invertebrates, using propensity scores to control for potential confounding factors (eg. climate, geology). Propensity scoring effectively reduced the collinearity between land cover and potential confounding variables, reducing the potential for covariate bias in estimated treatment–response relationships compared to conventional multiple regression.

Macroinvertebrate richness was significantly greater at sites with a higher proportion of improved pasture in their catchment or riparian zone, with these effects probably mediated by increased algal production from mild nutrient enrichment. In contrast, macroinvertebrate richness did not change with arable land cover, although sensitive species representation was lower under higher proportions of arable land cover, probably due to greatly elevated nutrient concentrations.

Propensity modelling has great potential to address questions about pressures on ecosystems and organisms at the large spatial extents relevant to land-use policy, where experimental approaches are not feasible and broad environmental changes often covary. Applied to the effects of agricultural land cover on stream systems, this approach identified reduced nutrient loading from arable farms as a priority for land management. On this specific issue, the data and analysis presented here support the use of riparian or catchment-scale measures to reduce nutrient delivery to sensitive water bodies.

3.2. Introduction

With global agricultural production set to double between 2005 and 2050 (Tilman *et al.*, 2011), the reconciliation of food production and environmental protection is a key challenge for sustainable development (Robertson and Swinton, 2005). The difficulties of balancing the use and protection of natural resources were evident in the expansion of UK agriculture between 1940 and 1980, as intensification resulted in habitat simplification, environmental pollution and declines in a broad range of terrestrial and freshwater taxa (Robinson and Sutherland, 2002). Seen from an ecosystem perspective, agricultural services were gained at the potential expense of other ecosystem services, including carbon sequestration and water quality regulation (Dale and Polasky, 2007).

The effects of agriculture on freshwaters are of particular interest due to the conservation, economic and cultural importance of these systems (Dudgeon *et al.*, 2006). The ecosystem services provided by streams, including water supply, fisheries and recreation, can be affected by both arable and intensive pastoral land uses, the latter where high densities of livestock graze on fertilized and reseeded pasture (hereafter ‘improved pasture’). The mechanisms involved include altered flow regimes (Niyogi *et al.,* 2007), increased nutrient and inorganic sediment inputs (McDowell *et al.*, 2003), and altered bankside vegetation structure (Townsend *et al.*, 1997a). The effects of these combined changes on stream community structure are, however, highly variable. For example, improved pasture land cover has been associated with both lower (Quinn and Hickey, 1990; Liess *et al.*, 2012) and higher invertebrate richness and sensitive species representation, compared to reference grasslands (Thompson and Townsend, 2004), with other studies finding no significant associations (eg. Riley *et al.*, 2003). One possible explanation for these variable results is that studies have often been of limited spatial extent and may not generalize to different regions (Knapp *et al.,* 2004). This lack of generality is a common concern in ecology, where studies are often too site-specific to guide environmental and land-use policies at the national-scale or regional-scale over which they are implemented (Donald *et al.*, 2006).

Whereas national-scale studies to assess the impacts of agricultural practices are well established for vertebrates such as birds (eg. Chamberlain *et al.*, 2000; Donald *et al.*, 2006), they are lacking for most other taxa, probably reflecting the difficulties of obtaining large-scale data. Fortunately, many nations have extensive environmental monitoring programmes and high resolution land cover imagery that could redress this gap. In England and Wales, for example, river monitoring data comprise records of water chemistry, macroinvertebrates and geomorphology from thousands of locations. These data provide an opportunity for large-scale analyses within realistic budgets and time-frames, whilst the statistical power afforded by the large sample sizes makes them a valuable adjunct to traditional field surveys (Vaughan and Ormerod, 2010). Beyond basic statutory reporting, however, there have been surprisingly few attempts to utilize these data to address large-scale ecological questions (eg. Murphy and Davy-Bowker, 2005; Vaughan and Ormerod, 2012).

A second challenge for research across large spatial extents is that there is often a complex pattern of collinearity between the variable of interest and other environmental variables. Across England and Wales, agricultural land cover correlates with environmental characteristics such as geology, soil type and climate, and trying to distinguish the impacts of agriculture is a major challenge (Scheiver *et al.,* 2007). Multiple regression is commonly used to investigate the effects of land use and attempt to control for these covariates. Collinearity between the covariates and the variable of interest, or amongst covariates, can, however, bias the estimated effect sizes from multiple regression and lead to unstable coefficient estimates with large standard errors, whilst complex relationships between the covariates increase the risk of model misspecification (Graham, 2003).

Fields including medicine, economics and social sciences face similar challenges in trying to quantify effect sizes and determine causal relationships from survey data, leading to the development of propensity score approaches (Dehejia and Wahba, 2002). The propensity approach attempts to mimic randomized controlled experiments by comparing the effect of the ‘treatment’ (eg. different land cover) in subsamples of the full data set that are closely matched on background covariates (eg. climate, geology). This comparison is commonly achieved by building a regression model to predict the probability or size of the ‘treatment’ based on the background covariates, and subdividing the data set into a small number of groups which have similar predictions (termed propensity scores): hence a similar distribution of the environmental covariates (Rosenbaum and Rubin, 1983). Within each group, the correlations between the covariates and the treatment are much weaker and so the effect of the treatment on response variables of interest can be modelled with reduced potential for confounding (Rosenbaum and Rubin, 1983). Both simulation and empirical studies have shown that the propensity approach can minimize bias in regression coefficients and allow changes in response variables to be ascribed more directly to the causal effect of the treatment variable (eg. Dehejia and Wahba, 2002; Imai and Van Dyk, 2004). Propensity scoring could be of great value to ecology, yet has been largely ignored with the notable exceptions of Yuan (2010), Bottrill *et al.* (2011) and Chessman (2013).

In this study, the propensity approach was used to analyse the effects of agricultural land cover on in-stream habitat, water chemistry and invertebrate community structure across England and Wales, making this one of the most comprehensive assessments of broad-scale agricultural effects and the first application of propensity modelling to assess the effects of land cover – a subject well-known for the challenges of collinearity (Van Sickle, 2003). In the highly modified UK landscape, there is little scope to compare agricultural land uses with semi-natural land cover or catchments that differ only in terms of a focal land cover type. Instead, streams with differing proportions of pastoral or arable land cover within their catchments or riparian zones were compared against a background mix of other land covers that typically occur within the same propensity score group. This comparison will indicate what the effects of contemporary changes in catchment land cover could be (i.e. the effect of increasing arable or pastoral cover relative to other land uses within the catchment). The aim was to quantify the effects of varying agricultural land cover at the national scale using characteristics that describe the physico-chemical conditions and biological structure of stream ecosystems. Changes in these characteristics would indicate alteration to ecosystem functioning with potential consequences for ecosystem service provision. Specifically the hypotheses tested were that increasing improved pastoral or arable land cover at the national scale would:

i) Increase nitrate and phosphate concentrations, stimulating increased in-stream vegetation;
ii) Increase sediment deposition;
iii) Simplify bankside vegetation;
iv) Lower invertebrate family richness and representation of taxa sensitive to organic pollution or low dissolved oxygen; and
v) Decrease the diversity of macroinvertebrate functional feeding guilds indicating the potential for impaired ecosystem functioning (Larsen and Ormerod, 2010).

3.3. Materials and methods

3.3.1. Physical habitat data

River Habitat Survey (RHS) is the standard method for recording the physical characteristics of rivers and streams in England and Wales (Seager *et al.,* 2012), covering channel morphology, bed and bank materials, flow types, vegetation in the channel and on the banks, surrounding land use and anthropogenic modifications at ten equidistant ‘spot-checks’ along a 500-m reach. The extent of features over the reach and presence of any additional features is recorded in a ‘sweep-up’ assessment (see Environment Agency, 2003 for a detailed description of the method). A national baseline survey was conducted in England and Wales during 2007–2008, with three reaches randomly selected within each 10-km Ordnance Survey grid square in England and Wales (Seager *et al.* 2012; Figure 3.1a).

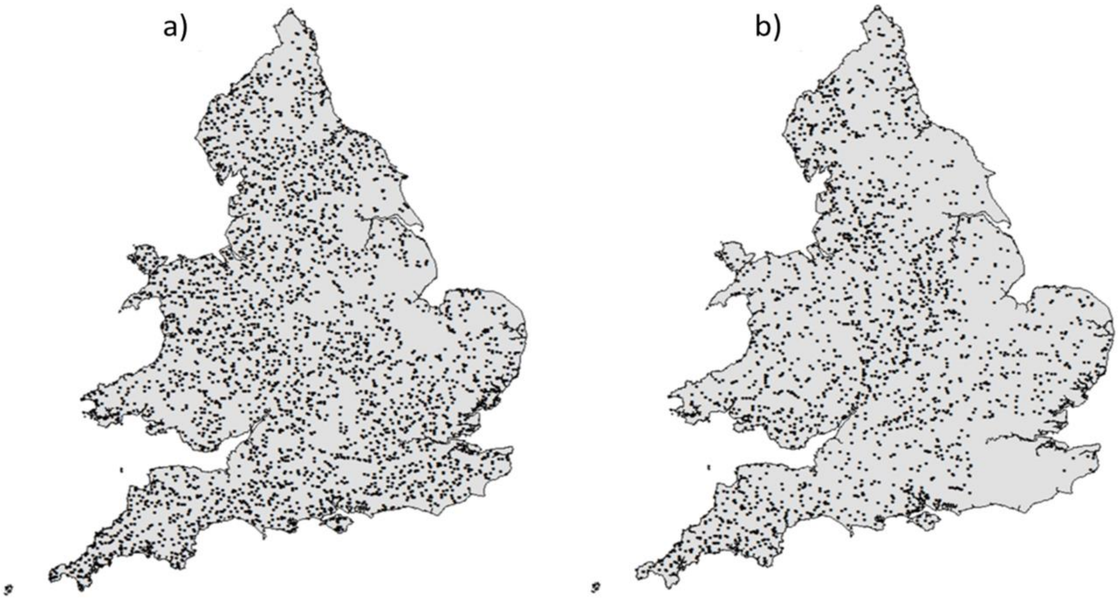

Figure 3.1 – Distribution of a) River Habitat Survey sites and b) Water chemistry/invertebrate monitoring sites used in analysis

Five response variables were derived from RHS data to capture key river characteristics that were hypothesized to be affected by agriculture (Table 3.1). Due to severe skews and U-shaped distributions, the response variables were dichotomized (Table 3.1; Vaughan *et al.*, 2013). Re-running analyses with alternative category thresholds confirmed that results were not sensitive to the precise thresholds selected (Table 3.1).

3.3.2. Macroinvertebrate and water chemistry data

Macroinvertebrate and water chemistry data were collected during routine monitoring by the Environment Agency in 2006. This year had a large sample size and was temporally consistent with the RHS data (2007–2008) and land cover imagery (2007; described below). Sampling sites were identified where water chemistry and biology were recorded within 500 m of each other and monthly chemistry samples were taken over the year preceding the invertebrate sample. To minimize the risk of spatial autocorrelation only one site per tributary was retained for analysis (n = 955, Figure 3.1b). Macroinvertebrates were collected using standard 3-minute kick samples and identified to family (Murray-Bligh, 1999). Presence/absence data from spring (March–May) and autumn (September–November) 2006 macroinvertebrate samples were combined and family richness and Average Score per Taxon (ASPT) calculated for each site. This latter index is a standard measure of community sensitivity to organic pollution, calculated by ascribing each family a score between 1 (tolerant) and 10 (highly sensitive) based on expert opinions, and averaging this score across all families present in a site (Armitage *et al.*, 1983).

Each family was assigned an affinity for different functional feeding guilds (FFGs) based on its morpho-behavioural methods of food acquisition, converting the species-level data of Schmidt-Kloiber and Hering (2012) to family-level using the method of Vaughan and Ormerod (2014). For each site the diversity of FFG affinities was calculated using Simpson's diversity index, producing a score between 0 and 1 where low values indicate dominance by a few feeding guilds while high scores indicate equitability across feeding guilds (Larsen and Ormerod, 2010).

Water chemistry data were used to indicate the influence of agricultural land cover on nutrient loading. The response variables were total oxidized nitrogen (abbreviated as nitrate because where both were recorded, nitrate approximated >99% of total oxidized nitrogen) and orthophosphate, analysed using standard methods (Standing Committee of Analysts, 1987, 1992). Annual medians were calculated for the 12 months preceding the 2006 spring invertebrate sample. Where ≥ 50% of these values were below detection limits, medians were estimated using regression on order statistics in R's NADA library (Lee and Helsel, 2005).

Table 3.1.- Explanation of response variables derived from River Habitat Survey data.

Habitat Characteristic	**Response variable**	**Alternative category thresholds**
Riparian Bankside trees	≥ 50% of spot-checks with broadleaf woodland within 5 m of bank top	≥ 40% and ≥ 60% of spot-checks
Macrophytes	≥ 1 spot-check with submerged, amphibious, emergent, rooted or floating-leaved vegetation or reeds	≥ 2 spot-checks
Filamentous algae	≥ 1 spot-check with filamentous algae	≥ 2 spot-checks
Silt/sand deposits	≥ 1 spot-check with sand and silt substrate	≥ 2 spot-checks
Sediment storage	Presence of point, side or mid-channel bars	

NB. Each site was categorized as Yes or No for each of the response categories

3.3.3. Catchment land cover

The proportions of arable and improved pasture land cover were determined for each RHS and invertebrate/water chemistry survey site from the 25-m resolution UK land-cover Map 2007 (Morton *et al.*, 2011). Catchment boundaries for each site were estimated from a 50-m resolution digital elevation model (Ordnance Survey Landform Panorama) using HydroTools (v.9; Centre for Research in Water Resources, University of Texas, TX, USA) in ArcInfo v. 10 (ERSI, Redlands, USA). The percentage of the catchment and the percentage of an upstream riparian strip (50 m either side of the channel for whole upstream network) under each land cover were determined using the Geospatial Modelling Environment (Beyer, 2011).

3.3.4 Statistical analysis

Propensity modelling involved four basic stages (Rosenbaum and Rubin, 1983): i) creating a model to predict the proportion of each site's catchment area under arable or improved pasture land cover from locational, climatic and geological variables; ii) stratifying the data set into groups with similar predicted proportions of arable or pasture land cover; iii) modelling the effect of agricultural land cover on response variables of interest within each propensity group; iv) calculating the average effect size and 95% confidence limits across all groups, weighted by the number of observations in each group.

Four propensity models were built to predict the percentage cover: one each for arable and pastoral, in the entire catchment and in the riparian strip. All site locations (RHS and invertebrate/water chemistry) were pooled for the propensity modelling (n= 3135). A range of potential confounding variables that covary with land cover on a national scale were identified: slope, altitude, mean annual rainfall (mm), temperature (°C), underlying solid geology, predominant soil texture and proportional catchment cover of urban land use and other agricultural land use (i.e. arable land for improved pasture models and *vice versa*). Climatic variables were derived from the 1961–1990 climatic averages mapped at 5-km resolution (UK Meteorological Office; Perry and Hollis, 2005). Geological and soil data were simplified from 1:625k geological maps (British Geological

Survey, 2007) into five lithological classes: hard (igneous and metamorphic), chalk, limestone, sandstone and other sedimentary (Emery *et al.*, 2003), and four soil classes: loam, clay, sand and 'other', to reduce over-fitting of the model. For all variables the mean value or the predominant category within the catchment/riparian strip was used as the predictor value. Generalized additive models (GAMs), using R's mgcv library, were used to describe the relationship between treatment land cover proportions and the predictor variables. Easting and Northing were also included using a tensor product smooth (Wood, 2006). As the relative influence of different covariates was not of interest, the models were not checked for collinearity, nor was model simplification implemented (Harrell, 2001). Predictions were made for all sampling sites using each of the four models, to give the respective propensity scores.

For each treatment land cover (arable/pasture, catchment/riparian strip), the data were split into five equally sized groups ('propensity groups') using the quintiles of the predicted probabilities (Rosenbaum and Rubin, 1983) and then separated into RHS and biology/chemistry data sets (Table 3.2). Although Rosenbaum (2002) states that five groups based on quintiles are appropriate for most data sets, all analyses were conducted with four and six groups to check that the number of propensity groups did not alter the conclusions (Tables S3.1 and 3.2).

Generalized linear models (GLMs) (binomial error distributions for RHS data) were used to describe the relationship between each response variable and percentage treatment land cover within each propensity group. Water chemistry variables were log transformed to meet model assumptions. The covariates used in the propensity model were also included in each model to account for remaining within-group variability and to allow any covariates that strongly influence the response variable to contribute to the model (Robins and Rotnitzky, 2001). Plots of residuals were used to check the model fits, alongside semivariograms (gstat library; Pebesma, 2004) to ensure that there was no residual spatial autocorrelation. For each response variable, the mean effect size across propensity groups was calculated, weighted by the proportion of observations within each subclass (Imai and Van Dyk, 2004). The effect sizes represent the change in the response variable for 1% increases in percentage agricultural land cover. For binomial models of habitat features these effect sizes are the odds ratios: values < 1 show a decrease in likelihood and > 1 an increase. A 95% confidence interval was calculated, over all *k* groups, according to the equation (Benjamin, 2003; Guo and Fraser, 2014):

$$CI = 1.96\sqrt{\left(\sum_{k=1}^{k} {se_k}^2 \left(\frac{n_k}{N}\right)^2\right)}$$

where *se* = standard error of group estimate, *n* = number of observations in group, N = total number of observations. Given the number of response models (20 for each of invertebrate/chemistry data and RHS data) confidence limits were extended using the method of Benjamini and Yekutiueli (2005) to control for the false discovery rate. Effects were considered statistically significant (at α = 0.05) if the interval did not span zero (invertebrates and water chemistry variables) or one for the odds ratios (RHS variables).

3.3.5 Evaluating the propensity approach

In the final stage, the propensity scoring approach was compared to conventional multiple regression (hereafter the ‘direct approach’). Generalised Linear Models were fitted between percentage treatment land cover and each of the response variables, using the same covariates as for the propensity scores. The efficacy of the propensity approach was evaluated by assessing the degree to which it had reduced confounding between the treatment land cover and covariates in response models compared to the direct regression approach. To achieve this, commonality analysis was performed for each response model in the ‘yhat’ package in R (Nimon, Oswald and Roberts. 2013) to give the unique and common contribution of each independent variable to the variance explained by each model. Commonality coefficients were averaged across the five propensity group models for each response variable to indicate the degree of confounding and compared to those from equivalent direct models using a paired t-test.

Table 3.2 – Number of sites per group after splitting the whole dataset into five groups based on percentiles of the propensity score for each land cover type then separating River Habitat Survey and water chemistry/invertebrate monitoring sites. Sites in group A have the lowest probability and sites in group E have the highest probability of having agricultural land use within their catchment.

	River Habitat Survey sites				Invertebrate/Chemistry sites			
Group	*Improved catchment*	*Improved riparian strip*	*Arable catchment*	*Arable riparian strip*	*Improved catchment*	*Improved riparian strip*	*Arable catchment*	*Arable riparian strip*
A	523	709	355	578	157	132	202	182
B	487	598	460	638	175	203	185	178
C	417	568	467	616	202	193	172	174
D	388	562	429	597	200	190	202	187
E	387	602	491	614	199	191	172	189

3.4. Results

3.4.1. Propensity models

The proportion of agricultural land cover in the riparian strip and whole catchment were strongly correlated (Pearson's $r = 0.78$ and $r = 0.86$ for improved pasture and arable respectively). The arable land cover models explained 76% of the variation at the catchment scale and 64% within the riparian strip, and the mean correlation between land use and the environmental covariates was 58% lower within propensity groups compared to the entire data set in both cases (Table 3.3). At both scales, the predicted proportion of arable land cover increased as the proportion of improved pasture and urban land use decreased, as altitude and rainfall decreased, and towards the east on chalk geology with loamy soils (Figure 3.2). Improved pasture was less predictable: models explained 45% of the variation at the catchment scale and 36% within the riparian strip. For the majority of covariates the correlation with improved pasture across the whole data set was low, but was still reduced by 24% (catchment) and 55% (riparian strip) by the propensity approach (Table 3.3). The predicted proportion of improved pasture land cover in the catchment and riparian strip was higher in the south-west, and increased as the proportion of arable and urban land cover, temperature, altitude and rainfall decreased (Figure 3.3).

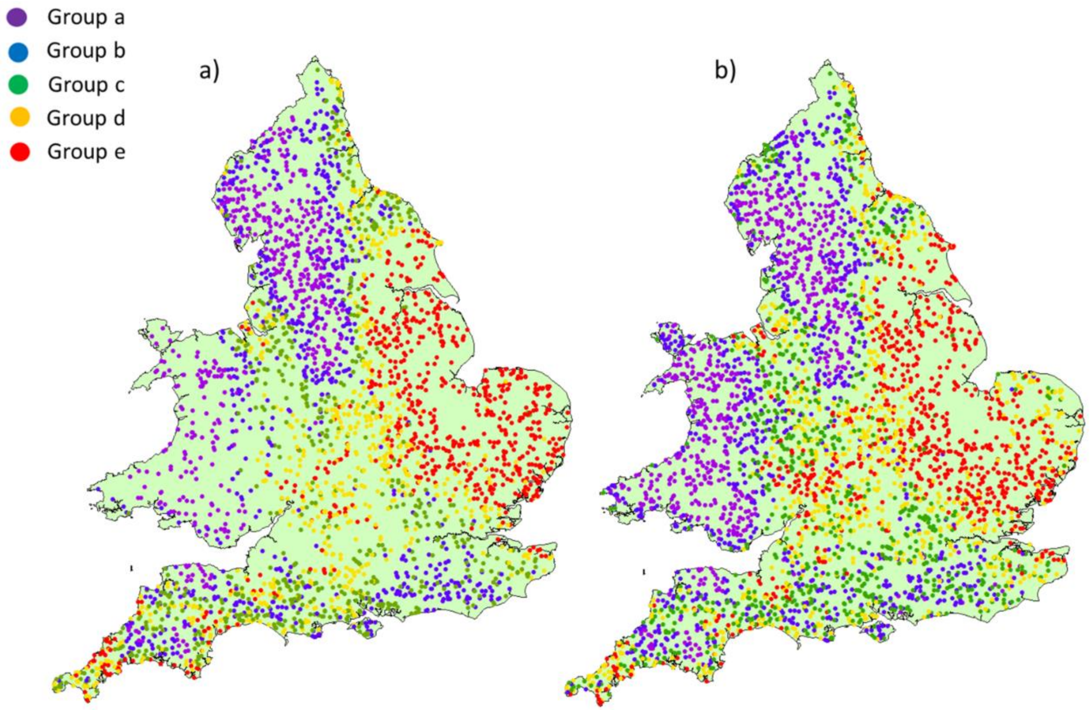

Figure 3.2 – Distribution of sites grouped by predicted proportion of arable land cover in a) the catchment and b) an 50 m upstream riparian strip. Predicted values are derived from a propensity model, based on climatic, locational and geological factors. Sites in Group A have the lowest predicted proportion of arable land cover and sites in Group E the highest. See table 3.2 for further explanation.

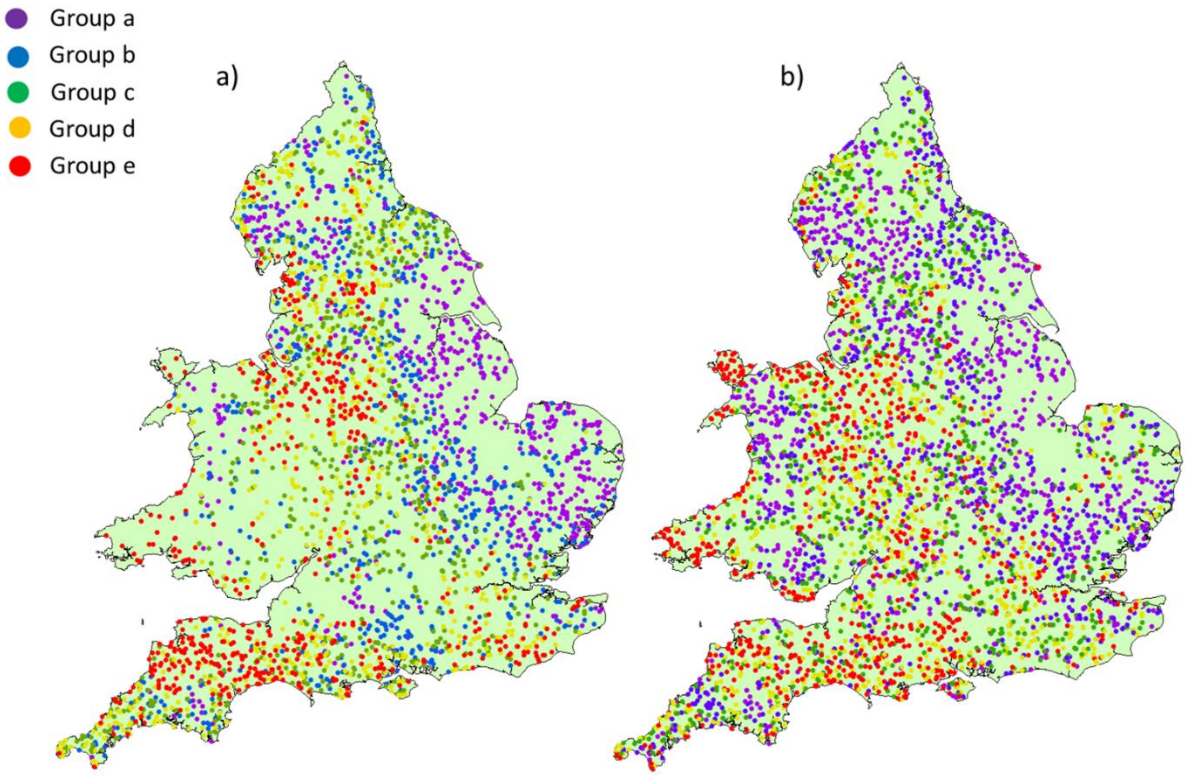

Figure 3.3 – Distribution of sites grouped by predicted proportion of improved pasture land cover in a) the catchment and b) a 50 m upstream riparian strip. Predicted values are derived from the propensity model, based on climatic, locational and geological factors. Sites in group A have the lowest predicted proportion of improved pasture land cover and sites in group E the highest. See table 3.2 for further explanation.

Table 3.3 – Absolute magnitude of Pearson correlations coefficients between environmental covariates and treatment land covers across the whole data set and within propensity groups.

Covariates	Arable catchment		Arable riparian strip		Improved pasture catchment		Improved pasture riparian strip	
	Whole data set	Group average	Whole data set	Group average	Whole data set	Group average	Whole data set	Group average
Altitude	0.44	0.15	0.37	0.10	0.11	0.13	0.16	0.12
Slope	0.21	0.08	0.20	0.08	0.07	0.06	0.11	0.04
Temperature	0.46	0.24	0.39	0.23	0.15	0.19	0.22	0.13
Rainfall	0.62	0.13	0.56	0.12	0.03	0.13	0.15	0.08
Easting	0.46	0.08	0.41	0.10	0.29	0.08	0.13	0.05
Northing	0.20	0.19	0.10	0.16	0.14	0.15	0.16	0.01
Other agricultural land cover	0.30	0.23	0.24	0.17	0.30	0.09	0.24	0.10
Urban land cover	0.10	0.12	0.07	0.07	0.10	0.12	0.09	0.10

3.4.2. Effects of agriculture based on the propensity approach

Estimated effects of agriculture on physical habitat were similar in direction and magnitude for land cover measured at the catchment and riparian scales (Figure 3.4). Sites with a higher proportion of their catchment or riparian strip under either improved pasture or arable land cover had a significantly higher likelihood of containing silt or sand deposits. Sites with a higher proportion of either land cover in the riparian strip, or a higher proportion of arable cover in the catchment, had a significantly lower occurrence of bankside trees (Figure 3.4). Neither improved pasture nor arable land cover had a significant relationship with the likelihood of occurrence of macrophytes, filamentous algae or stable sediment deposits (in-channel bars; Figure 3.4).

Phosphate concentrations showed no significant relationships with arable land cover at either spatial scale, but had a significant positive relationship with improved pasture at the catchment scale. Phosphate concentrations were, on average, 0.2 mg L^{-1} higher in catchments with 100% improved pasture cover compared to catchments with no improved pasture. Nitrate concentrations increased with both arable and improved pasture, especially when the land cover was measured at the catchment scale (Figure 3.5): catchments with 100% treatment land cover were estimated to have nitrate concentrations that were 7.6 mg L^{-1} higher for improved pasture, and 12.3 mg L^{-1} for arable compared to catchments with no agriculture.

Invertebrate richness increased with the proportion of improved pasture at catchment and riparian scales. The estimated effect size translated to six (catchment) or eight (riparian) extra families in sites with 100% improved pasture than in sites with no improved pasture, compared to an average of 23 nationwide in 2006 (Vaughan and Ormerod, 2012). The representation of taxa sensitive to organic pollution (ASPT) increased with improved pasture cover at the riparian, but not catchment, scale (Figure 3.5). Richness did not show a significant response to arable land cover at either scale, but a declining ASPT score indicated a lower representation of sensitive species, although this was only significant at the riparian scale. Although feeding guild diversity was significantly higher under improved pasture at the riparian scale the effect size was very small and there was no significant response to arable land cover (Figure 3.5).

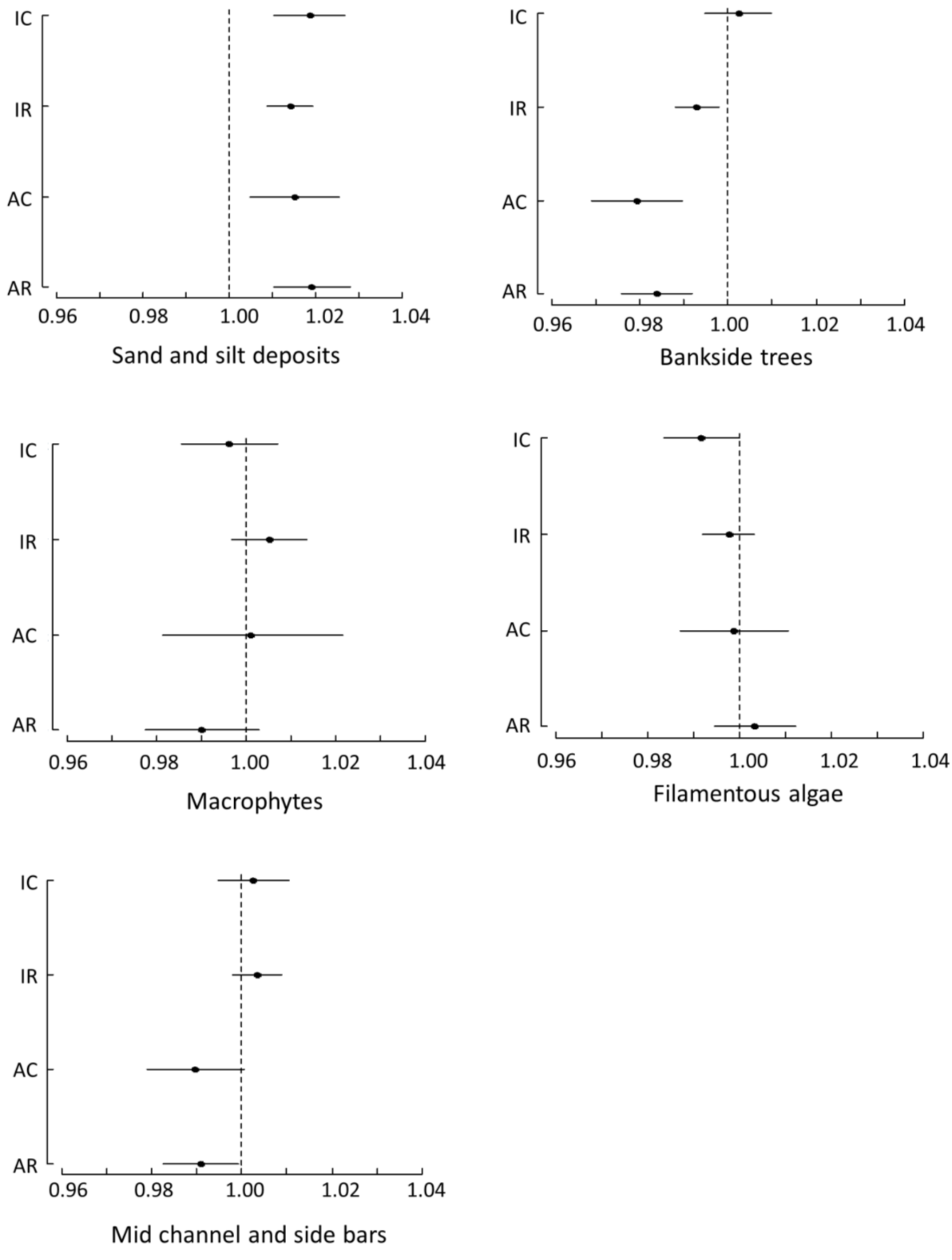

Figure 3.4 - Changes in the likelihood of occurrence (odds ratios) of habitat characteristics, based on the propensity approach, for each percentage increase in the proportion of the treatment land covers improved pasture in the catchment (IC), improved pasture in riparian strip (IR), arable farming in catchment (AC) and arable farming in riparian strip (AR) compared to the background mix of other land uses. Horizontal bars show 95% confidence intervals across the five propensity groups. Values of 1 = no change.

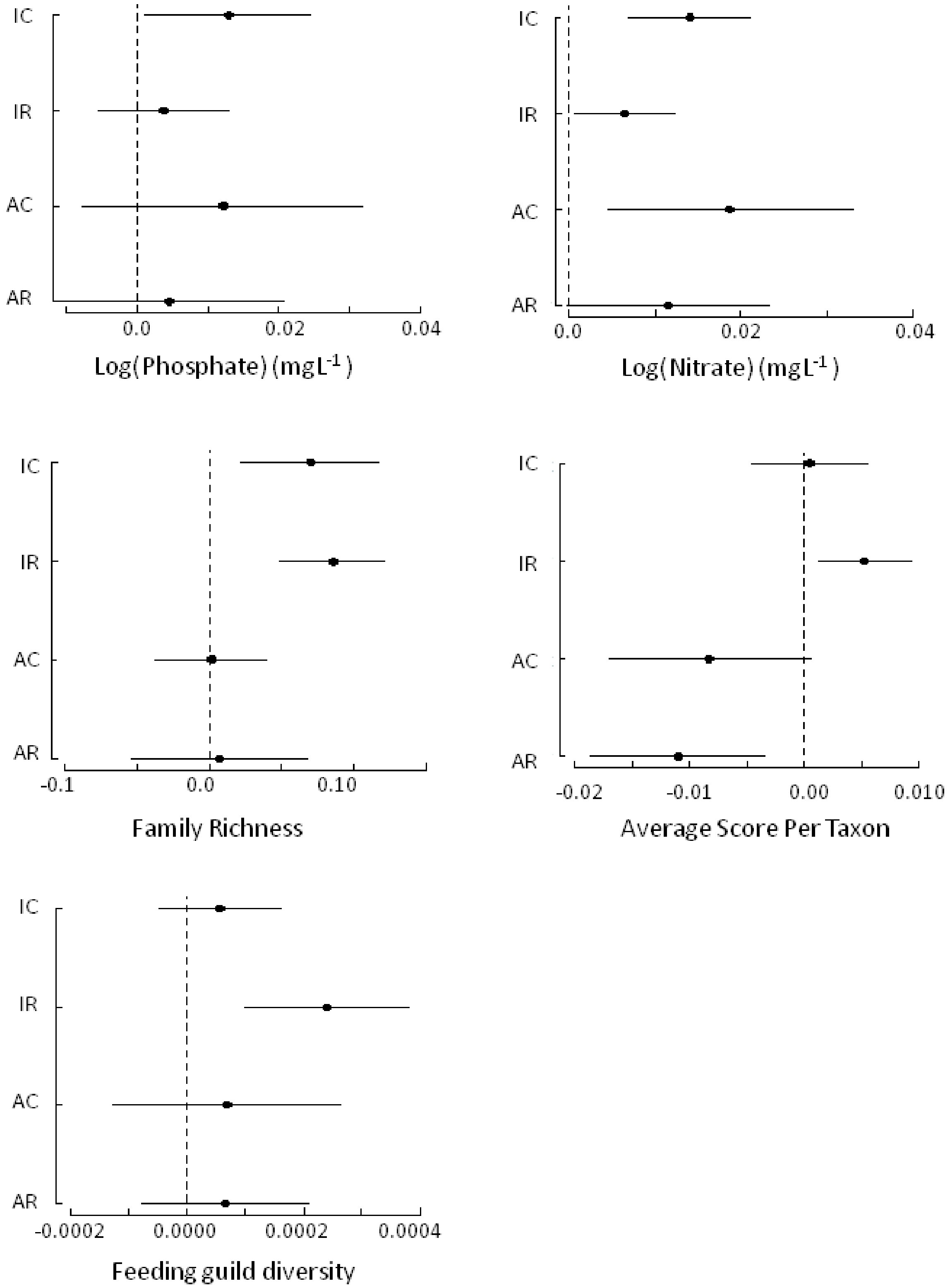

Figure 3.5 - Changes in water chemistry and invertebrate community variables based on the propensity approach, for each percentage increase in the proportion of the treatment land covers, improved pasture in the catchment (IC), improved pasture in riparian strip (IR), arable farming in catchment (AC) and arable farming in riparian strip (AR) compared to the background mix of other land uses. Horizontal bars show 95% confidence intervals across the five propensity group.

3.4.3. Comparison with direct models

Commonality analysis showed that there was little confounding between improved pasture land cover and covariates in direct response models (Figure 3.6), consistent with the low correlations between land cover and covariates across the whole data set (Table 3.3). Although the propensity approach did reduce the amount of variance shared between the treatment land use and covariates, the magnitude of this reduction was small and insignificant (Figure 3.6). The magnitude of confounding was much greater in models of responses to arable land cover. The propensity approach effectively reduced commonality coefficients across all response variables (Figure 3.6). Direct models suggested that land cover had a significant effect more frequently than what was determined with propensity models: 75% of the models tested compared to 45% of models using the propensity approach (Tables S 3.1 and S 3.2).

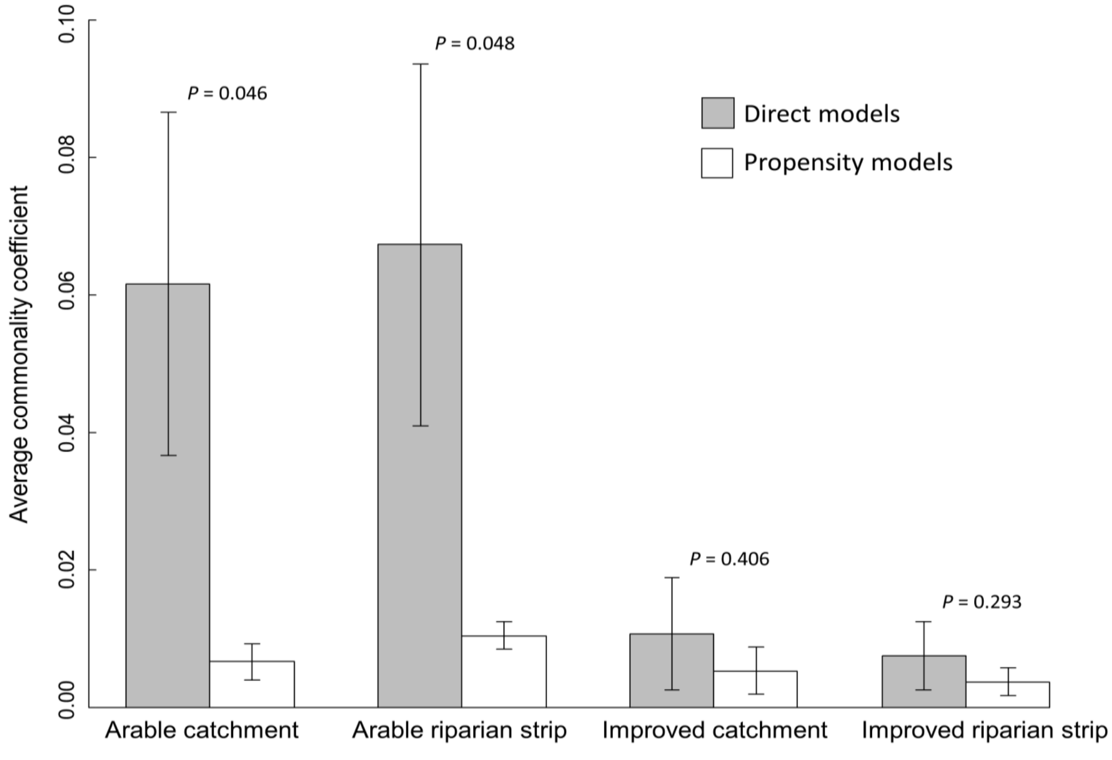

Figure 3.6- Differences in confounding between direct and propensity models. Bars show the commonality coefficients for each treatment land cover (the contribution to the regression effect that is shared with other covariates) averaged across all 10 response variables ± standard error. P values are the result of paired t-tests comparing commonality coefficients of propensity and direct models.

3.5. Discussion

A large body of literature illustrates how land cover can affect stream ecosystems, including recent experiments that have increased mechanistic understanding of the effects of single stressors and their interactions (eg. Matthaei *et al.,* 2010). The practical difficulties of manipulating catchment land cover experimentally, however, means that studies examining the aggregate impacts of agricultural land cover must rely on observational data. Typically, these studies compare catchments with differing land covers, matched as far as possible on other covariates. Despite minimizing differences between catchments these studies often encompass variability in confounding factors such as catchment elevation or microclimate (eg. Townsend *et al.,* 1997a; Riley *et al.,* 2003). Further, the majority of land-use studies are restricted to small geographic areas with similar site characteristics, which may reduce their generality to other regions and limit their utility for guiding national-scale environmental policy.

Here, national monitoring data allowed one of the largest studies of agricultural effects on stream systems to date, both in spatial extent and sample size (but see Meador and Goldstein, 2003; Carlisle and Hawkins, 2008). There are, however, important limitations when using monitoring data. Firstly, there is limited detail recorded at each location; RHS data provided relatively crude measures of physical habitat (eg. fine sediment loading), whilst invertebrate data were available only at family level, obscuring species-level responses. The difficulties in assigning traits at the family level (*cf.* species or genera) may account for the lack of ecologically significant responses in feeding guild representation observed in this study. More generally, the land-cover categories used in this study cover a range of management practices (eg. differences in stocking density, fertilizer application and pesticide use), which may differentially affect stream ecosystems. In combination, these limitations are likely to reduce the ability to detect significant responses to land cover change and increase the uncertainty associated with the modelled effects. Despite these limitations, the unrivalled sample size and spatial coverage of these data sets makes them valuable for large-scale assessments (Vaughan and Ormerod, 2010). First, the propensity method and then the ecological implications of the findings are discussed.

3.5.1. Evaluating the propensity approach

The benefits of propensity scoring have been confirmed by both theoretical studies and successful application in several fields, including, recently, in ecology (Yuan, 2010; Bottrill *et al.,* 2011; Chessman, 2013). Propensity scores have the ability to control for a large array of covariates by combining them into a single score, whereas attempts to control covariation through experimental design are restricted to relatively few covariates (Dehejia and Wahba, 2002). As demonstrated here, grouping data by propensity scores reduces the correlations between the treatment and covariates relative to the whole data set. Therefore, compared to conventional regression models, propensity modelling i) reduces the potential for covariate bias in estimated treatment–response relationships, ii) increases the likelihood that treatment–response relationships can be represented by linear models, reducing the risk of model misspecification or the need for complex models and, iii) makes models more robust to extrapolation by minimizing their reliance upon the particular distribution of the background covariates in the data set (Imai and Van Dyk, 2004; Vansteelandt and Daniel, 2014). Set against these advantages are the additional stage of data analysis required in propensity modelling, and limited benefit when covariates are poor predictors of the treatment variable (Weitzen *et al.,* 2005).

The few ecological studies to apply propensity modelling have shown an effective reduction in the strength of covariate bias (Yuan, 2010; Bottrill *et al.,* 2011). Here, the efficacy of the propensity approach differed between arable and improved pasture land cover. The propensity model explained much of the variation in arable land cover and effectively restricted its collinearity with other covariates within each propensity group. Thus, the variance explained by the shared effects of arable land cover and other covariates was substantially reduced; limiting bias in the coefficient estimates (Imai and Van Dyk, 2004). The benefits for improved pasture were less clear, with a smaller reduction in collinearity and similar model results for propensity and direct methods. The key difference was that collinearity was much lower in the original data set, indicating less potential for confounding between pasture and environmental covariates, which may indicate that improved pasture is less closely tied to large scale environmental conditions in the UK than arable land cover, or that may have been overlooked important confounders. The latter seems less likely given the range of environmental covariates, alongside geographical position, that was considered. The division of ‘improved grassland’ from semi-natural grasslands may be indistinct (Morton *et al.,* 2011), whilst the distribution of reseeded grasslands may depend on decisions taken by individual land owners at smaller spatial scales than the environmental data used here. Whatever the reason, the propensity approach offered little advantage over traditional regression methods for improved pasture. Thus, the most obvious applications for propensity modelling will be when there is strong collinearity between the treatment variable and known environmental covariates, as frequently occurs in large-scale ecological studies, and which are also the conditions under which controlling for such covariates is of greatest importance.

3.5.2. Effects of agricultural land cover on stream ecosystems

Whilst many studies have considered the effects of arable or pastoral land cover on streams, surprisingly few have studied both simultaneously (but see Kyriakeas and Watzin, 2006). This study also differed from most previous work by comparing arable and pasture to the mix of other land covers in the highly modified landscapes of England and Wales, rather than to semi-natural ‘reference’ conditions, increasing its relevance to decisions about rural policy and changing land cover.

Invertebrate richness and the representation of sensitive species were higher under improved pasture, whereas sites with arable land cover in their riparian zone had a lower representation of sensitive taxa but no change in species richness, suggesting a turnover from sensitive to tolerant families with increasing arable land cover. These results, on a national scale, are contrary to predictions and to a previous small-scale comparison which showed lower sensitive species representation in both arable land and pasture compared to reference grasslands, with greater impacts in pasture (Kyriakeas and Watzin, 2006).

As predicted, both agricultural types increased the frequency of silt/sand deposits and elevated nitrate concentrations. The change in fine sediment cover was similar for both agricultural types; a fourfold increase in the odds of occurrence between sites with 0 and 100% agricultural land cover. The impact of this increase on invertebrates will depend on the initial sediment cover but as sensitive families have been shown to decline at a sediment threshold of 20% cover (Burdon *et al.*, 2013) the estimated increase in fine sediment has the potential to have detrimental effects on invertebrate communities.

Nutrient enrichment was greater under arable land cover than improved pasture: catchments with no agriculture had on average 2 mg L^{-1} nitrate, increasing to 9.5 mgL^{-1} in catchments with 100% improved pasture and 14 mg L^{-1} in sites with 100% arable land cover. Therefore, the differences in invertebrate responses to arable and pasture land cover are attributed to the greater magnitude of nitrate enrichment from arable land cover. Nitrate adversely affects sensitive macroinvertebrates at concentrations exceeding 8.8 mg L^{-1}, which were predicted in catchments with more than 50% arable land cover (Camargo *et al.*, 2005). Unmeasured physico-chemical changes, such as increased pesticide concentrations, may also have contributed to the decline in sensitive invertebrate taxa (Schriever *et al.,* 2007).

The results suggest that the magnitude of the nitrate enrichment from improved pasture, coupled with increases in light availability associated with riparian vegetation loss, had a subsidy effect on invertebrate communities through supplementation of autochthonous food resources (Liess *et al.,* 2012). Although this analysis did not show the predicted increase in filamentous algae and macrophytes with nutrient enrichment, it is likely that these are insensitive indicators of in-stream production and that elevated nutrients increased the nutritional quality of algae or the availability of epilithic algae for grazing invertebrates (Niyogi *et al.*, 2007).

Increases in autochthonous food resources would be expected to increase invertebrate abundances due to increased energy availability (e.g. Riley *et al.*, 2003). Increases in abundance may also explain the increase in sensitive species' representation with pastoral land cover observed here; rare pollution-sensitive species are more likely to be identified in a sample as their absolute abundance increases, even if their relative abundance remains unaltered.

Several studies have demonstrated a ‘subsidy-stress’ response with pastoral development, in which invertebrate richness increases with initial nutrient enrichment until a threshold beyond which further enrichment and excessive sedimentation result in reduced richness (Niyogi *et al.*, 2007). The present results suggest that on average, current levels of pastoral intensity subsidise macroinvertebrate communities. The magnitude of this effect , an increase of six (catchment) and eight (riparian) families between sites with no improved pasture and 100% improved pasture land cover, is likely to have consequences for biotic interactions and ecosystem functioning. Further research is needed to determine the consequences of these changes in invertebrate communities and the intensity at which pastoral farming begins to deleteriously impact on macroinvertebrate diversity.

Although responses to agricultural land cover were largely similar in direction and magnitude whether land cover was measured at the riparian or catchment scale, nutrient concentrations showed slightly greater effect sizes at the catchment scale. This suggests that total contributing area is the best predictor of nutrient delivery (Roth, Allan and Erikson, 2006), especially in areas where buffering from riparian vegetation is low, as predicted in agricultural sites. Conversely, macroinvertebrate responses to arable land cover were larger when land cover was measured at the riparian scale. This supports the results of both Richards *et al.* (1997) and Peterson *et al.* (2011) who found in-stream biota to have stronger relationships with riparian land use than catchment scale land use, due to riparian scale measurements capturing effects with higher connectivity to the stream channel.

In summary, the propensity approach applied here has furthered the understanding gained from previous observational and manipulative studies by estimating the effect sizes of likely cause-effect relationships between changing proportions of agricultural land cover and key metrics of stream biological condition across a full range of natural complexity. This approach identifies the land management priority of reducing nutrient loading from arable farming and highlights the need for further research into the effects of pastoral land-use intensity. More broadly, this analysis illustrates the potential of propensity modelling to resolve the effects of large-scale ecosystem pressures with greater confidence, and thus to guide land-use policy.

3.6. Supporting Information

Table S 3.1- Modelled responses of river habitat characteristics to changes in the proportion of agricultural land cover in the catchment or upstream 50 m riparian strip with the data set split into 4, 5 or 6 strata based on percentiles of propensity scores and using the whole dataset ('Direct models'). Displayed values are odds ratios, the change in likelihood of occurrence of the habitat feature for each percent increase in the proportion of the treatment land cover ± 95% confidence limit values.

Land cover	Number of propensity groups	Macrophytes	Filamentous algae	Sand and silt deposits	Stable deposits	Bankside trees
Improved pasture catchment	*5*	0.996 ± 0.011	0.992 ± 0.008	1.018 ± 0.010	1.002 ± 0.008	1.002 ± 0.008
	4	0.998 ± 0.011	0.993 ± 0.008	1.018 ± 0.009	1.003 ± 0.009	0.999 ± 0.009
	6	0.999 ± 0.012	0.991 ± 0.009	1.018 ± 0.011	1.002 ± 0.009	1.002 ± 0.008
	Direct	1.000 ± 0.008	0.992 ± 0.006	1.016 ± 0.006	1.009 ± 0.006	1.000 ± 0.005
Improved pasture riparian strip	*5*	1.005 ± 0.008	0.998 ± 0.006	1.014 ± 0.005	1.003 ± 0.005	0.993 ± 0.005
	4	1.006 ± 0.007	0.998 ± 0.006	1.015 ± 0.006	1.003 ± 0.005	0.993 ± 0.005
	6	1.006 ± 0.010	0.998 ± 0.006	1.015 ± 0.005	1.003 ± 0.006	0.993 ± 0.006
	Direct	1.006 ± 0.006	0.997 ± 0.004	1.012 ± 0.004	1.002 ± 0.004	0.993 ± 0.004
Arable catchment	*5*	1.001 ± 0.020	0.999 ± 0.012	1.015 ± 0.010	0.989 ± 0.011	0.987 ± 0.010
	4	1.004 ± 0.019	0.996 ± 0.011	1.015 ± 0.010	0.988 ± 0.009	0.980 ± 0.011
	6	1.007 ± 0.042	0.996 ± 0.018	1.013 ± 0.015	0.986 ± 0.016	0.975 ± 0.019
	Direct	1.014 ± 0.007	1.004± 0.005	1.013 ± 0.005	0.987 ± 0.005	0.984 ± 0.005
Arable riparian strip	*5*	0.990 ± 0.013	1.003 ± 0.009	1.019 ± 0.008	0.991 ± 0.008	0.984 ± 0.008
	4	0.997 ± 0.012	1.001 ± 0.009	1.020 ± 0.008	0.991 ± 0.008	0.982 ± 0.008
	6	0.995 ± 0.015	1.002 ± 0.010	1.019 ± 0.009	0.991 ± 0.009	0.983 ± 0.009
	Direct	1.008 ± 0.005	1.001 ± 0.004	1.013 ± 0.004	0.988 ± 0.004	0.982 ± 0.004

Table S 3.2 - Modelled responses of water chemistry and invertebrate community metrics to changes in the proportion of agricultural land cover in a site's catchment or upstream 50 m riparian strip with the data set split into 4, 5 or 6 strata based on percentiles of propensity scores and using the whole dataset ('Direct models'). Displayed values are change in response value for each percent increase in the proportion of the treatment land cover ± 95% confidence limit values.

Land-cover	Number of propensity groups	log(Phosphate)	log(Total Oxidised Nitrogen)	Richness	ASPT	Feeding guild diversity
Improved pasture catchment	*5*	0.013 ± 0.012	0.014 ± 0.007	0.069 ± 0.047	0.001 ± 0.005	$2.40e^{-4} \pm 1.38e^{-4}$
	4	0.013 ± 0.011	0.012 ± 0.007	0.083 ± 0.045	0.001 ± 0.005	$2.08e^{-4} \pm 1.48e^{-4}$
	6	0.09 ± 0.012	0.013 ± 0.007	0.082 ± 0.049	0.002 ± 0.005	$2.46e^{-4} \pm 1.48e^{-4}$
	Direct	0.010 ± 0.004	0.013 ± 0.002	0.075 ± 0.015	0.002 ± 0.002	$1.17e^{-4} \pm 4.28e^{-5}$
Improved pasture riparian strip	*5*	0.004 ± 0.009	0.007 ± 0.005	0.085 ± 0.036	0.005 ± 0.004	$5.61e^{-5} \pm 1.02e^{-4}$
	4	0.007 ± 0.009	0.007 ± 0.005	0.072 ± 0.035	0.004 ± 0.004	$5.75e^{-5} \pm 1.07e^{-4}$
	6	0.006 ± 0.009	0.007 ± 0.006	0.075 ± 0.037	0.004 ± 0.004	$6.14e^{-5} \pm 9.49e^{-5}$
	Direct	0.006 ± 0.003	0.008 ± 0.002	0.084 ± 0.014	0.004 ± 0.002	$6.72e^{-5} \pm 3.89e^{-5}$
Arable catchment	*5*	0.012 ± 0.020	0.019 ± 0.014	0.00 ± 0.038	-0.008 ± 0.007	$6.81e^{-5} \pm 1.92e^{-4}$
	4	0.008 ± 0.010	0.017 ± 0.011	0.040 ± 0.058	-0.008 ± 0.007	$1.28e^{-4} \pm 1.40e^{-4}$
	6	0.020 ± 0.025	0.017 ± 0.018	-0.025 ± 0.088	-0.022 ± 0.012	$8.85e^{-5} \pm 2.44e^{-4}$
	Direct	0.011 ± 0.004	0.016 ± 0.002	0.007 ± 0.015	-0.007 ± 0.002	$2.75e^{-5} \pm 4.18e^{-5}$
Arable riparian strip	*5*	0.004 ± 0.016	0.012 ± 0.012	0.006 ± 0.059	-0.011 ± 0.007	$-5.8e^{-5} \pm 1.71e^{-4}$
	4	0.002 ± 0.015	0.010 ± 0.011	0.016 ± 0.056	-0.010 ± 0.007	$5.74e^{-5} \pm 1.39e^{-4}$
	6	0.005 ± 0.017	0.012 ± 0.012	0.022 ± 0.058	-0.010 ± 0.008	$8.36e^{-5} \pm 1.31e^{-4}$
	Direct	0.013 ± 0.003	0.017 ± 0.002	-0.021 ± 0.014	-0.009 ± 0.002	$-7.3e^{-5} \pm 3.82e^{-4}$

4. The effects of intensifying pasture on the structure and functioning of stream macroinvertebrate communities.

This chapter combines data I collected for this thesis with previously published data collected by Stefano Larsen in 2009.

4.1 Summary

On a global scale, terrestrial and aquatic ecosystems are being fundamentally altered by anthropogenic activities. The ability to identify and predict ecosystem-level effects is, however, limited by a lack of understanding of how community structure changes along anthropogenic stress gradients, particularly where this change is non-linear, and the consequences of these changes for ecosystem functioning. Here, the effects of agricultural intensification, one of the most widespread stressors of aquatic systems, on macroinvertebrate communities were assessed in 60 headwater streams. The aims were to: i) characterise the effects of pastoral intensification on the physical habitat of the streams; ii) identify how intensification affected community structure and trait representation; and iii) combine trait and community structure information to assess how inter-specific competition and environmental filtering structure the community as intensity increases and appraise the resultant changes in functional diversity.

Streams draining more intensive pasture had higher nutrient concentrations, including nitrate and potassium, trace metals and fine sediment cover. Taxon richness showed a non-linear response to pastoral intensity, declining beyond a threshold intensity level. Below this threshold intensity, communities were dominated by sensitive taxa such as *Ephemoptera*, *Plecoptera*, *Trichoptera* and riffle beetles and had a greater functional richness than expected at random, consistent with the theory that the community was structured by competitive exclusion. Above this threshold, taxon richness decreased with intensity due to the loss of taxa with traits poorly adapted to the physico-chemical conditions associated with agricultural intensification. This alleviated competitive exclusion produced a turnover of taxa, with dipterans, isopods and planarians, which were absent from low intensity sites, dominating. The environmental filtering effects of

agricultural stressors resulted in high intensity sites having a pool of functionally similar species with high representation of resistance/resilience traits. Reductions in functional richness exceeded declines in species richness, indicating that impairment to stream ecosystem functioning and biological integrity from pastoral intensification may be greater than expected from traditional structural measures.

4.2. Introduction

Anthropogenic activities are altering the structure and functioning of terrestrial and aquatic ecosystems fundamentally (Millenium Ecosystem Assessment, 2005), reducing species diversity, increasing species' dominance (Odum, 1985) and increasing temporal and spatial variability (Warwick and Clarke, 1993). Although measures such as richness, sensitive species representation and diversity indices are widely used for detecting the effects of anthropogenic stressors (Cairns *et al.*, 1993; Dale and Beyeler, 2001), such community-level metrics are limited in two ways. Firstly, ecological responses to stressors may be complex and non-linear, especially in the presence of multiple stressors, confounding simple interpretation of established indices (Rapport and Whitford, 1999; Townsend *et al.*, 2008). Examples include subsidy-stress responses, where abundance or diversity may initially increase along a stress gradient before declining (Odum, 1979), and threshold responses, where metrics do not respond until a critical level of perturbation is reached (May, 1977; Larsen and Alp, 2015).

The second limitation is that the consequences of changes in community-level metrics for ecosystem functioning are poorly understood. Being based on taxonomy, most metrics ignore the functional roles of species in ecosystems – roles which depend on traits such as feeding behaviour and reproductive strategy, and which in turn determine interactions with other species and the environment (Tilman and Downing, 1994; Díaz and Cabido, 2001). Changes in the array of traits present may affect ecosystem functions such as stability, productivity and nutrient cycling (Tilman *et al.*, 1997; Dukes, 2001), but may pass undetected if trait diversity or representation is not, or only weakly, correlated with changes in taxonomy. For example, anthropogenic stress may replace a pool of species displaying diverse traits with a pool displaying similar traits and high redundancy, causing functional richness to fall much more quickly than species richness

(Cornwell *et al.* 2006). Such changes can indicate the mechanisms causing the observed responses to anthropogenic stress gradients, in this case a shift from a primary role for inter-specific competition to increased 'environmental filtering' (Hardin, 1960; Zobel, 1997, Larsen and Ormerod, 2014). The relative importance of such mechanisms along anthropogenic stress gradients could provide fundamental insights into ecosystem responses to anthropogenic stressors, beyond those afforded by structural metrics alone (Mason *et al.* 2007; Mouchet, 2010).

This study investigates the structural and functional responses of stream macroinvertebrate communities to agricultural intensification, the most widespread anthropogenic land use and a leading cause of ecosystem alteration and biodiversity loss in aquatic systems (Dudgeon *et al.*, 2006). The focus is upon the intensification of livestock pasture, which impacts streams via increased fine sediment flux, nutrient enrichment and altered stream hydrology (eg. Niyogi *et al.*, 2007). Although changes in stream biodiversity in response to agricultural intensification are widely documented, the form of the relationship and underlying mechanisms appear to vary. Reported changes in macroinvertebrate richness and abundance accompanying agricultural intensification have included no effects (Niyogi *et al.*, 2007), a linear increase (Riley *et al.*, 2003) and decrease (Townsend *et al.*, 1997a), and subsidy-stress responses (Braccia and Voshell, 2007; Wagenhoff *et al.*, 2012). Alternatively, the community may show initial resilience before effects become apparent (i.e. a threshold effect; May, 1977) as demonstrated in response to nutrient enrichment (Larsen and Alp, 2015) and sedimentation (Burdon *et al.*, 2013). These inconsistencies may represent differences in the range of land use intensity spanned within the study systems: further studies covering broad gradients of in-stream conditions are required to clarify the community-level response (Townsend *et al.*, 2008).

There is a greater consensus on the effects of pastoral intensification on functional traits' representation. Resilience traits (eg. asexual reproduction, multivoltism and effective dispersal capacity), detrital feeding habits, burrowing behaviour and smaller body sizes increase with pastoral intensity, whilst crawling or free swimming behaviour, gill respiration and oviposition at the water surface decline (Richards *et al.*, 1997; Dolèdec *et al.*, 2006; Larsen and Ormerod, 2010). The influence of these changes on functional richness, and hence the consequences for ecosystem functioning, have not been explicitly tested. This study evaluates how changes in trait representation are likely to influence stream functioning and infers the mechanisms causing alteration to taxonomic and trait composition. Assessing functional responses to pastoral intensification is essential for land use management that aims to maintain biological integrity (Tilman, 2001; Dìaz and Cabido, 2001; Petchey and Gaston, 2002).

The effects of pastoral intensification were assessed in 60 headwater streams in South Wales. 'Intensity' is a multifaceted concept and was defined in this study from in-stream physico-chemical conditions including nutrient concentrations and fine sediment cover. Survey locations spanned a gradient of pasture development, from low intensity, extensive grazing to heavily improved pastures, allowing the competing hypotheses of linear, threshold and subsidy-stress responses to be tested for both taxonomic and trait metrics. The aim was to identify how pastoral intensification affected community structure, trait representation and the components of functional diversity, from which the relative roles of inter-specific competition and environmental filtering in structuring the community could be inferred. It is predicted that as intensity increased: i) overall macroinvertebrate richness and abundance would show a subsidy-stress response but, within that, sensitive taxa would show a monotonic decline, leading to a primarily nested pattern of species loss, and ii) the representation of resistance traits would increase but, overall functional richness and evenness would decrease, with an increasing role of environmental filtering (*cf.* inter-specific competition) in structuring the community.

4.3. Methods

4.3.1. Study Sites

This study was conducted on upland tributaries of the Usk, Wye, Neath and Tawe rivers in South Wales (Figure 4.1). The region is underlain by a homogeneous geology of sandstones and mudstones of the Old Red Sandstone series (Larsen *et al.*, 2009). Soils are a blend of brown earths and silty clay loam. Watercourses within the region are fairly uniform in base-cation availability (Larsen *et al.*, 2009).

Twenty-nine headwater streams (2nd and 3rd order) were selected across a gradient of pastoral intensity. Initial site selection was based on the proportion of improved pasture within the catchment due to the difficulties of obtaining accurate data on land use practices (eg. stocking density, fertiliser inputs; Delong and Brusven, 1998). The catchment of each potential site was delimited from a 50 m resolution digital elevation model (Ordnance Survey Landform Panorama), using ARC HydroTools (v.9; Centre for Research in Water Resources, University of Texas, TX, USA) in ARCGIS 10 (ERSI, Redlands), and the extent of pastoral land cover estimated from a field-scale resolution land cover map (Countryside Council for Wales, 2004). Catchments were selected that had >75% pastoral land cover but differed in the proportion of improved (fertilised and reseeded with high stocking densities) and unimproved (unfertilised native grass species supporting low densities of livestock) pasture (Jackson, 2000). The categories of 'acid grassland', 'marshy grassland' and 'wet heath' were combined to represent unimproved pasture, whereas improved pasture was recorded directly (Countryside Council for Wales, 2004). Fencing was absent between pasture and streams, and where other land cover types were present in a catchment they did not occur next to the channel. Sampling reaches were mostly a mix of cobbles and pebbles and were matched as far as possible on depth, width and altitude.

4.3.2. Macroinvertebrate and habitat sampling

Benthic macroinvertebrates were sampled in May 2012 using three minute kick-samples (two minutes in riffles, one minute in margins; Bradley and Ormerod, 2002) using a standard net (0.25 x 0.20 m; 1 mm mesh). All microhabitats were covered in proportion to their availability to provide the most representative sample of the whole community. Samples were preserved in 70% ethanol, before macroinvertebrates were removed from the sample in the laboratory and identified to genus, or a higher taxonomic level where this was not practicable (Table S4.1).

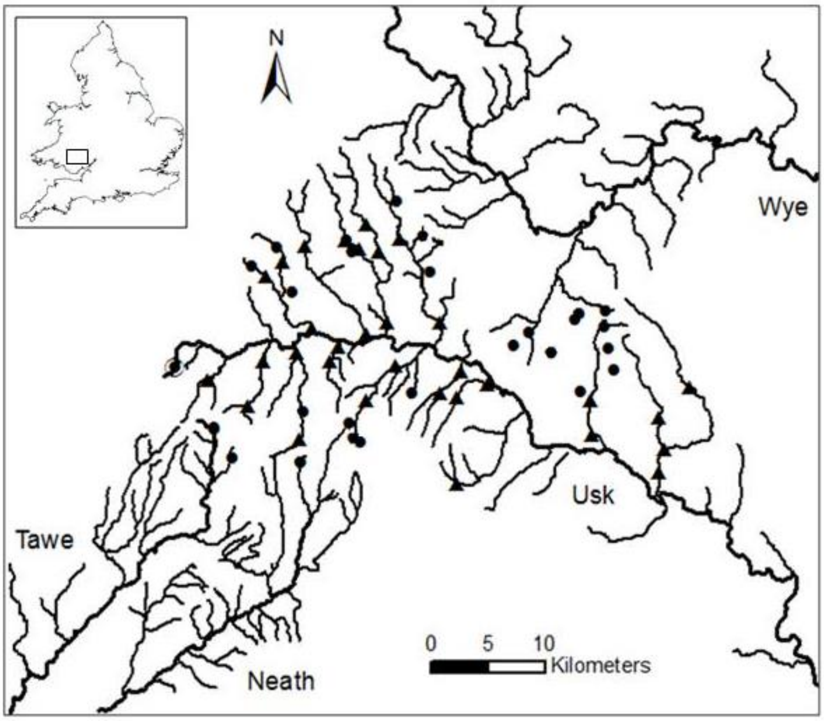

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Figure 4.1 - Location of sampling sites on tributaries of the rivers Usk, Wye, Tawe and Neath in South Wales. Circles show sites surveyed in May 2012 and triangles show sites surveyed in 2006.

A range of physico-chemical variables was recorded to quantify the intensity of pastoral land use effects on streams (Table 4.1). Water chemistry was assessed by taking a 150 ml water sample from a riffle in opaque sterilised bottles (SciLabware, HDPE screw cap bottle). The sample was frozen on return to the laboratory and ionic concentrations were determined using ion chromatography for anions (Dionex DX-80 Ion Analyser; Thermo Fisher Scientific, Inc.) and inductively coupled plasma mass spectrometry for cations (Thermo Elemental X-Series ICP-MS; Thermo Fisher Scientific, Inc.). In addition, three measures of pH and conductivity were taken in each site using a handheld probe (HANNA instruments, pH/EC/TDS model HI98129). Physical habitat was assessed over a 100 m reach (Table 4.1). Every 10 m, the bank material, bank profile, predominant substrate, canopy cover and bank-top vegetation complexity were recorded following River Habitat Survey protocols (Environment Agency, 2003). The width and depth at five equally spaced points across each transect were also recorded and the total length of bank undergoing active fluvial erosion or livestock poaching was measured. Over the area covered by the kick samples, flow velocity was measured at three locations in the fastest current and three locations in the margin. Bed coverage by fine sediment (< 2 mm) was estimated in 5% increments within ten 0.25 m² quadrats which alternated between the channel centre and margin (Zweig and Rabeni, 2001). In addition, the amount of resuspendable sediment was determined by pushing an open drum (25 cm diameter, 0.0625 m^2) into the substratum, disturbing the sediment to 2 cm depth for 15 seconds and capturing a 300 ml sample (Larsen *et al.*, 2009). Three replicate samples were combined to create a 900 ml bulk sample from which sediment between 0.025 and 1 mm was filtered. Ash free dry mass was then determined to distinguish organic and inorganic components (Riley *et al.*, 2003).

Table 4.1 – Description of physical habitat characteristics surveyed in 2012 and 2013.

Environmental variables	Units	Description
Depth	Metres (m)	Five measures across each of 10 transects
Wetted width	Metres (m)	Measured at ten transects
Bank material	RHS categories	Every 10m: Boulder, Cobble, Gravel or Earth
Substrate	RHS categories	Every 10m: Bedrock, Boulder, Cobble, Pebble, Sand or Silt
Bank top vegetation	RHS categories	Every 10m: Complex, Simple, Uniform Bare
Canopy cover	0, <33 %, >33 %, >66 %	Three transects within kick sample area
Bank profile	RHS categories	Every 10m: Vertical, Composite, Steep, Gentle
Conductivity	Parts per million (ppm)	Three replicates in kick sample area
Extent of fluvial erosion	Metres (m)	Length of bank undercut
Extent of bank poaching	Metres (m)	Length of bank trampled by livestock
Flow velocity	Metres per second (ms^{-1})	Three measures mid-channel and three in margin
pH	None	Three replicate measures in kick sample area
Resuspendable sediment	Grams per litre	Weight of resuspendable inorganics (25µm to 1 mm) (May 2012 only)
Fine sediment cover	Percent (%)	Average fine sediment cover from 10 quadrats in mid-channel and margin
Water chemistry	mg L^{-1}	7 Anion and 18 cations (cations May 2012 only)

4.3.3. Additional data

To strengthen the comparisons of alternative models of invertebrate responses, previously published data from 31 sites surveyed in 2006 (Larsen *et al.*, 2009) were combined with the dataset from the current survey. These sites were all second-order tributaries of the River Usk (Figure 4.1) that drained areas of rough pasture, moorland and improved pasture and had characteristics that closely matched those of sites in the 2012 survey (Larsen *et al.*, 2009; Table 4.2). Catchment land cover was determined in the same manner as in the 2012 study (Larsen *et al.*, 2009). As in the 2012 survey, invertebrates were sampled using a three minute kick sample. In 2006, sampling was restricted to glide and riffle habitats, excluding the margins. Although riffle/glide and margin samples have been shown to have large overlap in species composition, the slight differences between sampling technique may account for some differences between survey years (Bradley and Ormerod, 2002).

Table 4.2 - Range of site characteristics across sites sampled in May 2012 and previously in 2006 (data from Larsen et al., 2009).

Year		Altitude (m)	Mean width (m)	Mean depth (m)	Flow velocity (m/s)	Woodland catchment cover (%)
2012	Minimum	152	1.0	0.05	0.16	0
	Maximum	420	3.4	0.15	0.60	7.1
2006	Minimum	190	1.5	0.04	0.22	0
	Maximum	400	6.5	0.25	0.55	34

4.3.4. Data analysis

All analyses were performed in R version 3.0.1 (R Development Core Team, 2013) and the significance level for all tests was $\alpha = 0.05$.

4.3.4.1 Defining pastoral land use intensity

A principal component analysis (PCA) was performed on the 44 physico-chemical variables recorded. The first principal component (PC1) explained 29.7% of total variance and was highly correlated with percentage of the catchment under improved pasture (Pearson's r = 0.82, Figure 4.2). The variables with the strongest loadings on this axis were consistent with the expected effects of intensification (eg. nitrate concentration, extent of bank poaching, fine sediment cover; Figure 4.3, Table 4.3). All other components explained < 10% of the variation and were not related to agricultural intensity. PC1 scores were therefore interpreted as measure of pastoral intensification (hereafter 'intensity score'.)

To estimate the intensity score for sites sampled in 2006, a Generalised Additive Model (GAM) was fitted to model PC1 scores based on two variables recorded in both surveys – fine sediment cover and percent improved pasture in the catchment. A tensor product smooth was used for the latter due its sigmoidal relationship with PC1 score (Wood, 2006; Figure 4.2). The model had an R^2 value of 0.77 and was used to predict intensity scores for the 2006 sites.

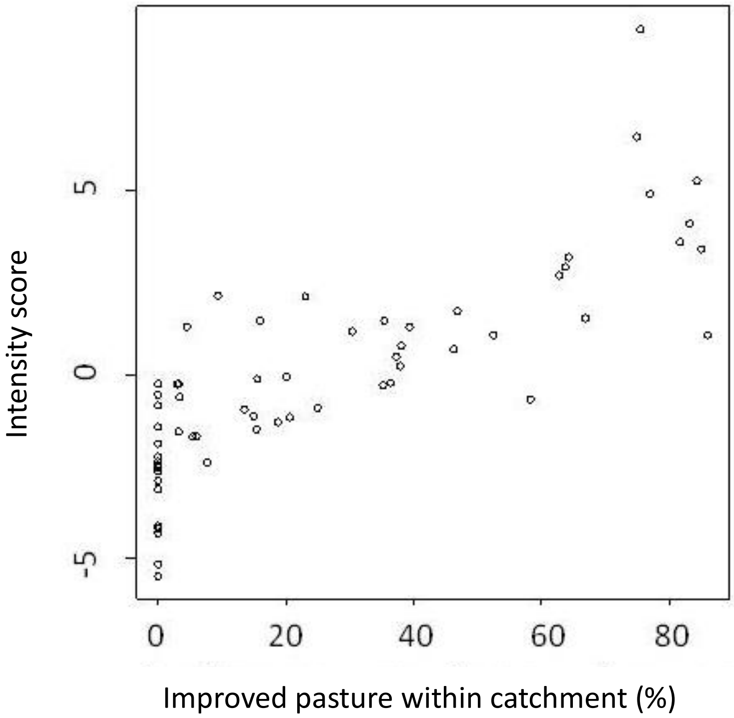

Figure 4.2 – Relationship between an index of land use intensity based on in-stream condition and the percent of the catchment area under improved pasture land cover.

Table 4.3 - Loading coefficients for Axis 1 of a Principal Components Analysis of all physico-chemical variables measured at each sampling site.

Variable	**Axis 1**
Calcium	0.25
Conductivity	0.24
Vanadium	0.24
Sulphate	0.24
Potassium	0.23
Sodium	0.23
Chloride	0.22
Magnesium	0.22
Nitrate	0.22
Chromium	0.21
Canopy cover	0.21
Length of bank poaching	0.20
Inorganic sediment	0.18
Organic sediment	0.18
Fine sediment cover	0.17
Bed sediment calibre	0.17
Extent of all erosion	0.17
Bank material calibre	0.15
pH	0.15
Gentle banks	0.14
Selenium	0.14
Artificial substrate	0.14
Iron	0.09
Nickel	0.09
Average width	0.05
Average flow velocity in thalweg	0.03
Cobalt	0.01
Vertical banks	0.01
Fluoride	-0.01
Average flow velocity at margin	-0.02
Bromide	-0.02
Phosphorus	-0.03
Steep banks	-0.03
Manganese	-0.04
Bedrock substrate	-0.04
Zinc	-0.05
Arsenic	-0.05
Undercut banks	-0.06
Bank vegetation score	-0.07
Composite banks	-0.09
Aluminium	-0.09
Copper	-0.10
Average depth	-0.12
Lead	-0.15
Cadmium	-0.15

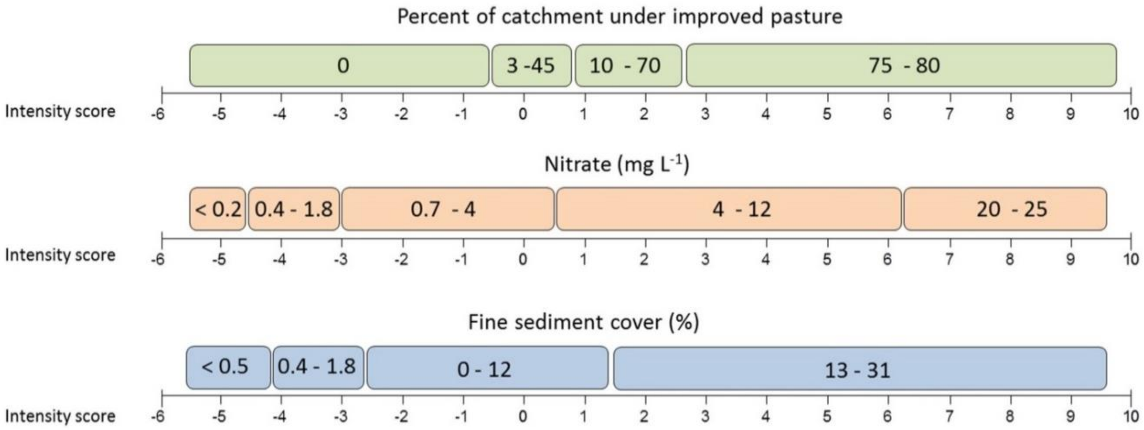

Figure 4.3- Relationship between intensity score, a derived index of pastoral intensity, and key indicators of agricultural intensity. Blocks show the range of values present in sites with a given intensity score. Breaks occur where sites with a given intensity score always have a higher value than sites with lower intensity scores.

4.3.4.2 Invertebrate community structure

The response of invertebrate community structure to pastoral intensity was assessed using four common metrics: i) taxon richness; ii) total abundance; iii) rarefied richness; and iv) Simpson's evenness. Richness was rarefied using Hurlbert's (1971) formulation in the *vegan* package in *R* to estimate the number of species expected based on the smallest sample size (311 individuals; Oksanen *et al.*, 2013). To assess the relative evidence for linear, subsidy-stress or threshold relationships, community structure was modelled in relation to intensity score using null (intercept-only), linear, second order polynomial and piecewise linear regressions, respectively. The evidence for alternative models was assessed using changes in the Akaike Information Criterion (delta AIC; Burnham and Anderson, 2004). Piecewise linear regressions were fitted using breakpoints identified from the segmented package (Muggeo, 2008). Models also included: i) survey year, to account for any differences between 2006 and 2012, and ii) altitude to adjust for variation in elevation across the intensity gradient. The fit of all models was checked using residual plots, alongside semivariograms to ensure that there was no spatial autocorrelation ('*gstat*' and '*sp*' package; Pebesma, 2004, 2005). The analyses were also run separately on the 2006 and 2012 surveys to confirm that they showed similar responses (results not shown), which also meant that an interaction between survey year and intensity score did not need to be included in the models.

To determine how taxa that differ in their sensitivity to organic pollution and sedimentation responded to the intensity gradient, taxa were assigned to three groups (low, medium and high) for their sensitivity to each of these stressors. Group assignments were based on recently revised Biological Monitoring Working Party scores for organic pollution (low = 0 - 4, medium = > 4 -7, high > 7; Paisley *et al.*, 2014) and Proportion of Sediment Sensitive Invertebrate scores for sedimentation (low = categories C and D, medium = B and high = A; Extence *et al.*, 2013). The changes in abundance and richness of each group with increasing intensity score were modelled as described above.

Community composition was analysed using Non-metric Multidimensional scaling (NMDS) in two dimensions with R's *vegan* library separately for each survey year (Oksanen *et al.*, 2013). Bray-Curtis similarities were used due to their ability to deal with zero-skewed data (Bray and Curtis, 1957), and abundance data were fourth-root transformed prior to analysis to down-weight the influence of the most abundant taxa (Clarke and Warwick, 2001). Rare species, occurring at fewer than 5% of sites, were removed from analysis to reduce noise in the dataset (Gauch, 1982).

The approach of Baselga *et al.* (2012) was employed to determine whether differences in species assemblage across the stress gradient were due to nestedness or species turnover, across the whole dataset and separately for each survey year. The *betapart* package in R (Baselga *et al.*, 2013) was used to calculate the Sørensen index, which shows total beta diversity, and Simpson's index of dissimilarity (Simpson, 1943), which accounts only for the turnover component of diversity. Nestedness was then computed by subtracting Simpson's beta diversity from total beta diversity. The relationship between site pairwise dissimilarities in beta diversity components and pairwise dissimilarity in intensity scores were examined using multiple regression models for distance matrices with *p*-values were calculated through permutation tests (1000 runs), using the *ecodist* package in *R* (Lichstein, 2007; Goslee and Urban, 2007)

4.3.4.3. Trait representation and Functional diversity

For each survey year, changes in functional composition along the land use gradient were assessed using eight behavioural and morphological traits, which reflected aspects of resistance and resilience (resistance forms, locomotion method, potential number of reproductive cycles per year), life history (maximum size, reproductive method, life cycle duration) and general biology (feeding guild, respiration method). Each trait was divided into several categories (39 in total, Table S4.2) and the affinity of each taxon to each category was described by a score from 0 (no affinity) to 5 (high affinity). These were then standardised, so the scores from the individual categories for a single trait summed to one for each taxon. Taxon affinities were obtained from Bis and Usseglio-Poltera (2004), and Schmidt-Kloiber and Hering (2012). Trait profiles for each site were calculated by multiplying the relative scores for each trait with the relative abundances of the taxa expressing the trait (Dolèdec *et al.*, 2006, Table S4.4). The resulting site-trait profiles were ordinated using a fuzzy correspondence analysis (fCA) to visualise the differences in trait representation between sites (Usseglio-Polatera *et al.*, 2000). Axis scores from the fCA were correlated against intensity scores.

Three components of functional diversity were calculated for all 60 sites based on the eight traits following Villèger *et al.* (2008): i) functional richness (F_{ric}), a measure of the volume of functional space occupied by the community, ii) functional evenness (F_{eve}), a measure of how regularly species are distributed within this space and; iii) functional divergence (F_{div}) which measures the divergence in the distribution of species abundance in this space. Linear, quadratic and threshold models for the relationships between functional diversity components and intensity score were compared as described above. This analysis was repeated using only resistance/resilience traits, as they are expected to respond more directly to stress gradients (Townsend *et al.*, 1997a).

To investigate the mechanisms structuring communities along the intensity gradient, functional richness was compared to the values expected due to changing species richness (Villeger *et al.*, 2008). For each of the 60 locations, values for expected F_{ric} were obtained by randomly selecting the observed number of species from the whole species pool (without replacement) and calculating the average functional richness from 10^4

randomizations at each level of species richness. The ratio of observed to expected functional richness was expressed using the index of variance (IV) (Mason *et al.*, 2008):

$$IV = 2[\text{Observed}/(\text{Observed} + \text{Expected})] - 1$$

Negative IV values show coexisting species have trait profiles that are more similar than expected at random, indicating environmental filtering (Mason, 2008). Positive values show greater than expected F_{ric}, consistent with competitive exclusion (Mason *et al.*, 2008). These values were calculated separately for the 2006 and 2012 datasets but results were combined to assess the relationship between IV and pastoral intensity using linear quadratic and threshold models.

4.4. Results

4.4.1. Response of macroinvertebrate community structure to pastoral intensification

Richness changed non-linearly along the intensity gradient (Figure 4.4a). Piecewise regression best described the relationship between intensity score and species richness (Table 4.4), with an estimated break-point of -0.75: below this, richness increased non-significantly (t = 1.72, $p = 0.10$, n = 25) and above it decreased significantly (t= -3.75, $p < 0.001$, $R^2 = 0.27$, n = 35) (Figure 4.4a). There was some evidence for a threshold relationship between rarefied richness and intensity score but as ΔAIC of this model was only 0·77, the linear model could not be rejected (t= -0.76, $p = 0.004$, $R^2 = 0.36$) (Table 4.4, Figure 4.4b). Neither metric showed a significant response to altitude or survey year. Log abundance and evenness did not change significantly along the intensity gradient (t= 1.17, $p = 0.24$ and t = 0.38, $p = 0.71$, respectively, Table 4.4). The equivalent analyses with taxa sub-divided into 'low', 'medium' and 'high' sensitivity groups for fine sediment (PSI score) or organic pollution (BMWP score) confirmed that there was no evidence that abundance varied across the gradient (lowest AIC = null model in every case). Richness of taxa with medium or low sensitivity to either stressor did not respond to pastoral intensity (Table 4.5) but the richness of taxa 'highly' sensitive to either stressor showed strong evidence for non-linear declines with increasing intensity (both ΔAICs ≥4.98 compared to linear models). The estimated thresholds were similar to that for total richness, suggesting that the most sensitive taxa were responsible for the community level decline in richness (organic pollution t = 1.61, $p = 0.12$ and t = -4.31 $p <$

0.001, for the linear and quadratic terms, $R^2 = 0.33$; sedimentation t = 1.16, $p = 0.26$, t = -3.95, $p < 0.001$, $R^2 = 0.40$; Table 4.5). Again, survey year and altitude were not significant in these models.

Table 4.4 – Comparisons of null, linear, quadratic and threshold models of the relationship between pastoral intensity score and macroinvertebrate community metrics. Null models included survey year and altitude but not intensity score.

Invertebrate response	Null models δAIC	Linear δAIC	Quadratic δAIC	Threshold δAIC
Richness	12.62	8.18	3.38	0
Rarefied richness	10.52	0.77	0.88	0
Log(Abundance)	0.64	0	0.02	0.97
Evenness	0	2.00	2.70	4.44

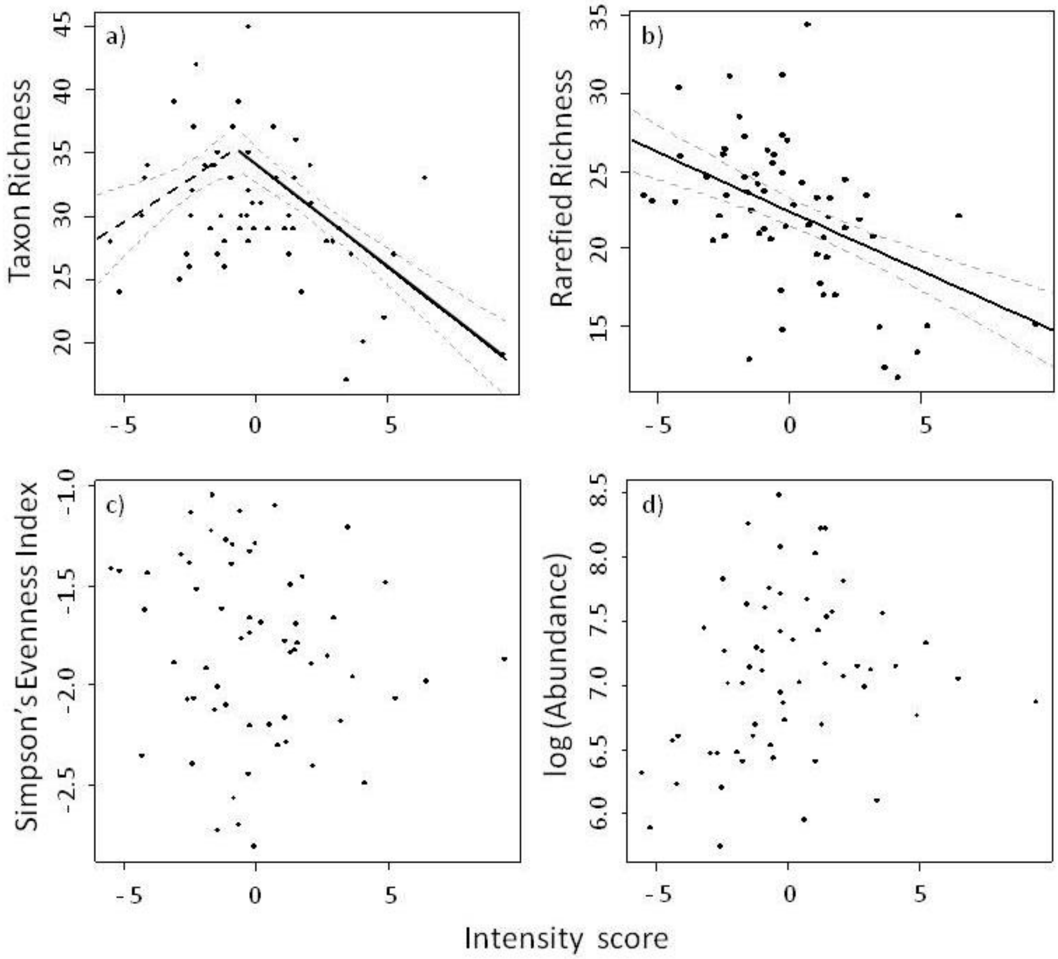

Figure 4.4 – Modeled relationships between invertebrate metrics and pastoral intensity scores. Black lines show model predictions holding altitude at its mean value and using the 2012 sample year (dashed where non-significant) and dashed grey lines show standard errors.

Table 4.5 - Comparisons of null, linear, quadratic and threshold models of relationships between pastoral intensity score and groups of taxa with 'low', 'medium' and 'high' sensitivity to organic pollution and sedimentation. Group assignments were based on recently revised Biological Monitoring Working Party scores for organic pollution (low = 0 - 4, medium = > 4 -7, high > 7; Paisley et al., 2014) and Proportion Sediment Sensitive Invertebrate scores for sedimentation (low = categories C and D, medium = B and high = A; Extence et al., 2013).

Stressors	Sensitivity group	Invertebrate response	Null δAIC	Linear δAIC	Quadratic δAIC	Threshold δAIC
Organic pollution	Low	Richness	0	1.89	3.81	4.05
		Abundance	0	0.33	2.26	4.32
	Medium	Richness	0	0.69	2.23	1.83
		Abundance	0	0.69	0.73	2.27
	High	Richness	13.15	6.51	1.24	0
		Abundance	0	1.95	3.48	4.72
Sedimentation	Low	Richness	0	1.36	0.82	2.16
		Abundance	0	0.08	1.94	1.63
	Medium	Richness	0	0.69	2.16	1.41
		Abundance	0	0.69	2.34	3.15
	High	Richness	18.28	7.57	2.59	0
		Abundance	0	1.67	1.05	2.49

An apparent land use effect on community composition was only evident in the NMDS ordination for the 2012 data. The NMDS ordination separated the invertebrate community along two axes, with a stress score of 0.152 (Figure 4.5). Intensity score increased along Axis 1, which represented a shift from communities dominated by riffle beetles, Ephemoptera, Plecoptera and Trichoptera (*EPT*) species and Sialidae to communities dominated by Asellidae, Planaria, dipteran larvae and molluscs (Figure 4.6). There was no relationship between intensity scores and Axis 2 values but flow velocity increased with Axis 2 score. The variability between sites with similar intensity scores increased along the intensity gradient.

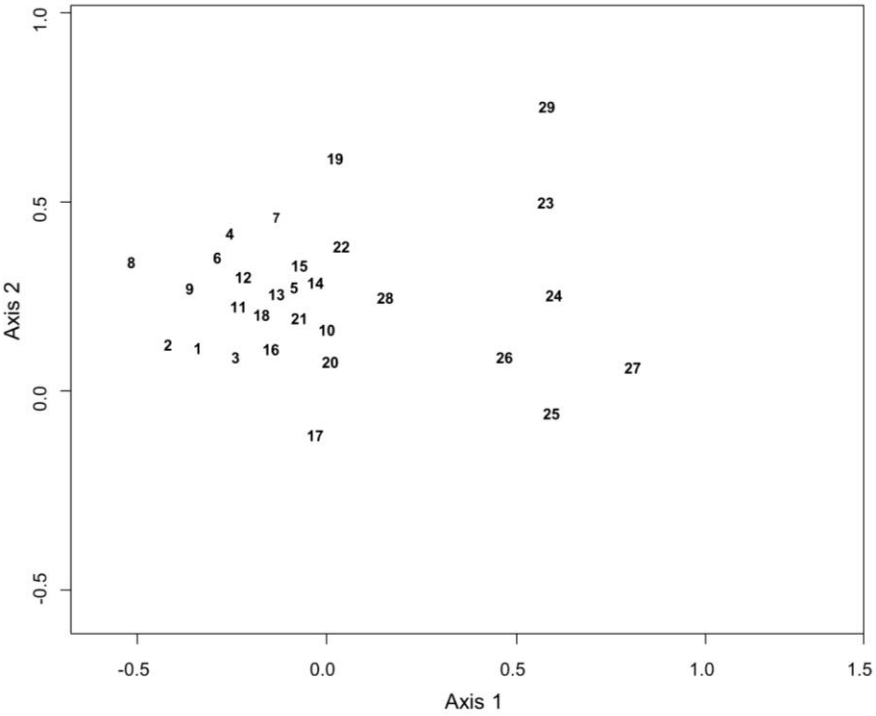

Figure 4.5 – Site loadings from Non-metric Multidimensional Scaling Analysis of invertebrate taxonomic composition. Sites are numbered based on rank of pastoral intensity score (1 lowest, 29 highest).

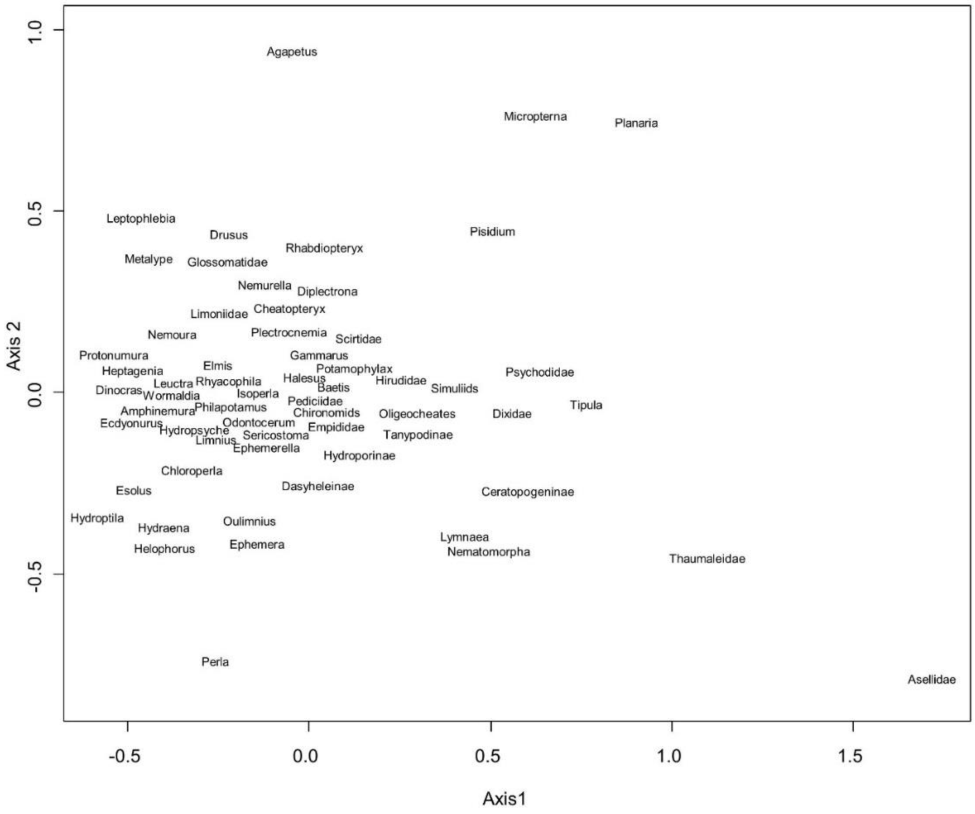

Figure 4.6 – Results of Non-metric Multidimensional Scaling Analysis of invertebrate taxonomic composition across 29 sites spanning a gradient of pastoral intensity.

The change in community composition across the land use gradient resulted in a total Sørensen beta diversity of 0.915 across all sites. This was almost entirely due to species turnover (97.2 %), with little evidence of nestedness (2.80 %). The same pattern was observed when each survey year was analysed separately: nestedness accounted for 6.5% of betadiversity in the 2006 survey and 5.8% in the 2012 survey. Total community dissimilarity, turnover and nestedness between sites showed significant positive relationships with pairwise dissimilarities in intensity scores (F = 303.0, $p < 0.001$, $R^2 = 0.15$; F= 472.5, $p = 0.001$, $R^2 = 0.22$; and F = 108.7, $p = 0.01$, $R^2 = 0.06$, respectively). Because species richness had a threshold relationship with intensity score, this analysis was repeated for the subset of sites above and below the threshold value (intensity score = -0.7). The results in these subsets were near-identical to the full data set: beta diversity was 0.70 for the low intensity sites and 0.80 for the high intensity sites, and in both subsets turnover accounted for >90% of beta diversity.

4.4.2. Response of trait representation and functional diversity to pastoral intensification

For 2012 data, fuzzy correspondence analysis separated traits along two main axes which explained 84% of the total variance (Figure 4.7). Intensity score was negatively correlated with Axis 1 (Pearson's $r = -0.50$, $p = 0.006$) and positively correlated with Axis 2 ($r = 0.59$, $p = 0.001$). Sites with low pastoral intensity were clustered with positive Axis 1 scores and negative Axis 2 scores (Figures 4.7 and 4.8, Sites 1-16). These sites had a high representation of univoltine and semivoltine species with gathering and grazing feeding behaviour, reproduction via free or fixed eggs or clutches, and crawling locomotion. Mirroring the greater variation in taxonomic composition at high pastoral intensity, there was high variability in trait profiles between high intensity sites with some sites having high representation of shredding behaviour, ovoviviparous reproduction and long life cycles (Figures 4.7 and 4.8, Sites 17, 19, 22, 29), whilst others had a high representation of filter feeders with spiracle or tegument respiration and temporary attachment (Figures 4.7 and 4.8, Sites 21, 23, 24, 27). Generally, plurivoltine species with no resistance forms, asexual reproduction and endobenthic behaviour were common between sites with high intensity scores. No other measured environmental variables showed significant relationships with either axes. There was no significant relationship between trait profiles and intensity score in the 2006 dataset.

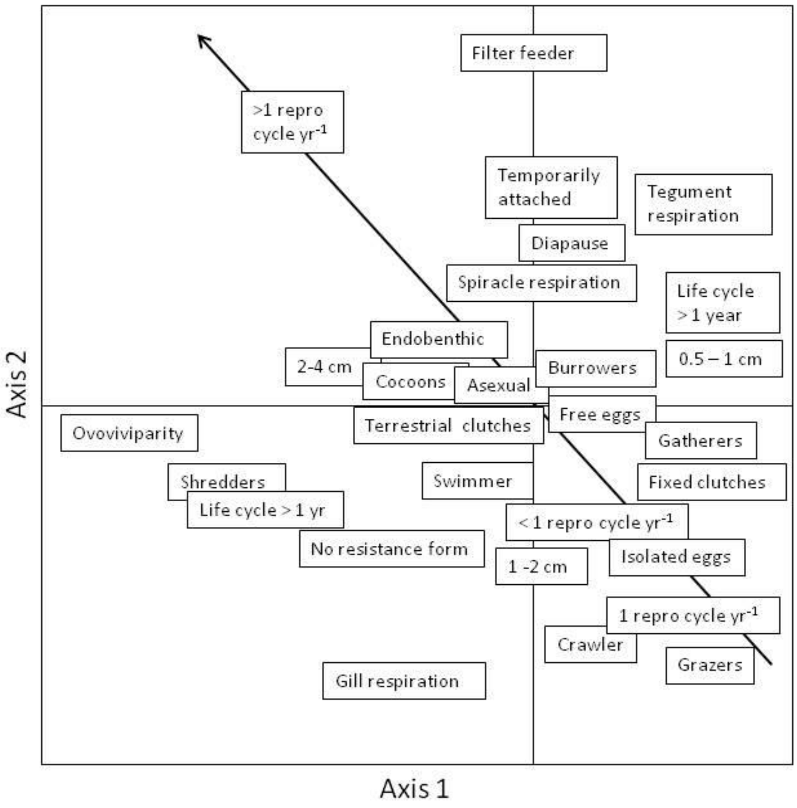

Figure 4.7 – Fuzzy correspondence analysis plot of the representation of 39 trait categories from 29 sites spanning a gradient of pastoral intensity. For clarity not all traits are shown. Bold arrow shows increasing pastoral intensity scores.

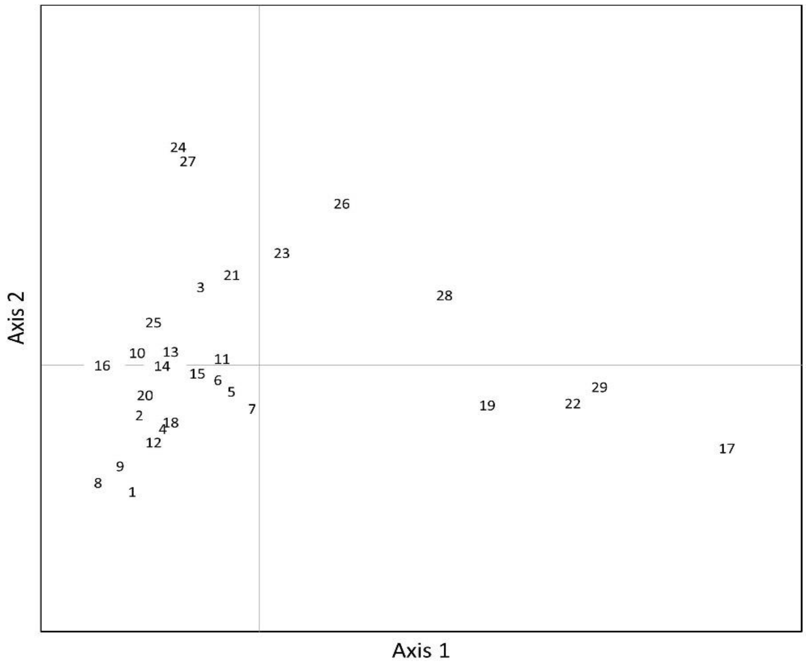

Figure 4.8 - Fuzzy correspondence analysis plot of the 2012 sites based on trait composition. Sites are numbered based on rank of pastoral intensity (1 lowest, 29 highest).

Functional richness showed a threshold response to increasing pastoral intensity, following the same pattern as taxon richness; a non-significant increase to intensity scores of -0.3 (t = 1.32, $p = 0.20$, $R^2 = 0.02$) and a significant decline with further increases in intensity (t = 4.72, $p < 0.001$, $R^2 = 0.42$, Figure 4.9a). Both F_{eve} and F_{div} showed a significant linear increase with intensity score (t = 2.59, $p = 0.001$, $R^2 = 0.67$; t = 3.00, p =0.004, $R^2 = 0.48$, respectively, Figure 4.9 b and c). Survey was not significant in the F_{ric} model but the 2006 survey had significantly higher F_{div} and F_{eve} than the 2012 survey (t = 10.79, $p < 0.001$; t = 6.9, $p < 0.001$, respectively). The interaction between intensity score and survey year was not significant for any of the response variables. The same response patterns were observed when the analysis was repeated using only resistance/resilience traits.

There was no compelling evidence to differentiate a non-linear and linear model to describe the relationship between F_{ric}IV and intensity score (Table 4.6). With increasing intensity score, functional richness decreased relative to random expectation (t = 2.63, p =0.01, $R^2 = 0.12$) with no significant effect of survey year (t= 1.66, $p = 0.10$). This randomly expected F_{ric} may be inflated by the species turnover along the land use gradient, as expected values were calculated from a large species pool. To determine if this was the case, observed and expected functional richness were calculated separately for subsets of sites with intensity scores above and below the threshold in F_{ric} response (intensity score = -0.3) using only the species present in each subset. F_{ric} values in the low intensity group were greater than random expectation and F_{ric}IV showed no significant relationship with intensity score (t = 1.54, $p = 0.14$; Figure 4.9d). F_{ric} values in the high intensity group were lower than random expectation and F_{ric}IV significantly declined with increasing intensity score (t = 3.21, $p = 0.003$, $R^2 = 0.22$, Figure 4.9d) with survey year being insignificant (t =2 .03, $p = 0.06$).

Table 4.6 – Comparisons of null, linear, quadratic and threshold models of relationships between pastoral intensity score and components of functional diversity.

Invertebrate response	Null models δAIC	Linear δAIC	Quadratic δAIC	Threshold δAIC
Functional richness	11.43	3.57	1.48	0
IV of Functional richness	5.71	0.85	0	0.35
IV of Functional richness in subsets of Intensity score < -0.3	0	0.57	0.65	1.37
IV of Functional richness in subsets of Intensity score > -0.3	4.17	0	1.91	2.90
Functional Evenness	4.65	0	0.72	0.64
Functional Divergence	6.77	0	0.19	0.14

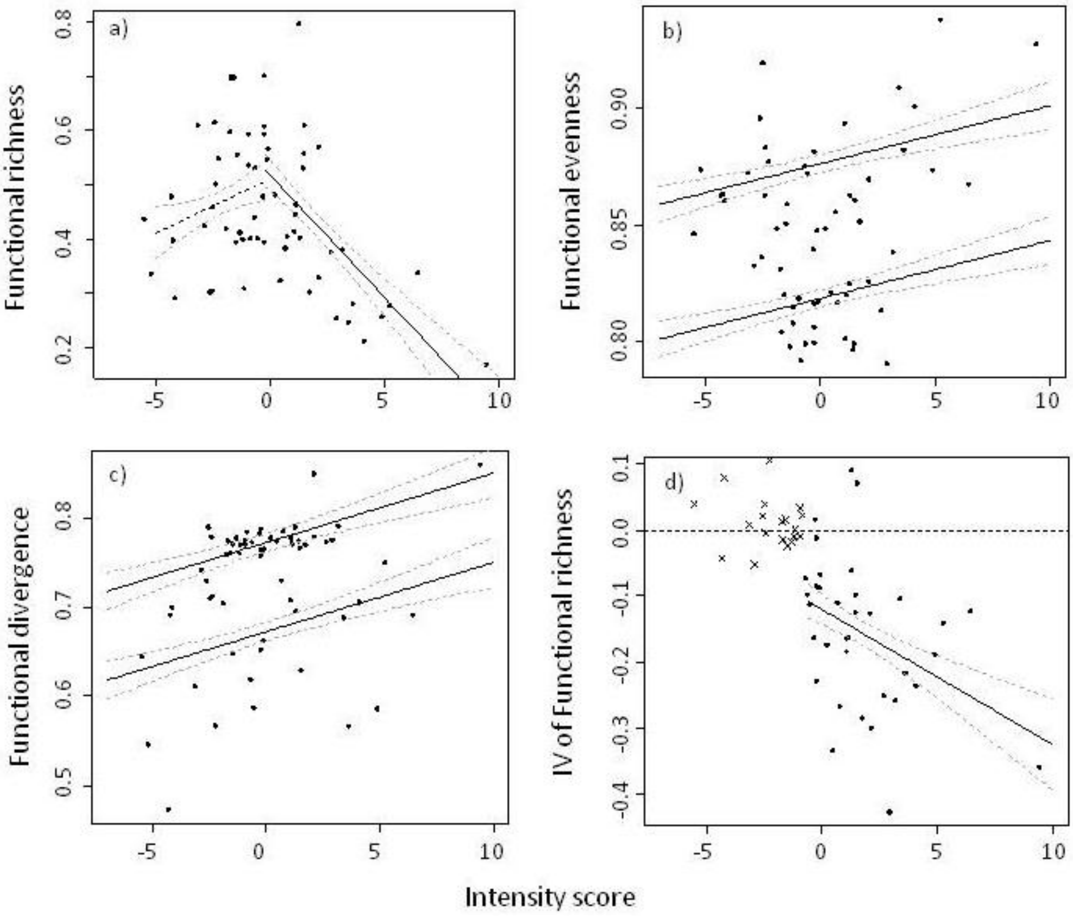

Figure 4.9 – Relationships between functional diversity components and pastoral intensity scores. Black lines show modelled predictions (dashed where non-significant) and dashed grey lines show standard errors. Two lines are shown where survey year was significant, with 2006 having higher values than 2012 in both cases.

4.5. Discussion

The physico-chemical changes associated with pastoral intensification resulted in a turnover of macroinvertebrate taxa, and a decline in both taxon and functional richness above a threshold intensity scores of approximately -0.5: which equates to around 40% catchment land cover under improved pasture, 4 mg L^{-1} nitrate and 8% fine sediment cover (Figures 4.2 and 4.3). There was evidence of increasedenvironmental filtering along the stress gradient of land use intensity. In combination, the present results suggest that land use change has significant, non-linear effects on both biodiversity and ecosystem functioning in streams.

4.5.1. Physico-chemical effects of pastoral intensification

In-stream conditions were used to quantify pastoral development due to the difficulties of defining and measuring land use intensity. The non-linear relationship between the resulting intensity index and percentage of the catchment under pastoral land cover suggests that proportional cover, without consideration of management practices, is a poor indicator of pastoral intensity (*cf.* Harding *et al.*, 1999). Although use of this intensity index limits comparison with other studies and direct application to land use management, it affords a more representative measure for evaluating conceptual models of pastoral land use effects.

The pastoral intensity score captured changes in fine sediment, nitrate concentration, trace metals, salts and canopy cover allowing assessment of the aggregate impacts of these co-varying stressors, which are likely to be unpredictable from their isolated effects (Townsend *et al.*, 2008). The observed physico-chemical changes along the land use gradient were consistent with the effects of livestock trampling, bank erosion, and application of both organic and inorganic fertilisers (Trimble and Mendel, 1995; Jarvie *et al.*, 2008). Surprisingly, phosphate levels were undetectable in all study streams. This may reflect a reduction in phosphate mobilisation under the baseflow sampling conditions or may be due to rapid uptake of available phosphates by in-stream producers (Jarvie *et al.*, 2008). Information on toxic chemicals from insecticides and sheep dips were not available in this study but may have contributed to the observed responses.

4.5.2. Response of macroinvertebrate community structure to pastoral intensification

Taxon richness initially increased with pastoral intensity before declining, consistent with the predicted subsidy-stress response. The initial increase, which was probably a result of mild nutrient enrichment increasing algal food resources (Bernot *et al.*, 2006), was, however, statistically non-significant and could not be differentiated from a threshold response. Rarefied richness declined across the gradient, suggesting that the initial increase in taxon richness was partially attributable to a sampling effect: there was a weak, non-significant increase in overall macroinvertebrate abundance, which would tend to increase the probability of detecting rare species (Magurran 2004). The lack of a significant response in abundance, contrary to the predicted subsidy-stress response, may be partially attributable to the kick-sample technique employed. Although kick sampling is an effective sampling method for community composition, the variability between sites in the area of stream bed sampled and the ease of dislodging substrate means that this technique produces only rough estimates of abundance.

The decline in richness with increases in pastoral intensity beyond the threshold level is likely to be due to the combined effects of increased nitrate concentrations and fine sediment. This assertion is supported by the observed threshold response to intensity by species with high sensitivity to sedimentation and organic pollution, but not by low and medium sensitivity species. Sedimentation reduces both habitat and feeding niche heterogeneity by smothering interstitial habitat, reducing stable substrates for algal attachment and abrading primary producers (Niyogi *et al.*, 2007; Burdon *et al.*, 2013). The high nutrient concentrations observed in several sites can result in excessive growth of epilithon with reduced palatability and nutritional quality for invertebrate consumers (eg. increased representation of cyanobacteria, fungi and bacteria) (Braccia and Voshell, 2007) and can lead to declines in dissolved oxygen (Skinner *et al.*, 1997).

These results are in agreement with the results of Braccia and Voshell (2007), and Wagenhoff *et al.* (2011), who also found marked declines in richness after weak initial increases along gradients of pastoral intensity. Further, these results are consistent with the increases in richness with increased pastoral intensity observed by Riley *et al.* (2003)

over a nitrate concentration gradient from 0.0035 to 0.34 mg L^{-1} and the lack of significant change in invertebrate richness observed by Niyogi *et al.* (2007) over a nitrate gradient of 0.005 to 1.8 mg L^{-1}. In the present study the non-significant increases in richness occurred over nitrate concentrations ranging from 0.1 to 2.9 mg L^{-1} and declines occurred as nitrate concentrations increased from 2.8 to 25.9 mg L^{-1}. This is the first study to assess macroinvertebrate responses to the wide range of nitrate concentrations in UK upland pastoral streams (Jarvie *et al.*, 2008) and illustrates how the observed effects of land use change depend on the portion of the land use intensity gradient considered.

4.5.3. Response of trait representation and functional diversity to pastoral intensification

The difference in taxonomic and trait composition between sites with similar levels of pastoral intensity increased along the intensity gradient. This supports previous studies showing increased variability in community structure in response to stress (eg. Clarke and Warwick, 1993) and may reflect differences in the dominant stressors between sites or differences in the trajectory of stress response in sites with differing species pools.

The response of FD components to increasing pastoral intensity suggests that different mechanisms acted to control the macroinvertebrate community above and below the intensity score threshold. Below the threshold, greater functional richness than expected at randomand low functional evenness and divergence (Figure 4.10) may indicating that biotic interactions structure the community (eg. competitive exclusion from niches with low availability such as depositional habitats) (Mason *et al.*, 2008).

In sites above the intensity threshold, divergence and evenness of functional strategies increased with intensity, and functional richness declined more rapidly than taxonomic richness indicating a non-random loss of traits and an increased role of environmental filtering (Mason *et al.*, 2008; Figure 4.9). This result supported predictions and is likely to be caused by sediment and nutrient stress restricting the range of viable functional strategies (Burdon *et al.*, 2013; Lange *et al.*, 2014). Taxa with traits poorly adapted to the conditions, such as those with low resistance/resilience (no resistance form, univoltine and semivoltine, long life cycles) and susceptibility to sedimentation (crawling behaviour, feeding by grazing, large sizes and reproduction via free or fixed eggs) or elevated nitrate (gill respiration and crawling behaviour; Lange *et al.* 2014) were filtered out, leaving a pool of functionally similar species (Poff, 1997; Cornwell *et al.*, 2006). These changes in trait representation with pastoral intensity are consistent with previous studies (Dolèdec *et al.*, 2006, Braccia and Voshell, 2007; Larsen and Ormerod, 2010), but were only evident in one of the survey years considered here (2012). This discrepancy may reflect the minor differences in the sampling technique between the survey years and suggests that the largest changes in trait representation occur in the margins, which were not sampled in 2006.

The limitations in the methods employed to analyse trait profiles in this study may explain why the observed trait responses were relatively minor. Firstly, trait affinities were inferred rather than measured at each site. Characteristics such as ingestion rates or size distributions may have varied between sites for a given species. Secondly, the methods were not able to account for the interactions between traits, which occur as a result of ecological and evolutionary constraints predisposing certain trait to occur in concert (Verberk *et al.*, 2013). Environmental filtering will act upon the whole subset of traits possessed by an organism such that interactions between traits may have obscured the response of individual traits to the stress gradient. Further, a trait's adaptive significance will depend on the combination of other traits the organism possess (Verberk *et al.*, 2013).

These results provide an indication of the mechanisms causing the observed species turnover, which, although contrary to the predicted nested loss of species that is prevalent along gradients of anthropogenic stressors (Gutiérrez-Cánovas *et al.*, 2013), has been shown previously in response to agricultural stress gradients (eg. Harding *et al.*, 1999; Niyogi *et al.*, 2007). Low intensity sites had a high representation of sensitive taxa such as *EPT* species and riffle beetles but several taxa were absent. This result, combined with the higher than expected F_{ric} may indicate that biotic interactions determine community assembly, and it is suggested that this is competitive exclusion from depositional habitats which had low availability in streams with low pastoral intensity.

The ability of trait-based approaches to detect controls on community structuring is, however, limited (Adler *et al.*, 2013), and the processes determining species assemblages in low intensity sites warrant further investigation. Although a higher than expected functional diversity does indicate a role of niche partitioning in structuring the community it cannot indicate which coexistence mechanisms are most important e.g. resource partitioning, or temporal or spatial variation in conditions (Adler *et al.*, 2013). Further, trait based analyses do not consider the role of intra-specific competition in influencing community structure. In heterogeneous habitats, the niche partitioning that promotes species coexistence will result in an increased strength of intra-specific competition relative to that of inter-specific competition (Cross and Benke, 2002).

In high intensity sites, isopods, gastropods, diptera larvae, planarians and oligochaetes, which were largely absent from low intensity sites, dominated the community. As these taxa thrive in depositional habitats, this result supports the hypothesis of competitive exclusion from low intensity sites. Sensitive species declined in high intensity sites in response to stressors (*cf.* Niyogi *et al.* 2007; Braccia and Voshell, 2007), resulting in a turnover of species along the land use gradient. Although this turnover resulted in small overall declines in taxon richness, the non-random loss of species with specific life history and behavioural traits may be of concern for biodiversity conservation. Further, the consequent decline in functional richness exceeded declines in species richness, indicating that impairment of ecosystem functioning by pastoral intensification may be greater than predicted from traditional structural measures (Tilman, 2001; Mouchet *et al.*, 2010). Overall, the results presented here demonstrate that the changes in biodiversity associated with intensive pastoral agriculture are likely to result in impaired stream ecosystem functioning at levels of intensification that are already widely exceeded worldwide.

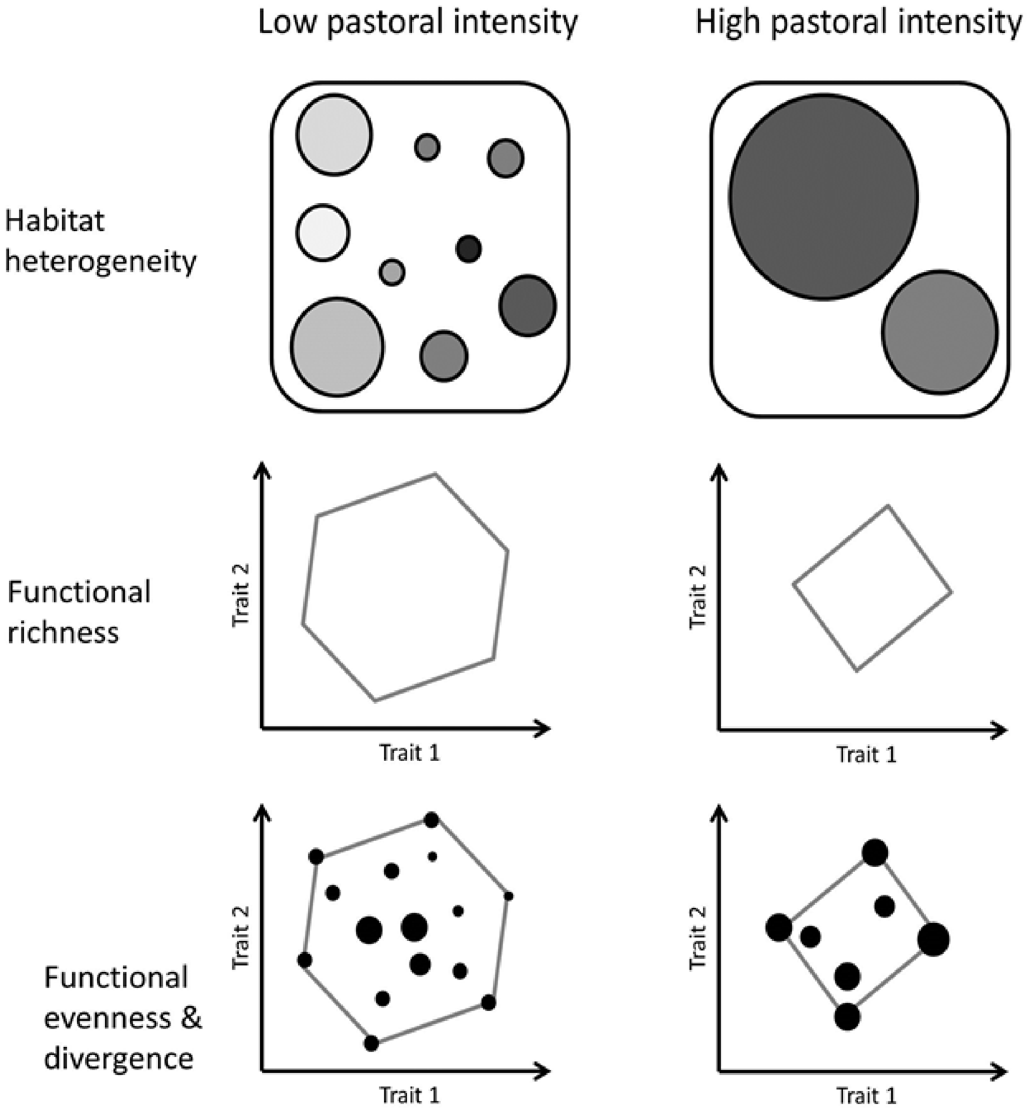

Figure 4.10 – Effects of pastoral intensification on components of functional diversity of macroinvertebrate communities. Heterogenous habitats in low intensity sites can support a diverse range of overlapping trait profiles, which occur in differing proportions. This results in communities with high functional richness and low functional evenness and divergence. Sediment deposition as a result of pastoral intensification reduces the heterogeneity of habitat types within the stream, producing large areas of homogenous habitat. Environmental filtering produces communities with low functional richness and high divergence between few distinct habitat types.

4.6. Supporting material

Table S4.1 -List of taxa recorded in kick samples.

Class/Group	Order	Family	Genus
Arachnida	Trombidiformes	Hydrachnidiae	
		Lymnaeidae	
		Planorbidae	
Bivalvia	Veneroida	Sphaeriidae	
Gastropods		Hydrobiidae	
Hirudinaeta/	Arhynchobdellida	Eropobdellidae	
Clitella	Rhynchobdellida	Glossiphoniidae	
Insecta	Coleoptera	Curculionidae	
		Elmidae	*Elmis*
			Esolus
			Limnius
			Oulimnius
			Riolus
		Dryopidae	
		Dytiscidae	*Agabus*
			Colymbetinae
			Hydroporinae
		Helophoridae	*Helophorus*
		Hydraenidae	*Hydraena*
		Hydrophilidae	*Enochrus*
		Scirtidae	
	Diptera	Ceratopogeninae	
		Chironomidae	
		Dasyheleinae	
		Dixidae	
		Empididae	
		Limoniidae	
		Pcychopteridae	
		Pediciidae	
		Psychodidae	
		Tabanidae	
		Thaumaleidae	
		Tipula	
	Ephemeroptera	Baetidae	*Baetis*
		Caenidae	*Caenis*
		Ephemeridae	*Ephemera*
		Ephemerellidae	*Ephemerella*
		Heptagenidae	*Ecdynonurus*
			Heptagenia
			Rhithrogena
		Siphlonuridae	*Siphlonurus*
			Ameletus
		Leptophlebiidae	*Habroleptophlebia*
			Leptophlebia
			Paraletophlebia

Class/Group	Order	Family	Genus
	Hemiptera	Veliidae	
	Lepidoptera	Crambidae	
	Megaloptera	Sialidae	
	Odonata	Cordulegasteridae	
	Plecoptera	Chloroperlidae	*Chloroperla*
		Leuctridae	*Leuctra*
		Nemouridae	*Amphinemura*
			Nemoura
			Neumurella
			Protonemoura
		Perlidae	*Dinocras*
			Perlodes
		Perlodidae	*Isoperla*
			Perlodes
		Taeniopterygidae	*Brachyptera*
			Rhabdiopteryx
	Trichoptera	Brachycentridae	*Brachycentrus*
		Goeridae	*Silo*
		Glossomatidae	*Agapetus*
			Glossoma
		Hydropsychidae	*Diplectrona*
			Hydropsyche
		Hydroptilidae	*Hydroptila*
			Allotrichia
		Lepidostomatidae	*Lepidostoma*
		Limnephilidae	*Anabolia*
			Chaetopteryx
			Drusus annulatus
			Ecclisopteryx
			Halesus
			Micropterna
			Potamophylax
		Odontoceridae	*Odontocerum*
		Philopotamidae	*Philopotamus*
			Wormaldia
		Polycentropidae	*Plectrocnemia*
		Psychomyiidae	*Metalype*
			Tinodes
		Rhyacophilidae	*Rhyacophila*
		Sericostomatoidea	*Sericostoma*
Nematomorpha			
Oligochaetes			
Turbellaria	Tricladida	Planariidae	*Planaria*

Table S4.2 – Trait categories used in analysis of functional composition

Trait	**Trait category**
Life history	
Maximum size (cm)	< 1
	1 - 2
	2 – 4
	> 4
Life cycle duration (years)	≤ 1
	> 1
Number of repro cycles per year	Plurivoltine
	Univoltine
	Semivoltine
Reproductive technique	Ovoviviparity
	Free eggs
	Free clutches
	Fixed or cemented eggs
	Fixed or cemented clutches
	Clutches in vegetation
	Terrestrial clutches
	Asexual
Resistance and resilience	
Resistance form	Eggs, statoblasts
	Cocoons
	Housing against desiccation
	Diapause or dormancy
	No resistance form
Locomotion and substrate relation	Flier
	Swimmer
	Crawler
	Burrower
	Interstitial
	Attached
General biological characteristics	
Respiration	Tegument
	Gill
	Plastron
	Spiracle
Feeding Habits	Gatherer
	Shredder
	Scraper
	Filter feeder
	Predator
	Parasite

5. Impacts of pastoral intensification on basal resource utilisation in streams

5.1. Summary

Agricultural intensification causes major changes in physical habitat, water quality and biodiversity in streams, but effects on ecosystem functions, such as primary and secondary productivity and nutrient cycling, are poorly understood. Because many such functions are governed by energy fluxes, valuable insights about anthropogenic stressors on stream food webs could arise from integrated measures of basal energy resources such as in-stream primary production and terrestrial detritus. Nutrient addition, increased sediment delivery and altered riparian tree cover could all affect stream energetics in intensively managed pasture.

This study aimed to determine how increasingly intensive pastoral agriculture affects the importance of allochthonous versus autochthonous energy sources to stream macroinvertebrates in 28 UK headwater streams across four seasons. The quantity of algal and detrital food resources were measured and their nutritional quality appraised using C:N ratios. Changes in the relative abundances of invertebrates from different functional feeding guilds, coupled with stable isotope analyses of primary consumers (Baetidae and Gammaridae) and predators (*Rhyacophila dorsalis* and *Dinocras cephalotes*), were used to estimate the utilisation of alternative basal resources.

The relative abundance of detrital feeding invertebrates increased in response to greater resource availability along the agricultural intensity gradient, whilst grazing invertebrates declined due to the negative effects of fine sediment. Stable isotopic analysis was unable to resolve changes in the contributions of algae and detritus to consumer diets with increasing intensity. Isotopic data did, however, indicate that methane-derived carbon, entering food webs through apparent methanotrophy, contributed up to 33% of carbon assimilated by a generalist primary consumer (Baetidae) at intensified sites, probably due to local anoxia and thickened biofilms in sediment patches. There was little evidence of seasonal variability in basal resource use.

In concert, these data are consistent with substantial modifications to functional diversity and energetic pathways in stream ecosystems as a consequence of agricultural stressors, especially sedimentation. The contribution of methane to invertebrate communities has not previously been observed in upland streams and demonstrates that pastoral intensification could radically alter emergent ecosystem properties such as secondary production and nutrient processing in these ecosystems.

5.2. Introduction

Agricultural intensification is one of the most widespread anthropogenic stressors on freshwater ecosystems and has resulted in fundamental alterations to the physico-chemical characteristics of streams and rivers (Allan, 2004). Substantial biodiversity loss and taxonomic changes have been reported (Dudgeon *et al*., 2006), but associated changes in ecosystem functioning have received less attention despite providing a more sensitive indicator of ecosystem condition, ecosystem service provision and perturbation (Gessner and Chauvet, 2002; Sandin and Solimini, 2009). Thus, incorporating measures of ecosystem functioning into routine biomonitoring may increase the capacity to identify agricultural effects on streams that may be overlooked by more traditional bio-assessment (Gessner and Chauvet, 2002; Sandin and Solimini, 2009).

Measures of basal energy resources can be valuable functional descriptors in freshwater systems because they integrate indicators of ecosystem function and are sensitive to a range of anthropogenic stressors (Young and Collier, 2009). Energy enters freshwater ecosystems in two distinct forms: autochthonous material from in-stream primary producers (algae, macrophytes, and autotrophic bacteria) and allochthonous material entering the stream as detritus from terrestrial systems (Bott, 1996; Wallace *et al.*, 1997). More recently, a third energy source has been identified in lowland streams, where stable isotope analysis has revealed significant contributions of methane-derived carbon to riverine food webs (Jones and Grey, 2011). The production of methane in anoxic sediment and its metabolism by methanotrophic bacteria at oxic-anoxic interfaces has long been understood, but the contribution of these methanotrophic bacteria to invertebrate diets has only recently been explored. There is increasing evidence of methane-derived carbon in a range of invertebrate taxa including chironomids (Jones and Grey, 2011), oligochaetes (Hershey *et al.*, 2006), coleopteran larvae (Kohzu *et al.*, 2004) and trichopteran larvae (Trimmer *et al.*, 2009), but evidence for the phenomenon has so far been restricted to lowland rivers and is unlikely in well-oxygenated upland streams. The relative utilisation of these alternative basal resources, with their differing nutritional quality (Cross *et al.*, 2005), regulates other ecosystem properties, such as nutrient processing rates, secondary production and system stability, and therefore provides a fundamental descriptor of ecosystem integrity (Bunn *et al.*, 1999; Meyer *et al.*, 2007; Kominoski and Rosemond, 2012; Wolkovich *et al.*, 2014).

A simple measure of basal resource use can be gained from assessing macroinvertebrate feeding behaviour. Macroinvertebrates are dominant primary consumers and key conduits for energy and nutrient transfers in streams (Wallace and Webster, 1996). The relative abundance of different functional feeding guilds (FFGs) is assumed to represent the relative dependence of the community on its preferred nutritional resource, as increased availability of energy from a given basal energy resource will permit increased secondary production of its consumers (Thompson and Townsend, 2005). The representation of invertebrate predators is also informative, indicating the effects of changes in basal energy resources for higher trophic levels. Functional feeding guild ratios are straightforward to compute and interpret, and could be readily incorporated into stream monitoring programmes (Covich *et al.*, 1999; Merrit *et al.*, 2002). The disadvantage of this approach, however, is that resource use may be more variable than assumed from FFG classifications, which were designed to categories mouthpart morphology rather than food source (Merrit and Cummins, 1978). Actual ingestion rates of different food sources by a given species may vary between sites based on availability and quality of sources (Lauridsen *et al.*, 2014). Simple FFG analysescannot account for site-specific differences in the ingestion rates of the generalist consumers that often comprise a large fraction of the invertebrate community (Chapman and Demory, 1963). This can be achieved by stable isotope analysis (SIA) in which the ratios of carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N) in the tissues of consumers are used to determine the assimilation of food sources with distinct isotopic signatures (Peterson and Fry, 1987; Post, 2002). Stable isotope analysis can discern resource switching or seasonal diet variability which can act to stabilise stream food webs against the impacts of perturbations (Woodward and Hildrew, 2002; Wolkovich *et al.*, 2014) but has the disadvantages of being relatively expensive and difficult to interpret. Maximising the the value of FFG and SIA analyses as indicators of agricultural perturbation depends on improving mechanistic understanding of how they respond to the multitude of interacting stressors associated with agricultural intensification.

There is comprehensive evidence of the effects of individual stressors related to agriculture (eg. sedimentation, nutrient enrichment) on basal resource quality and quantity, and FFG representation (Hladyz *et al.*, 2011). Their combined effects on basal resource utilisation, both by the overall macroinvertebrate community and by generalist taxa, however, remain poorly understood. Fine sediment inputs caused by livestock trampling abrade algal cells, smother biofilm growth and reduce the availability of bed substrate suitable for algal attachment, increasing grazer mortality (Broekhuizen *et al.*, 2001; Parkhill and Gulliver, 2002; Gücker *et al.*, 2009). Conversely, nutrient enrichment, from inputs of inorganic fertilisers and faecal material, increases algal biomass and enhances the nutritional quality of both algal and terrestrial material (decreased carbon:nitrogen and carbon:phosphorus ratios; Young and Huryn, 1999; Riley *et al.*, 2003; Cross *et al.*, 2005) with consequent increases in grazing invertebrates (Liess *et al*., 2012). These effects are non-linear, however, with higher nutrient concentrations resulting in dominance of fungi and senescent algal cells in epilithon, reducing palatability for grazers and increasing the resource for detrital feeders (Braccia and Voshell, 2007).

All previous efforts to determine the aggregate impacts of agriculture on basal resources have assessed systems in which intensification of agriculture coincides with clearance of riparian vegetation (eg. Young and Huryn, 1999; Townsend *et al.*, 1997a; Hagen *et al.*, 2010; Hladyz, *et al.*, 2011), such that reductions in terrestrial detritus inputs and increases in the amount of light available for in-stream photosynthetic production coincide with increased fine sediment and nutrient concentrations (Townsend *et al.*, 1997a; Harding and Winterbourn, 1995). Agricultural intensification may, however, occur independently of riparian management, and, in fact, farmers may retain or increase riparian cover for bank protection, livestock forage or as part of agri-environment initiatives to safeguard streams against climatic change and diffuse pollution (Lee *et al.*, 2003; Ormerod, 2009). In England and Wales, for example, the River Habitat Survey Baseline in 2008-9 revealed that over 50% of river reaches in intensively managed pastures (re-seeded and fertilised) have near-continuous tree cover on their banks compared to 36% in semi-natural grasslands (Appendix 5.1, Figure S5.1; Seager et al. 2012). The degree to which this increasing riparian cover modifies the relationship between agricultural intensification, in-stream basal resources and the utilisation of those resources remains unexplored, despite the importance of understanding how riparian management affects stream ecosystem structure and function (Townsend and Riley, 1999; Benke *et al.*, 2001; Thomas *et al.*, 2015).

Seasonal variation is an additional, neglected dimension to the impact of agricultural intensification on ecosystem functioning, particularly in upland streams. The temporal dynamics of stream energy transfers in general are poorly resolved (Tavares-Cromar and Williams, 1996) despite strong seasonality in both abiotic and biotic characteristics that are likely to produce temporal variation in the trophic base of stream ecosystems (Ledger and Hildrew, 1998). Determining the degree of seasonal variability in macroinvertebrate communities and their resource use will reveal how consistent the observed responses to pastoral intensification are through the year, extending current understanding gained from studies which focus on spring or summer. This could provide novel insights into the effects of anthropogenic stress on ecosystem stability.

Here, measures of basal resource quality and quantity, and macroinvertebrate feeding guild representation were combined with stable isotopes analysis of a common consumers (baetid mayflies) and two predatory invertebrate taxa (*Rhyacophila dorsalis* and *Dinocras cephalotes*) to test the hypothesis that increasing agricultural intensity, in the presence of riparian tree cover, will alter the quality and quantity of basal energetic resources in streams, and consequently the utilisation of different energy pathways across trophic positions. Seasonal variability in these measures was assessed in ten of these sites, using two primary consumer taxa for stable isotope analysis (Baetidae and Gammaridae). The specific predictions tested were that increasing agricultural intensity would result in:

a) The macroinvertebrate community becoming increasingly reliant on allochthonous resources due to the association between pastoral intensity and riparian tree cover which will result in an increase availability of detrital material and declines in algal production due to light limitation;

b) An increased magnitude of seasonal variability in resource quality and quantity, and hence relative utilisation of different basal resources.

Hypothesis (a) is opposite to that made at the outset of this study (Section 1.1, hypothesis 3) which predicated an increased reliance on autochthonous material with agricultural intensification. The hypothesis was modified based on the observed association between pastoral intensity and tree cover across the sampling sites.

5.3. Methods

5.3.1. Study sites

This study was conducted on twenty-eight headwater streams (2nd and 3rd order) on upland tributaries of the Usk, Wye, Neath and Tawe rivers in South Wales. Pastoral agriculture was the dominant land cover in all catchments (> 75 %) but catchments differed in their proportions of unimproved pasture (unfertilised native grass species supporting low densities of livestock; 0 - 100% catchment cover) and improved pasture (fertilised and reseeded with high stocking densities; 0-86% catchment cover) (Jackson, 2000). Full details of the study locations are given in Chapter 4. Riparian tree cover increased with agricultural intensity. Low intensity sites tended to have isolated trees, usually hawthorn (*Crataegus monogyna*), whereas high intensity sites had semi-continuous lines of tree cover, dominated by alder (*Alnus glutinosa*) and ash (*Fraxinus excelsior*).

5.3.2. Macroinvertebrate samples

All 28 sites were sampled in May 2012 and ten sites, selected to span the gradient of pastoral intensity, were also sampled in February, June, September and December 2013. The latter selection was based on the intensity score described in Section 4.3.4.1. On each sampling occasion, a three-minute kick-sample of benthic macroinvertebrates was taken using a 1 mm mesh size D-frame net (0.25 x 0.20 m), covering all micro-habitats in proportion to their occurrence. All samples were preserved in 70% ethanol until processing when they were rinsed through a 500 µm sieve and all individuals removed, identified to genus, or a higher taxonomic resolution where this was not practicable, and counted (see Chapter 4, Table S4.1 for a taxon list).

5.3.3. Stable isotope samples

Baetidae and Gammaridae were selected to represent generalist primary consumers because they were present in all sites and can utilise both allochthocous and autochthonous basal resources (Moore, 1975; Schmidt-Kloiber and Hering, 2012). *Rhyacophila dorsalis* and *Dinocras cephalotes* were selected as representative generalist predators. *Rhyacophila dorsalis* was, on average, the most abundant generalist predator across all sites, and whilst *D. cephalotes* was only present in 15 of the 28 sites, it was the most abundant predator within these sites.

In May 2012, a single bulk sample (8-10 individuals) of Baetidae and of each predator (if present) was obtained from each site. In seasonal samples, three replicate bulk samples of both Baetidae and Gammaridae were collected from each site. On all sampling occasions three replicate samples of the potential food sources were also collected from each site: terrestrial detritus (decaying broadleaf leaves or riparian grasses, in proportion to availability at the site) and benthic algae (filamentous algae or scrapings of epilithon). All samples were stored in screw top plastic vials which were frozen at -20°C on return to the laboratory. Gut clearance was not performed as tissues and gut contents are highly similar (Jardine *et al.*, 2005), and isotope ratios can change as animals are held in confinement (Kaehler and Pakhomov, 2001).

Upon thawing, all stable isotope samples were rinsed with distilled water and non-target materials such as silt or chironomids were removed with forceps. Stable isotope studies typically use unpurified epilithon scrapings but these are likely to contain a mix of bacteria, diatoms, algae and terrestrial organic matter, resulting in high variation in autochthonous signals and reduced separation between allochthonous and autochthonous resources (Hamilton *et al.* 2005). Epilithon samples were purified to remove detritus by centrifuging with colloidal silica solution (1.27g cm^{-3} Ludox TM-40) for 10 minutes at 1000 rpm using gradual acceleration (Hamilton *et al.*, 2005). The supernatant containing the lighter algal material was used in further analysis leaving heavier detrital material to be discarded.

All samples for stable isotope analysis were freeze-dried at -60 °C for 48 h. The dried material was then ground to a powder-like consistency and weights required for analysis (1 ± 0.2 mg for invertebrate tissue, 3 ± 0.2 mg for plant material) were packaged within tin capsules. Dual $\delta^{13}C$ and $\delta^{15}N$ was performed at the University of California, Davis Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, U.K.), which has a reported long term standard deviation of 0.2‰ for δ13C and 0.3‰ for δ15N respectively (as determined from laboratory standards).

5.3.4. Measures of basal resource quality and quantity

Canopy cover was used as a proxy for the availability of coarse particular organic matter (particles > 1 mm; CPOM). On all sampling occasions an estimate of percent canopy cover was obtained from three vertical photographs, taken from the centre of the stream along the area covered by the kick sample, using image analysis software (HabitApp, Macdonald and Macdonald, *in prep*.). To determine how well this measure reflected CPOM availability, the amount of CPOM in each site was measured directly in December 2013 and correlated against canopy cover. All terrestrial material exceeding 1 mm was collected from five 0.1 m^2 Surber samplers (mesh size 1 mm) randomly positioned on the stream bed. The substrate within each Surber was disturbed, allowing large pieces of CPOM to be collected by hand and smaller pieces to be picked off the net. The five samples were combined into a single bulk sample, rinsed, air dried for 14 days and weighed. The relationship between CPOM weight and canopy cover in December 2013 was strong and positive (Pearson's $r = 0.91$, $p = 0.01$), suggesting that canopy cover was a good proxy for CPOM availability, although there may be variability in this relationship among seasons.

On all sampling occasions, the availability of benthic epilithon was estimated by scrubbing the upper surface of ten cobbles (64 – 256 mm diameter), using a steel bristled brush, into a bucket containing 300 ml of stream water. Cobbles were systematically selected from three transects across the sampling reach, and any cobbles that were unsuitable for scrubbing or with moss cover were discarded until ten suitable cobbles were obtained. The mixture was transferred to an opaque screw top bottle and stored in a dark cool box (Jaarsma *et al.*, 1998), and a vertical photograph of the scrubbed cobbles was taken with a ruler in the frame, allowing calculation of the combined surface area in Image J analysis software (Rasband, 1997). Finally, an estimate of water borne organic matter volume at each site was obtained by holding a 53 µm seston net in the flow for 5 minutes. The net submersion depth and the flow velocity directly in front of the net were recorded, allowing calculation of the volume of water filtered by the net within the 5 minute period.

Immediately upon return to the laboratory, the slurry from epilithon samples was rinsed through a 500 µm sieve. Each sample was then thoroughly stirred and four 10 ml aliquots were filtered through pre-combusted, pre-weighed glass fibre filters (pore size 1.2 µm). Two filters were placed in centrifuge tubes, wrapped in aluminium foil to limit light exposure, and stored at -80°C until further processing for cholorophyll analysis and the other two were oven dried for 48 hours. Similarly, seston samples were sieved and filtered through pre-weighed filter papers (pore size 20 µm) and oven dried for 48 hours. All oven dried filters were weighed, placed in a muffle furnace for 5 hours at 550°C, and reweighed, allowing calculation of ash free dry mass (Jaarsma *et al.*, 1998).

Chlorophyll measures were used to quantify the availability of photosynthetically active algae in the epilithon. Upon thawing, filters for chlorophyll analysis were submerged in 15 ml of 90% ethanol and left overnight at 4°C (Nusch and Palme, 1975 *in* Hansson, 1988). The solution was then thoroughly mixed and passed through a coarse filter paper (pore size 20 µm) to reduce turbidity. Each sample was centrifuged at 4000 rpm for 10 minutes and the supernatant transferred to a vial for spectrophotometric analysis. The absorbance of each sample was recorded at 750 nm and 665 nm after calibrating the spectrophotometer with pure ethanol. Chlorophyll concentrations were calculated according to the equation of Marker *et al.* (1980) converted to be expressed per square centimetre of rock surface:

$$Chlorophyll\ \alpha\ (\mu g\ cm^{-2}) = s\ \frac{(11.99\ (A665\text{-}A750))\ x\ (V/a)}{R}$$

where $A665$ = absorbance at 665 nm, $A750$ = absorbance at 750 nm, s = ethanol volume (ml) and V = slurry volume (L), a = aliquot volume (ml) and R = rock surface area (cm^2).

Ratios of C:N, obtained from stable isotope analyses, were used as measures of resource nutritional quality. Low C:N ratios generally indicate higher nutritional value for consumers, as nitrogen-containing amino acids and proteins are normally limiting factors for organisms to grow (Bergström *et al.*, 2015). As expected, consumers in this study invariably had lower C:N ratios than their resources, suggesting resources with low C:N ratios provided a better stoichiometric balance, and hence better nutritional quality (Cross *et al.*, 2005).

5.3.5. Data analysis

5.3.5.1. Defining agricultural intensity

Agricultural intensity is a multi-faceted concept, influenced by many land management practices, such as stocking density and fertiliser applications, for which high resolution data are difficult to obtain. Therefore, this analysis used an index of in-stream physico-chemical conditions as a measure of agricultural intensity (hereafter 'intensity score'). The index was the first principal component of 44 variables describing nutrient concentrations, trace metals, bank poaching and sedimentation, and correlated with the proportion of the catchment with improved pasture land cover (see Section 4.3.4.1 for further details).

5.3.5.2. Basal resources and Functional feeding guild representation

Each invertebrate taxon was assigned an affinity to each FFG (grazer, shredder, gatherer, filter feeder, predator or parasite) based on their morpho-behavioural methods of food acquisition, using data from Schmidt-Kloiber and Hering (2012). The affinity scores summed to ten across all guilds and reflected the specificity of feeding behaviour and variation within a genus and over a taxon's lifespan. These affinities were standardised as proportions (i.e. summing to one across the six categories) and then at each site the affinities were multiplied by the taxon's relative abundance to give affinity-weighted abundances (Dolèdec *et al.*, 2006). These weighted abundances were then summed across all taxa present in a site for each guild to give relative FFG abundances (Chevenet *et al.*, 1994). The ratio of grazers to the sum of shredders, gatherers and filter feeders was calculated as an analogue for production:respiration ratios (Merrit and Cummins, 1996).

The relationships between intensity score and quality and availability of each basal resource, and between intensity score and FFG relative abundances, were modelled using generalised linear models (GLMs). Intensity was modelled using linear and quadratic terms, with appropriate error structures, and changes in the Akaike

Information Criterion (delta AIC) were used to assess whether the non-linear term was needed (Burnham and Anderson, 2004). The fit of all models was checked using residual plots, alongside semivariograms to ensure that there was no residual spatial autocorrelation (‘*gstat*’ and ‘*sp*’ package; Pebesma, 2004; Pebesma, 2005). A total of 13 models were fitted (three measures of quantity, four of quality and six FFGs against intensity score, Table 5.2) and so the level of statistical significance was adjusted to $\alpha = 0.042$ to control for the false discovery rate, following Benjamini and Yekutiueli (2005). Altitude was strongly negatively correlated with the intensity score (Pearson’s $r = 0.81$) and so was not included as a covariate in these models. To assess the relative influence of land use intensity and altitude on each response variable, commonality analysis was used to calculate their unique and joint contribution to the overall variance explained by a linear model for each response variable, using the ‘*yhat*’ package in *R* (Nimon, *et al.*, 2013).

Differences in the overall structure of FFG representation between sites was visualised using non-metric multidimensional scaling (NMDS) in two dimensions using Bray-Curtis similarities in the *R* ‘*vegan’* library (Bray and Curtis, 1957; Oksanen *et al.*, 2013). Vectors of abiotic variables were fitted to the ordination to show their relationships with FFG composition and the goodness-of-fit of these vectors was tested from 1000 permutations (Oksanen *et al.*, 2013).

Alternative hypotheses for the causal links between changes in physico-chemical conditions and agricultural intensification, basal resource characteristics and FFG representation were tested using confirmatory path analysis. This technique allows for the simultaneous assessment of multiple causal pathways, both direct and indirect, thus reducing errors of interpretation that can occur in regression analyses with highly correlated variables (Bizzi *et al.*, 2013). *A priori* models were created for the three most abundant feeding guilds (grazers, shredders and filter feeders) based on the hypotheses that the representation of each FFG reflected the quality and quantity of their food resource, which in turn were determined by canopy cover, nutrients and fine sediment concentrations (Figure 5.1). A negative relationship between each FFG and fine sediment was also hypothesised and undirected fixed paths were included between nitrate, sediment and canopy cover to account for their collinearity. High collinearity between exogenous variables, as observed here (> 0.6) is a common problem in path analysis and can result in significant Type 2 errors and coefficients with opposite signs to hypothesised relationships (Grewel *et al.*, 2004). Where relationships had the opposite sign to the hypothesised causal mechanism the hypothesised model was re-specified such that observed pathways were consistent with background information on the causal processes (Shipley, 2009).

Because of the relatively small sample size (n=28), Fisher's C statistic was used to assess goodness-of-fit of the hypothesised path model (Shipley, 2000a). This method is based on the concept of 'd-separation' (directed separation; Verma 1988, Geiger *et al.* 1990, Shipley 2000b) and assesses the conditional independence of the hypothesised model: if the hypothesised model is supported, the variables in the path diagram that are not directly causally connected should be independent, upon conditioning by another variable (Shipley 2000a). For example, in the model of grazer representation, periphyton quality and quantity are hypothesised to be independent, conditional upon nitrate. Pairs of unconnected variables in the hypothesised models and their conditioning variables were identified using the '*ggm*' library in *R* (Marchetti *et al.*, 2014) and their conditional independence tested using GLMs with appropriate error structures (Shipley, 2013). The outputs from all the independence claims associated with a model were combined to give the C-statistic, which was calculated following the equation:

$$C = -2\sum_{i=1}^{k}\ln(p_i)$$

where: p_i = the probability of each pair of variables being conditionally independent and k = number of independence tests. The C-statistic simultaneously tests all conditional independencies within the path diagram and follows a chi-square distribution with $2k$ degrees of freedom. Lack of significant difference ($p > 0.05$) between the observed and predicted pattern of independencies implies that the hypothesised causal pathways in the diagram are supported by the data (Shipley 2009). Path coefficients were calculated for each relationship in significant models using standardised data and appropriate error structures.

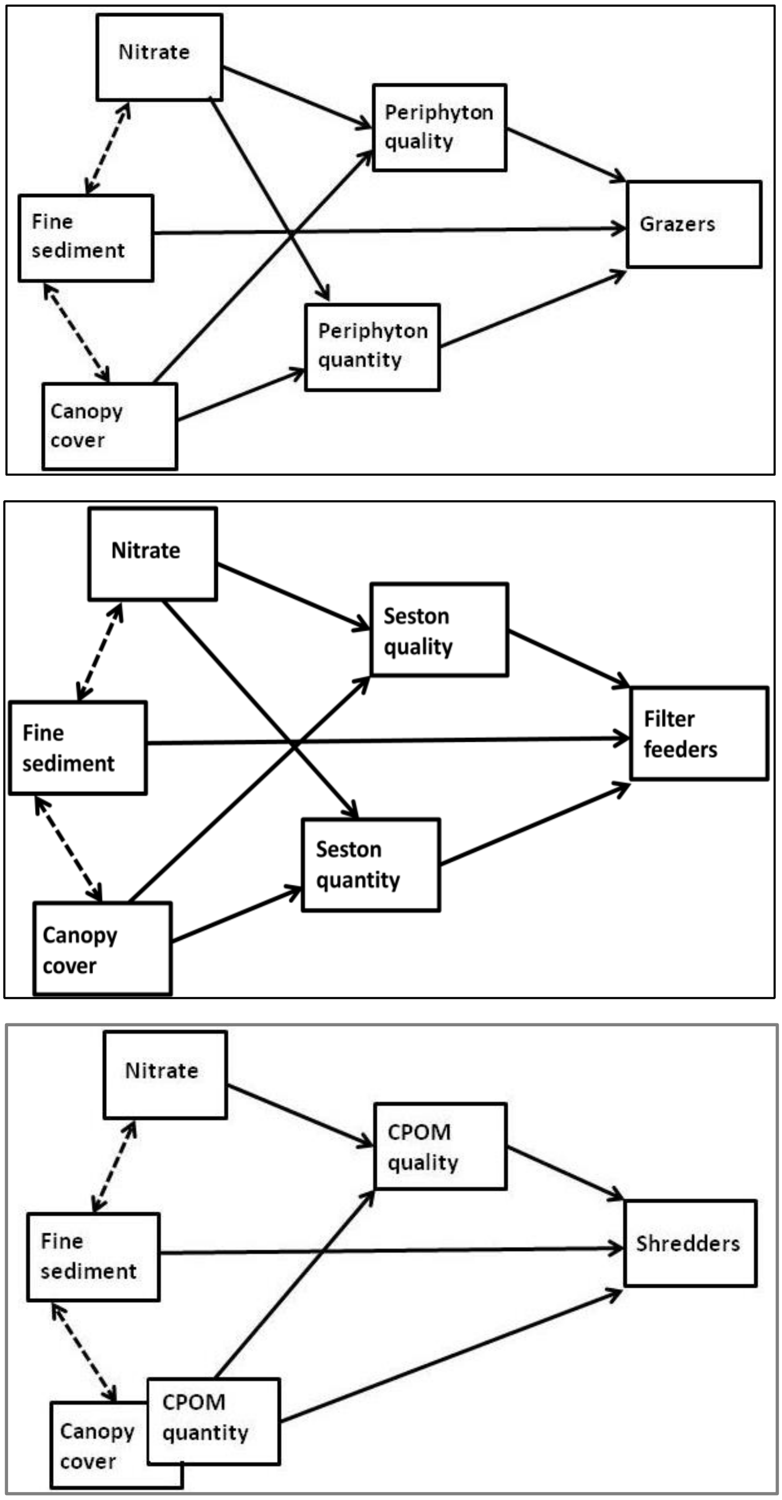

Figure 5.1 – Hypothesised path models of the proximal and distal controls on invertebrate functional feeding guild representation. Dashed lines shown the known correlations between measured exogenous variables. Solid lines connect variables that are predicted to be significantly related. It is hypothesised that the representation of each functional feeding is determined by the quality and quantity of its food source, which in turn is affected by nitrate concnetrations, sedimentation and canopy cover. A direct negative relationship between fined sediment and feeding guild representation is also predicted for each guild.

NB/ Canopy cover was used as a measure of Coarse Particulate Organic Matter (CPOM) quantity.

5.3.5.3. Seasonal patterns in functional feeding guild representation

Functional feeding guild compositions from all seasonal samples were plotted using NMDS, as described above. The area of the convex hull required to enclose the four seasonal samples from each site was determined as a measure of seasonal variability in FFG composition. Seasonal variability in the quality and quantity of CPOM, seston and epilithon were measured as the coefficient of variation (CV; standard deviation/mean) across the four seasonal samples. These measures of variation in FFG composition and the six basal resource variables were regressed onto the intensity score to test whether seasonal variation in basal resources and resource exploitation increased with land use intensity. Again, α was adjusted to account for the false discovery rate ($\alpha = 0.004$).

5.3.5.4. Stable isotopes analysis

Bayesian mixing models were used to estimate the contributions of benthic algae and terrestrial leaf litter to consumer diets in May 2012. As isotopic values of seston were invariably intermediate between terrestrial and in-stream production, being a combination of these materials, seston was not included as a food resource in mixing models. Consumer δ^{13}C values were corrected for lipid content because fractionation during lipids synthesis depletes C^{13} relative to other tissues, such that variation in lipid content between individuals may introduce bias in δ^{13}C estimates (DeNiro and Epstein, 1978; Post *et al.*, 2007). Lipid content is highly correlated to C:N ratio in invertebrates and can therefore be estimated using the equation (Post *et al.*, 2007):

$$\delta^{13}C_{normalised} = (\delta^{13}C_{untreated} - 3.32) + (0.99 \times C{:}N)$$

Lipid corrections were not considered necessary for basal resources as invertebrate consumers assimilate plant lipids. Further, Post *et al.* (2007) only recommend lipid corrections for plant sources with > 40% carbon, which applied to a minority of samples.

Because assimilated carbon and nitrogen are isotopically heavier than excreted carbon, consumers are isotopically enriched relative to their food sources (McCutchan *et al.*, 2003). This trophic enrichment was accounted for in mixing models using values calculated separately for each potential food source in each sampling period, according to the equations of Caut *et al.* (2009):

$$\Delta^{13}C = (-0.113(\delta^{13}C \text{ Food sources})) - 1.916$$
$$\Delta^{15}N = (-0.311(\delta^{15}N \text{ Food sources})) + 4.065$$

This resulted in estimated enrichment factors ranging from 1.4 to 2.7 for $\delta^{13}C$ and 3.2 to 4.1 for $\delta^{15}N$ (Table 5.1). These values are highly comparable to previously published general estimates (eg. Post, 2002; McCutchan *et al.*, 2003; Vander-Zanden and Rasmussen, 2001). For predator mixing models, which assessed the contribution of basal resources to assimilated predator tissue rather than the consumption of different prey species, trophic enrichment estimates and standard errors were doubled to account for fractionation between predators and prey species.

Table 5.1 – Trophic enrichment factors (mean ± standard error) used in stable isotope mixing models for primary consumers for each sampling period, calculated using equations from Caut et al. *(2009) for invertebrates.*

Sampling period	Trophic enrichment Factor $\delta^{13}C$		Trophic enrichment factor $\delta^{15}N$	
	Terrestrial litter	Algae	Terrestrial litter	Algae
May 2012	1.39 ± 0.17	1.82 ± 0.62	4.14 ± 0.72	3.61 ± 0.83
Feb 2013	2.10 ± 0.12	2.14 ± 0.82	3.91 ± 0.76	3.30 ± 1.40
June 2013	1.36 ± 0.11	2.19 ± 0.87	3.86 ± 0.73	3.56 ± 0.81
Sep 2013	2.10 ± 0.08	2.69 ± 0.83	3.98 ± 0.26	3.41 ± 1.16
Dec 2013	1.45 ± 0.16	1.83 ± 0.94	3.74 ± 0.63	3.23 ± 0.96

Mixing models to estimate the contributions of algae and terrestrial litter to consumer diets were run in the SIAR (Stable Isotope Analysis in R) package in R version 2.16 (Parnell and Jackson, 2015). For 2012 data, the *siarsolomcmcv4* function was used, based on 500,000 iterations with the first 50,000 discarded (Parnell *et al.*, 2010). Preliminary analysis revealed that a simple two-source model was not valid because in 14 of the 28 sites sampled in May 2012, Baetidae had a much more negative $\delta^{13}C$ signature than either of the measured food sources. In the majority of the remaining sites Baetidae had a greater reliance on the source with the most negative $\delta^{13}C$ signature, regardless of its identity.

In several sites, the $\delta^{13}C$ values of Baetidae (range -26 to -42‰) were more negative than is usual for consumers feeding on phototrophic sources (Kiyashko and Wada, 2001), falling below published estimates of potential food sources that were not sampled in the current study, including fine particulate organic matter, fungi, cyanobacteria and biofilm (eg. Finlay, 2001; Füreder *et al.*, 2003). The most plausible explanation is that Baetidae were ingesting carbon from chemotrophic sources. This is likely to be methane-oxidising bacteria (MOB), as has previously been demonstrated, even in mostly aerobic freshwater environments, because other chemotrophic bacteria, such as ammonia-oxidizing bacterium, do not have $\delta^{13}C$ as low as observed in this study (eg. Jones and Grey, 2004; Doi *et al.*, 2006; Deines *et al.*, 2007; Trimmer *et al.*, 2009).

Therefore, the relative contribution of MOB to consumer diets was assessed using two-source and three-source mixing models, assuming that MOB were present in all sites (Bunn and Boon, 1993; Jones and Grey, 2004). The three source mixing models used dual $\delta^{13}C$ and $\delta^{15}N$ values for CPOM, algae and MOB whereas the two-source mixing model only used $\delta^{13}C$ for MOB and the measured source with the most positive $\delta^{13}C$ value. Biogenic methane has $\delta^{13}C$ values ranging from -50‰ to -80‰ (Whiticar *et al.*, 1986) and MOB show a discrimination against ^{13}C of 16‰ (Summons *et al.*, 1994; Jones and Grey, 2004). Thus, both model structures were run with MOB $\delta^{13}C$ values of -66‰ and -96‰ to give the possible range of MOB contribution to consumer diets. It was assumed that, as primary autotrophs, MOB had the same $\delta^{15}N$ values as algae in each site (Cole *et al.*, 2011).

The relationship between pastoral intensity and contributions of MOB to both Baetidae and predator diets were explored using linear and quadratic generalised linear models (GLMs) as described above. Initial plots of estimated MOB contributions showed that the five sites on the Tawe and Neath rivers had much higher estimated MOB contribution than sites on the Wye and Usk with comparable levels of agricultural intensity. A term to separate the Wye/Usk and Tawe/Neath rivers was added into the model.

5.3.5.5. Seasonal variability in isotopic signatures

In five out of the ten sites that were sampled repeatedly in 2013, the positions of CPOM and algae on the $\delta^{13}C$ axis switched between seasons. Because the isotopic signatures of invertebrate tissue reflects assimilation over 1-3 months (Thomas and Crowther, 2015; based on body size of 1 – 10 mg at 10°C) this temporal variability in isotopic position of the basal resources prevents accurate estimation of their contributions to consumer diets (Fry, 2006). As in May 2012, Baetidae $\delta^{13}C$ values were frequently more negative than the sampled sources and often below -40‰. Therefore, the contribution of MOB to Baetidae diets were estimated as described above. Linear mixed effects models, with site as a random term, were used to determine whether the relationship between agricultural intensity and estimated contribution of MOB to Baetidae diets varied by season. These were performed using the 'nlme' library in R v.3.1 (Pinheiro *et al.*, 2015). The CV of MOB contribution was calculated as a measure of seasonal variability in each site and its relationship with agricultural intensity assessed using a linear model.

Gammaridae isotope values did not have more negative $\delta^{13}C$ values than the measured basal resources on any occasion, and in the majority of sites were closer to the source with the more positive $\delta^{13}C$ value (usually CPOM). There is therefore no reason to suspect that Gammaridae were consuming MOB but the high temporal variability and indistinctness of the CPOM and algal resources precluded the use of mixing models. To determine whether the isotopic niches of Baetidae and Gammaridae were significantly different, all individual consumers (from all sites/seasons) were plotted on a single $\delta^{13}C$-$\delta^{15}N$ biplot and the convex hull and standard ellipses areas for each of the two consumer taxa were calculated. Standard ellipses were fitted using Bayesian estimates with 100,000 repetitions (Jackson *et al.*, 2011) and the probability distributions of these estimates compared to determine whether the ellipse areas were significantly different.

5.4. Results

5.4.1. Basal resource quality and quantity and FFG representation

Biomass of organic epilithon was not significantly related to intensity score across the 28 streams (t = 0.234, *p* = 0.817, *d.f* = 27 Table 5.2) but chlorophyll had a significant non-linear relationship with pastoral intensity, initially increasing with intensity scores but declining in the highest intensity streams (Figure 5.2, t = 2.09 and -2.44, *p* = 0.039 and 0.022 for the linear and quadratic terms, $R^2 = 0.20$). The amount of organic seston increased monotonically with land-use intensity (t = 2.683, *p* = 0.013, $R^2 = 0.26$). Canopy cover increased with land use intensity but levelled off at 75% cover in sites with the highest intensity scores (quadratic term t = 2.31, *p* = 0.029, $R^2 = 0.72$; Figure 5.2).

The quality (as assessed from C:N ratio) of all three basal resources varied across the land use gradient (Figure 5.2). The quality of CPOM increased linearly with land-use intensity (t = - 4.82, *p* < 0.001, $R^2 = 0.49$), whilst both periphyton and seston showed evidence for quadratic relationships. The quality of periphyton was highest at intermediate intensity scores (quadratic term, t = 3.40, *p* = 0.002, $R^2 = 0.33$) whereas seston quality was lowest at intermediate scores (quadratic term; t = 2.19, *p* = 0.038, $R^2 = 0.39$) (Figure 5.2, Table 5.2). The quadratic relationships remained significant when the highest intensity site was removed.

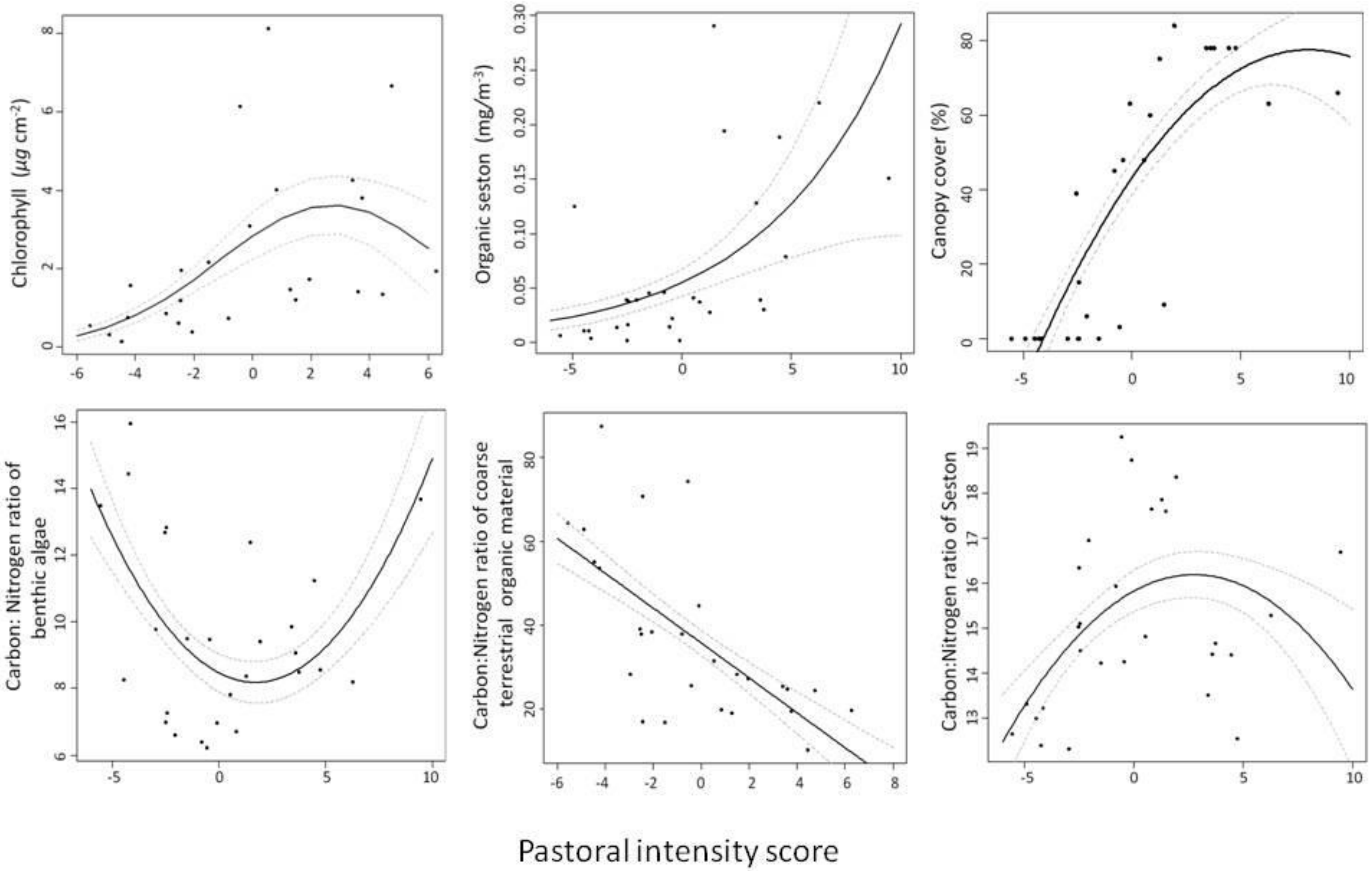

Figure 5.2- Models of the relationship between basal resource quality and quantity and pastoral intensity scores. Black lines show modelled predictions and dashed grey lines show standard errors.

All FFG measures changed significantly with pastoral intensity (Figure 5.3; Table 5.2). The representation of filter feeders and shredders increased across the land use gradient (t = 4.17, $p < 0.001$, $R^2 = 0.28$ and t = 2.134, $p = 0.042$, $R^2 = 0.11$, Figure 5.3), whilst grazer and collector-gatherer representation declined linearly (t = 5.50, $p < 0.0001$, $R^2 = 0.51$, Figure 5.3 and t= 2.41, $p = 0.023$, $R^2 = 0.14$). The magnitude of this decline was much greater for grazers than gatherers. Correspondingly, the ratio of grazers:detritivores declined with increasing intensity scores (t= -5.22, $p < 0.001$, $R^2 = 0.49$, Figure 5.3). Predator representation was apparently unrelated to pastoral intensity (Table 5.2).

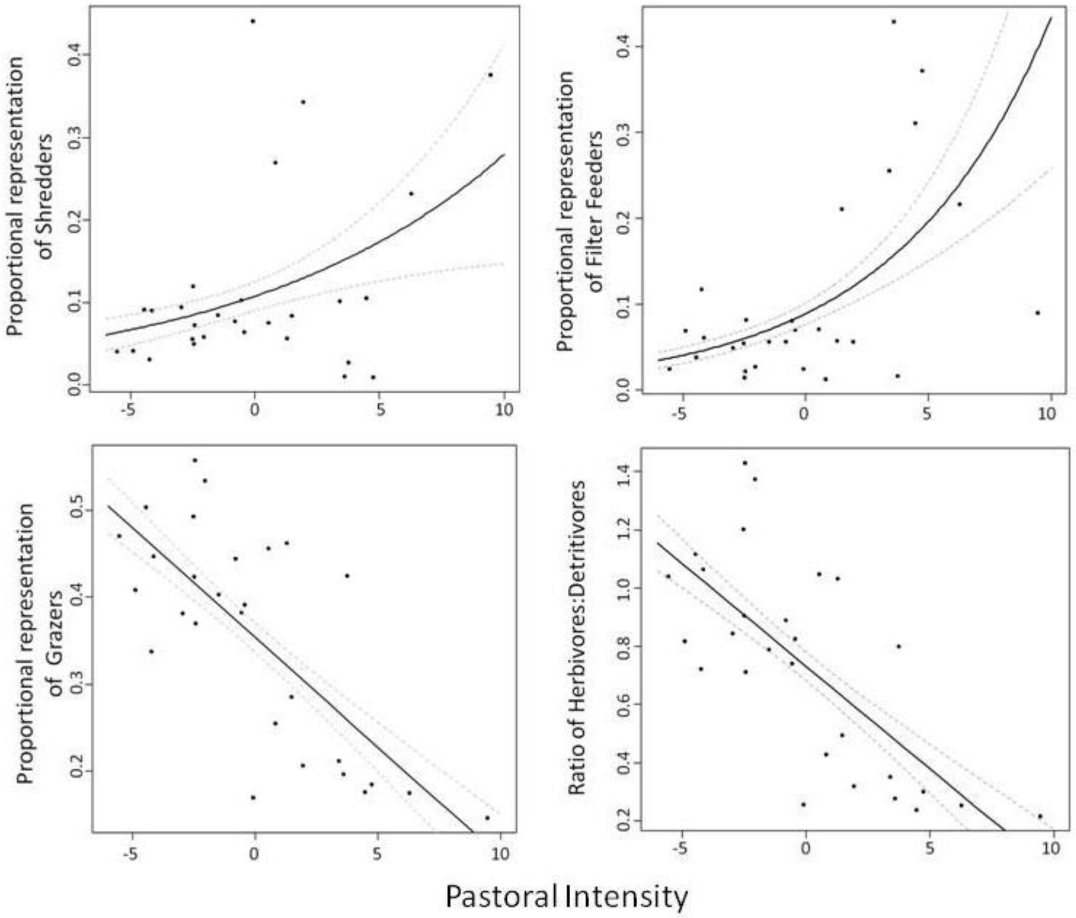

Figure 5.3- Models of the relationship between functional feeding guild representations and pastoral intensity scores. Black lines show modeled predictions and dashed grey lines show standard errors.

Table 5.2. - Comparisons of null, linear and quadratic models of the relationship between pastoral intensity score and measures of basal resource quality and quantity.

Response	Δ AIC null	Δ AIC linear	Δ AIC quadratic
Organic seston	8.56	0	2.00
Percent canopy cover	30.40	3.46	0
Chlorophyll	11.90	4.10	0
Organic epilithon	0	1.94	3.46
CPOM quality	13.92	0	0.08
Periphyton quality	7.00	8.63	0
Seston quality	3.38	2.92	0
Grazer representation	19.60	0	1.87
Filter feeder representation	11.97	0	1.99
Shredder representation	3.41	0	1.82
Gatherer representation	3.64	0	1.77
Predator representation	0	2.00	3.11
Production:Respiration	18.06	0	2.00

Commonality analysis showed that the joint effects of intensity score and altitude accounted for most of the explained variance in the majority of response variables (Figure 5.4). The unique contribution of intensity score was greater than that of altitude for all variables apart from canopy cover and CPOM quality.

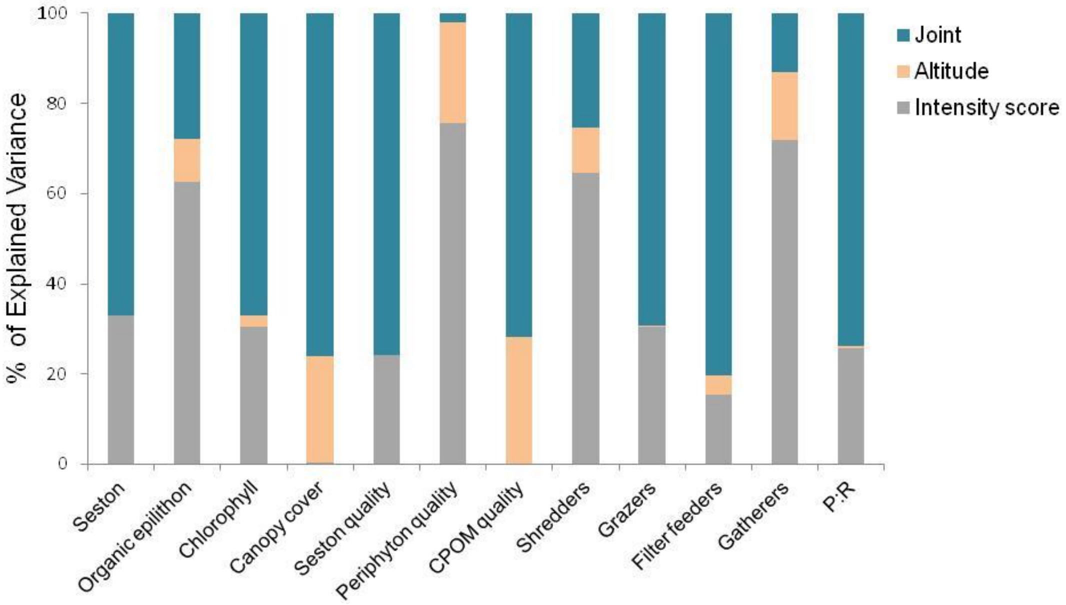

Figure 5.4 – Proportion of explained variance in linear response models accounted for uniquely by intensity score, uniquely by altitude and by their joint effects.

The NMDS had a stress score of 0.05 showing excellent agreement between the plot and the data. Axis 1 scores were highly correlated with intensity score (Pearson's r = 0.980, *p* < 0.001) and separated sites dominated by grazers and gatherers from sites dominated by filter feeders or shredders. Axis 2 separated filter feeders from shredders and was significantly correlated with seston quality: filter feeder representation increased with seston quality (Pearson's r = -0.959, *p* = 0.009; Figure 5.5).

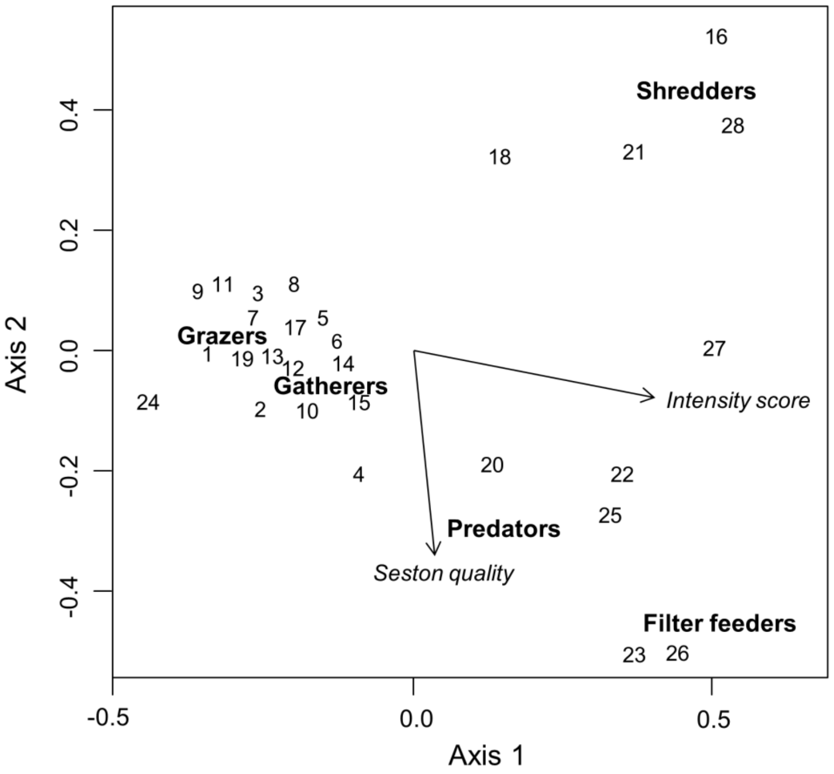

Figure 5.5 –Non-metric Multidimensional Scaling Analysis plot of functional feeding guild representation. Sites are numbered based on rank of pastoral intensity score (1 lowest, 28 highest). Arrows show the direction of significant correlations between vectors of abiotic variables and the ordination space.

5.4.2 Confirmatory path analysis

There were positive relationships between canopy cover and periphyton quality and quantity which did not reflect the hypothesised mechanism of light limitation (Hypothesis a). This suggests that the positive effect of nutrient enrichment, which was strongly collinear with canopy cover, overrides the expected negative effects of shading so this pathway was removed from the model of grazer representation and the coefficients recalculated (Grewel *et al.*, 2004). The remaining hypothesised causal relationships for grazer representation were consistent with observations (C = 4.94, *d.f.* = 8, *p* = 0.76). Grazer representation had a significant negative relationship with inorganic fine sediment and non-significant positive relationships with both periphyton quality and quantity. Nitrate was a significant predictor of periphyton quantity but not quality (Figure 5.6a).

The high collinearity between nitrate and sediment concentrations resulted in positive relationships between both shredder and filter feeder representation and fine sediments. This hypothesised negative relationship was removed from both path models and the coefficients recalculated. The hypothesised confirmatory path model for filter feeders was not consistent with observations (C = 15.512, *d.f.* = 8, *p* = 0.049) due to nitrate being significantly positively related to filter feeder representation, independent of seston quality and quantity (Figure 5.6b). Filter feeder representation was also positively related to both seston quality and quantity but only the relationship with quantity was significant (Figure 5.6b). Seston quantity significantly increased with nitrate concentration and canopy cover.

The hypothesised confirmatory path model for shredder representation was consistent with observations (C = 0.390, *d.f.* = 2, *p* = 0.823). Shredder representation was positively related to CPOM quantity (approximated by canopy cover) although this relationship was marginally insignificant. In turn, CPOM quantity had significant positive relationships with nitrate concentrations and canopy cover (Figure 5.6c).

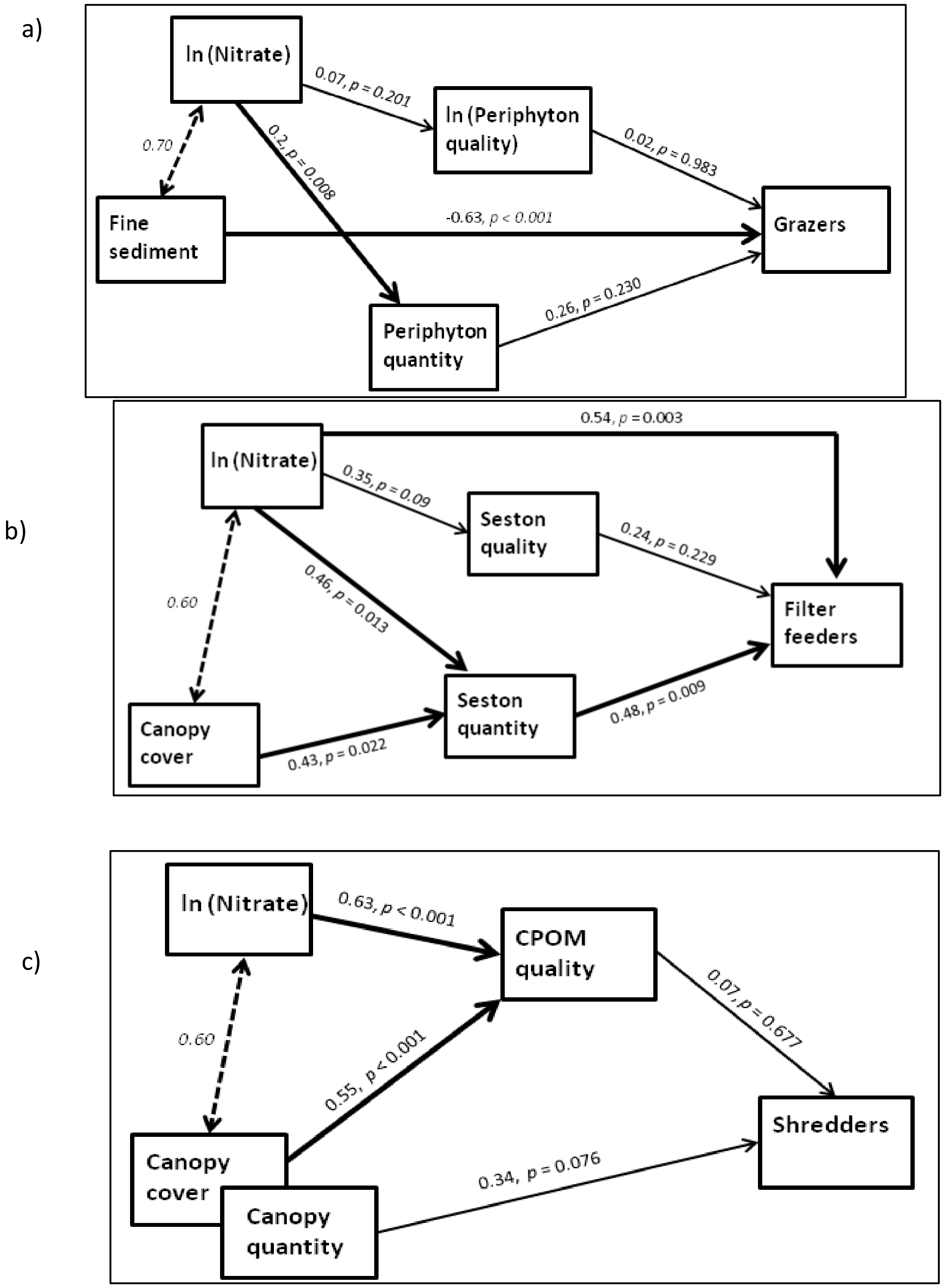

Figure 5.6 –Results of models of relationships between variables in hypothesised path models of the proximal and distal controls on invertebrate functional feeding guild representation for a) grazer representation, b) filter feeder representation and c) shredder representation. Numbers next to single headed arrows are standardised path coefficients and significance values. Thick lines show significant relationships (p < 0.05) and thin lines show non-significant relationships (p > 0.05). Numbers next to double headed dashed arrows are fixed correlation coefficients between measured exogenous variables.

5.4.3 Seasonal patterns in functional feeding guild representation

Convex hull area, a measure of the magnitude of seasonal variability in FFG representation, showed a quadratic relationship with intensity score although this relationship was not significant ($F_{2,7} = 2.99$, $p = 0.10$, $R^2 = 0.31$) and was driven by the low convex hull area of the highest intensity site, which was dominated by shredders/predators in all seasons. Removing this site resulted in a significant linear relationship between convex hull volume and intensity score ($t = 2.87$, $p = 0.02$, $R^2 = 0.39$), suggesting greater seasonal variability in FFGs in more intensive farmland. Low intensity sites had small convex hull areas and were dominated by grazer/gatherer communities in each season whereas sites with intermediate intensity scores showed greater variability in FFG composition across seasons (Figure 5.7). There was no evidence that variation in basal resource quality or quantity varied across the intensity gradient (Table 5.3).

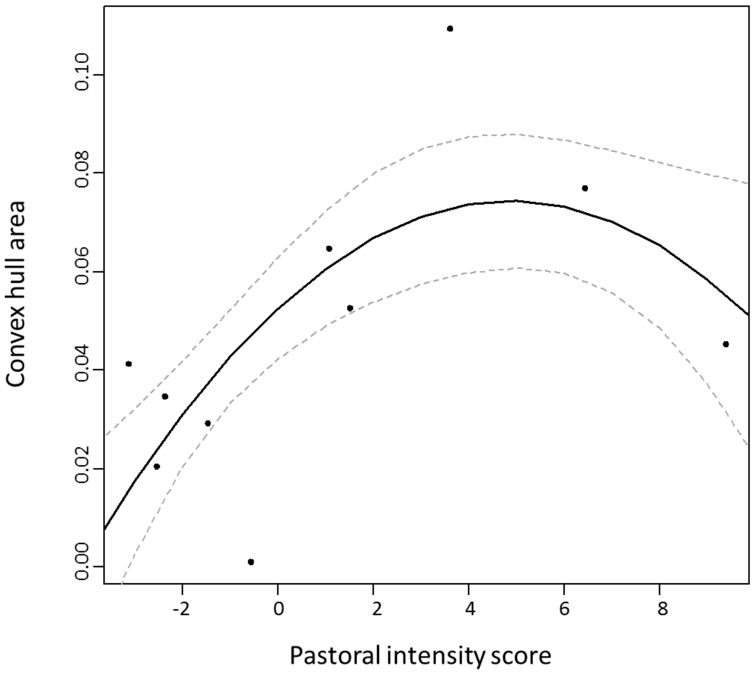

Figure 5.7 - Relationship between pastoral intensity scores and the area of a hull required to enclose four seasonal samples on a non-metric multidimensional scaling plot of functional feeding guild representation. Black lines show modelled predictions and dashed grey lines show standard errors.

Table 5.3 – Results of linear regressions between pastoral intensity and seasonal variability in basal resource quality and quantity (as measured by coefficient of variation across four seasonal samples).

Variable	Intensity score	
	Estimate	*p* Value
Periphyton quality	0.011	0.368
CPOM quality	-0.011	0.627
Seston quality	0.006	0.334
Organic seston	-0.019	0.337
Chlorophyll	0.029	0.166
Canopy cover	0.065	0.160

5.4.4. Stable isotope analysis

The estimated contributions of MOB to Baetidae diets were very similar for two-source and three-source mixing models (Pearson's r = 0.84, $p < 0.001$; Table 5.4). Models parameterised with MOB $\delta^{13}C$ values of -96‰ gave more conservative estimates than models using values of -66‰ but the correlation between these estimates was very high (Pearson's r > 0.98, $p < 0.001$; Table 5.4). Therefore, only results from 3-source models with MOB at -66‰ are presented. These models performed poorly at separating the contribution of CPOM and periphyton to consumer diets. No inferences are drawn from these estimates.

The estimated contribution of MOB to Baetidae diets increased significantly with intensity score (Figure 5.8, t = 3.79, $p < 0.001$, $R^2 = 0.38$). Sites on the Tawe and Neath had significantly higher MOB contributions than the sites on both the Usk and Wye (Figure 5.8; Tawe and Neath vs. Usk t = -3.63, $p = 0.004$ and Tawe and Neath vs. Wye, t = 4.14, $p = 0.002$), which were not significantly different from each other (t = 2.10, $p = 0.110$).

The average estimated contribution of MOB to predator diets was 8.9% (range 1.9 – 19.9%) and did not significantly differ between *Rhyacophila* and *Dinocras* predators (t = 0.635, $p = 0.532$). Estimated MOB contribution to predator diets showed the same significant differences between river systems as for Baetidae (t = 0.06, p = 0.049) but was not significantly related to pastoral intensity (t = -0.963, p = 0.346, R^2 = 0.04). There was a significant positive relationship between MOB contribution to Baetidae and to predator diets (t = 3.06, p = 0.006).

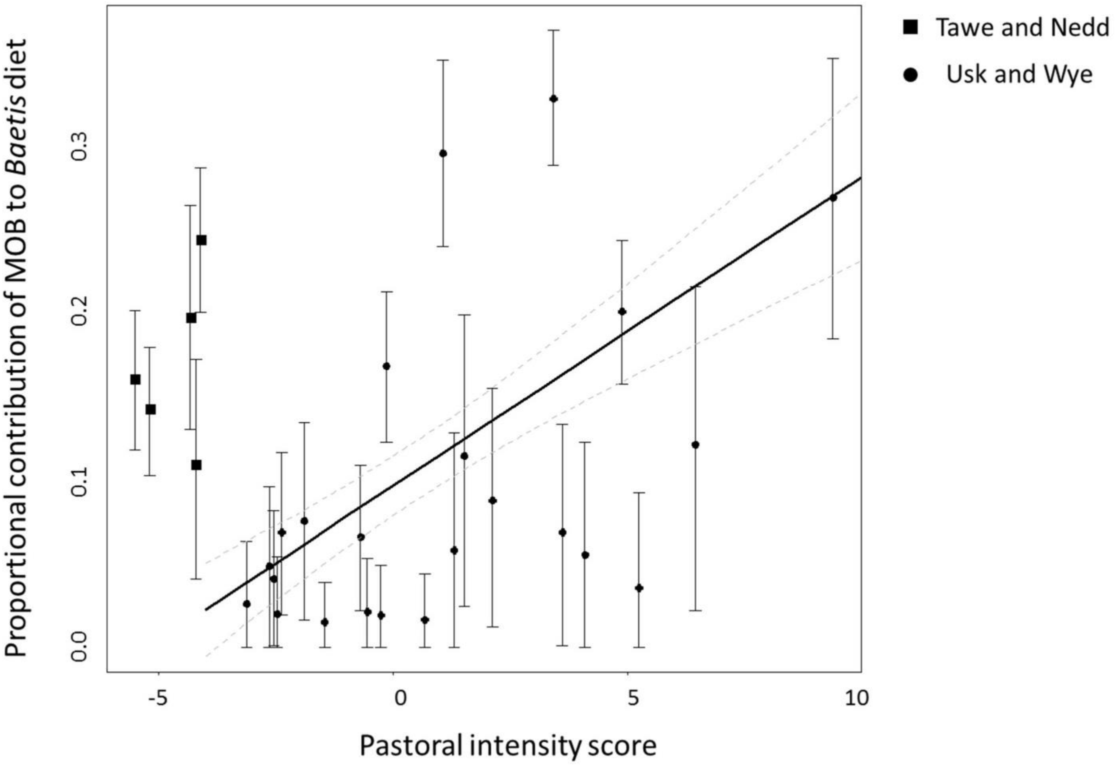

Figure 5.8– Relationship between an index of pastoral intensity and the proportional contribution of methane oxidising bacteria (MOB) to the diet of Baetidae consumers, as estimated from stable isotope mixing models. Error bars show 95% confidence interval of mixing model estimates. Black line shows the model prediction for the Wye and Usk rivers and grey dashed lines show model standard error.

Table 5.4 – Estimated contribution of methane oxidising bacteria (MOB) to the diet of Baetidae consumers from stable isotope mixing models using alternative δ13C signals for MOB and different combinations of potential sources.

Modelled sources	MOB $\delta^{13}C$ value (‰)	Mean MOB contribution (%)		Minimum MOB contribution (%)		Maximum MOB contribution (%)	
		Mean	95% CI	Mean	95% CI	Mean	95% CI
CPOM, Algae, MOB	- 66	10.9	5.9 – 16.1	1.5	0.0 – 4.0	33.2	29.2 – 37.4
	- 96	5.9	3.1 – 9.5	0.9	0.0 – 2.2	18.2	15.9 – 24.5
CPOM, MOB	- 66	14.4	10.3 – 8.3	0.5	0.0 – 1.5	35.2	30.8 – 39.6
	- 96	7.9	5.4 – 9.9	0.3	0.0 – 0.3	19.5	18.3 – 22.0

5.4.5. Seasonal variability in stable isotope signals

Mixed effects models showed no significant effect of season on overall MOB contribution to Baetidae tissues ($F_{3,24} = 2.20$, $p = 0.114$) nor a difference in the magnitude of the increase in MOB contribution with pastoral intensity score between seasons (intensity score $F_{1,8} = 14.54$, $p = 0.005$; intensity score:season $F_{3,24} = 1.12$, $p = 0.362$). The coefficient of variation of MOB contributions did not have a significant relationship with pastoral intensity ($t = 1.45$, $p = 0.19$).

The area of the convex hulls surrounding all consumer individuals on the $\delta^{13}C$- $\delta^{15}N$ biplot was 7.21 for Baetidae and 2.86 for Gammaridae. The Gammaridae convex hull fell almost entirely within the Baetidae convex hull, with the remaining Baetidae having more negative values than Gammaridae. The standard ellipse area was 2.33 for Baetidae consumers and 0.90 for Gammaridae, giving > 0.999 certainty that Baetidae isotopic niche was larger than that of Gammaridae (Figure 5.9).

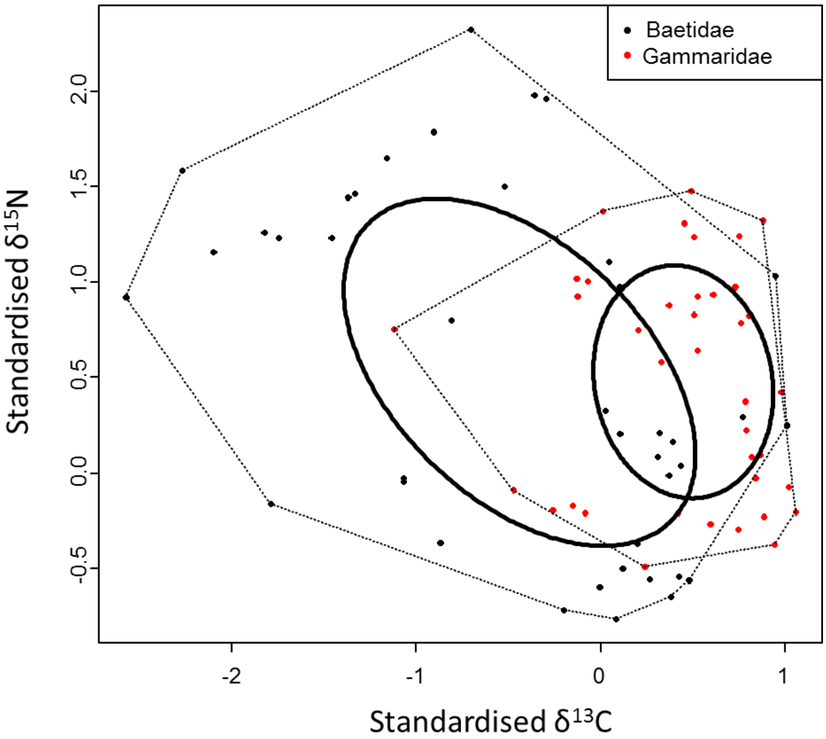

Figure 5.9 – Differences in the isotopic niches of Baetidae and Gammaridae consumers across four seasonal sample periods in ten sites differing in pastoral intensity. Black lines show the standard ellipse of each consumer and dotted lines show their convex hulls (Jackson et al., 2011).

5.5. Discussion

Sedimentation, nutrient enrichment and increased canopy cover associated with pastoral intensification at these sites appeared to alter the availability and quality of basal energetic resources, in turn changing the abundance of different invertebrate feeding guilds. With increasing agricultural intensity, the representation of detrital feeders increased, suggesting that macroinvertebrate communities became increasingly reliant on terrestrial energy resources. Further, stable isotope analysis indicated that methane-derived carbon contributed to the diet of generalist consumers, a finding which has not previously been observed in upland streams. The positive relationship between pastoral intensification and methane contribution suggests agricultural stressors significantly alter basal energy pathways with likely consequences for many emergent ecosystem properties, such as secondary production and nutrient processing rates (Wallace *et al.*, 1997).

5.5.1. Basal energy resources and FFG representation

Intensive upland pastoral farming frequently coincides with high riparian tree cover and this association is likely to strengthen as farmland streams are increasingly managed to reduce diffuse pollution and for climate change mitigation (Lee *et al.*, 2003; Thomas *et al.*, 2015). Although altitude was a potential confound in the relationship between tree cover and pastoral intensity, there was little evidence of altitudinal differences contributing to the observed changes in basal resource quality and FFG representation. As demonstrated here, increased tree cover will increase the availability of both CPOM and seston, and increase the nutritional quality of CPOM, where inputs of native grasses are replaced by Common Alder (*Alder glutinosa*) leaf litter (Hladyz *et al.*, 2011). Surprisingly, shredder representation did not respond to CPOM quality, but did hint at an increase with CPOM availability (p = 0.07). The lack of a direct measure of CPOM availability may have partly obscured this relationship.

Similarly, filter feeder representation increased with pastoral intensity, in response to an increase in resource availability but not quality. The non-linear relationship between agricultural intensity and seston quality suggests a change in the source of seston changes in high intensity sites, which may be from scenescent algae or faecal material

(Rosario *et al.*, 2002). Confirmatory path analysis showed nitrate to have a direct effect on filter feeders, which was not mediated by seston quality or quantity. This could indicate that nitrate was a better measure of overall food quality for filter feeders than the direct measure of seston quality used here, which is likely to have high spatio-temporal variability.

The observed increase in filter feeders with agricultural intensification is in agreement with previous studies, but the observed increase in shredders is contrary to studies conducted along an agricultural gradient with riparian clearance (Dolédec *et al.*, 2006; Braccia and Voshell, 2007; Townsend *et al.* 2008). Thus, the presence of riparian trees appears to modify the effects of agricultural land use on FFG representation. Interestingly, filter feeders and shredders did not co-dominate high intensity sites; sites were dominated by either filter feeders or shredders , and this was determined by seston quality. As seston is transported downstream, its quality is likely to be influenced by upstream land use, which may explain the high variability even between sites with seemingly similar levels of intensification.

Contrary to predictions, the quality and biomass of epilithic algae increased with initial pastoral intensification, presumably in response to mild nutrient enrichment (Harding *et al.* 1999; Benstead *et al.*, 2005; Greenwood and Rosemond, 2005). Although periphyton biomass and quality declined at high pastoral intensities, the lack of significant negative relationships between these measures and canopy cover, even within high intensity sites (results not shown) suggests that this was not a result of light limitation. This response may have been caused by excessive nutrient concentrations which can result in periphyton becoming dominated by low quality cyanobacteria, fungi and senescent cells (Braccia and Voshell, 2007). The range of nitrate concentrations encompassed in this study (0.1 to 25.9 mg L^{-1}) far exceeded the concentrations considered in previous studies that showed positive or neutral effects of nutrient addition on benthic algae in headwater streams (up to 0.1 mg L^{-1}; Dodds *et al.*, 2002; Greenwood and Rosemond, 2005). A full understanding of the effects of nutrients on algal resources will require further study over this wider range of concentrations.

Irrespective of changes in algal resource characteristics, the representation of grazing invertebrates declined across the intensity gradient due to negative effects of sedimentation. Fine sediment may have a disproportionate effect on grazers because they tend to be active crawlers/swimmers with delicate mouthparts and a lack of resistance traits. The loss of grazers and their associated traits in intensive pastoral streams will reduce functional diversity, potentially impairing ecosystem functioning (Tilman, 1997) and may be a useful 'early warning' indicator of agricultural effects.

In concert, the changes in FFG representation along the intensity gradient produced a linear decline in grazer:detritivore representation, giving support to the prediction that communities would become increasingly reliant on terrestrial sources. Because detrital material is of lower nutritional quality than algal material, these results indicate that pastoral intensification is likely to have implications for secondary production (Kominoski and Rosemond, 2012). Although predator abundance did not respond significantly to agricultural intensification in the present study, changes in predator biomass and the relative contribution of grazers and detritivores to predator diets warrants further exploration.

5.5.2. Stable isotope analysis

It was intended that stable isotope analysis would reveal whether generalist consumers altered their diet in response to changing characteristics of basal resources with pastoral intensification. Primary consumers were, however, assimilating carbon from unmeasured basal resources, preventing exploration of the initial hypotheses. This is a common problem in stream stable isotope studies (Dodds *et al.*, 2014) and is often a result of the differences in isotope signatures between the high quality algal fraction of periphyton that is preferentially selected by consumers and the bulk epilithon scrapings that are sampled (Dodds *et al.*, 2014). This was not the case in the present study because detrital and algal fractions of biofilm were separated by centrifuging (Hamilton *et al.*, 2005). Further, because this discrepancy occurred across sites and sampling periods, it cannot be attributed to high spatio-temporal variability in algal isotopic values (Dodds *et al.*, 2014). Baetidae $\delta^{13}C$ values were often lower than -40‰, which is unusual for freshwater food webs based on phototrophic sources (Kiyashko and Wada, 2001).

Therefore, it seems feasible that consumers were ingesting methane-derived carbon (Trimmer *et al.*, 2009), a phenonomon that is increasingly being recognised in freshwater systems (Stanley *et al.*, 2015).

Methane can enter stream systems from upwelling ground water or from local methanogenesis in anoxic conditions (Sanders *et al.*, 2007; Shelley, *et al.*, 2014). Even in primarily oxic habitats, reductive conditions can occur in sediment patches, in large amounts of leaf detritus or in the deep layers of thick biofilm, enabling methanogenesis (Grey *et al.*, 2004; Doi *et al.*, 2006). This methane diffuses upward into the water column or surface sediments, and is oxidised by MOB which occur at the anoxic/oxic interface (Bastviken, 2002; Jones and Grey, 2004; Trimmer *et al.*, 2009). There is increasing evidence of both detritivorous and grazing stream invertebrates consuming MOB (Bunn and Boon, 1993; Doi *et al.*, 2006; Deines *et al.*, 2007; Trimmer *et al.*, 2009). Kohzu *et al.* (2004) found *Helodes* beetle larvae to consume MOB in leaf litter packs in stagnant backwater pools and Trimmer *et al.* (2009) showed cased caddis larvae to obtain 11% of their carbon from grazing on MOB within epilithon.

Here, MOB were estimated to account for up to 33% of the carbon assimilated by Baetidae. As these estimates are from the least conservative of the acceptable models and assume presence of MOB in all sites they may overestimate the contribution of methane to Baetidae diets, particularly in sites with low contributions. Nevertheless, these results give strong indication that Baetidae assimilate carbon from methanotrophic biomass and the contribution of this to Baetidae tissues increases with agricultural intensity. It is likely that anoxic conditions, and associated MOB, are more common in agricultural streams due to the greater abundance of fine sediment deposits, leaf packs and thick biofilms (Doi *et al.*, 2006). This in-situ production of methane from localised anoxia can be viewed as an alternative source of autochthonous energy.

Intriguingly, some of the least intensive locations were also estimated to have relatively high contributions of MOB to Baetidae tissues. These were also the sites at the highest altitudes and were located on a different river system from the other sites. Without directly measuring methane concentrations, it is suggested that the poorly drained, anoxic moorland soils of these sites produce methane which enters the stream in throughflow and groundwater, and is oxidised by MOB (Jones and Mulholland, 1998).

This is the first study to suggest consumption of MOB by Baetidae. Trimmer *et al.* (2009) found Baetidae to have δ^{13}C values of -39‰ but attributed this to feeding on *Ranunculus*. There is, however, limited evidence that grazers consume macrophytes (Otto and Svensson, 1981) and macrophytes were very rare in the present study sites, suggesting that Trimmer *et al.*'s results may present further evidence of methane derived carbon in Baetidae tissues. Neither Trimmer *et al.* (2009) nor the present study found evidence of Gammaridae consuming methanotrophic food sources. Gammaridae had a smaller isotopic niche than Baetidae despite being more generalist consumers, although this may reflect the greater number of species within the *Baetis* genus than within *Gammarus*, with *Gammarus pulex* being the only species within these streams. More interestingly, the Gammaridae isotopic niche was more positive on the δ^{13}C axis than that of Baetidae. Because Gammaridae are predominantly reliant on detrital material whereas Baetidae diets contain a greater proportion of algal material, this result might indicate that the source of MOB is epilithon rather than in leaf packs. This speculation warrants further investigation.

Baetidae accounted for a large proportion of the invertebrate biomass in these streams and is known to feature prominently in the diet of many predators, including *D. cephalotes* and *Rhyacophila spp*. studied here. There was a positive relationship between estimated MOB contribution to Baetidae and predator tissues, with methane derived carbon accounting for up to 19% of predator carbon. Thus, methane-derived carbon could be an important energy source for the higher trophic levels in agricultural streams. Analysis of hydrogen stable isotopes across a range of consumers could reveal the extent to which methane-derived carbon fuels stream food webs (Deines *et al.*, 2009).

5.5.3. Seasonal variability

Methanotrophy has been shown to increase in prevalence during summer due to a reduction in algal growth under canopy shading (Trimmer *et al.*, 2009; Shelley, *et al.*,

2014) but in this study there was no evidence of differences in the contribution of MOB to Baetidae diets between seasons. In part, this may be due to the high temporal variability in periphyton $\delta^{13}C$ values, which reduced confidence in the mixing model results (Hadwen *et al.*, 2010), but may also reflect the lack of light limitation occurring in the study streams. In low intensity sites algal production was probably consistently nutrient limited and, in high intensity sites, the negative effects of sediment and excessive nutrients probably outweighed any effects of seasonal changes in light availability. This is supported by the lack of a significant change in the magnitude of seasonal variability in periphyton quality and quantity along the gradient of agricultural intensity and canopy cover.

There was, however, indication of differences in the magnitude of seasonal variability in FFG representation along the intensity gradient. Sites with medium pastoral intensity had the greatest seasonal variability in FFG representation as low intensity sites were consistently dominated by grazers/gatherers and the highest intensity site was consistently dominated by shredders or filter feeders as grazers were precluded by high sediment loads. These results suggest that low and high intensity sites may operate in alternative stable states whereas medium intensity sites show high temporal variability.

5.5.4. Conclusions

With increasing agricultural intensity and riparian cover macroinvertebrate communities changed from being grazer dominated to being dominated by detrital feeders, with little evidence of temporal variability in the magnitude of this effect. This result was largely attributable to fine sediment inputs negatively affecting grazers, highlighting reduction of sediment inputs to agricultural streams as a priority for land managers aiming to maintain stream ecosystem functioning (Townsend, 2008; Matthaei *et al.*, 2002).

In addition to changes in FFG, there was evidence for methanotrophy becoming an important third energy pathway in agricultural streams. In concert, these changes to the pathways of energy transfers in stream systems are likely to have consequences for wider ecosystem properties including secondary production and nutrient processing rates. Thus, FFG ratios and measures of methanotrophic inputs could be usefully employed as indicators of agricultural effects on ecosystem functioning.

5.6. Supporting Information

Appendix 5.1: Association between bankside tree cover and agricultural intensity

Data from the Baseline 2 (2008-9) River Habitat Survey were used to determine how the prevalence of riparian trees changes with pastoral intensity in the UK (see Environment Agency, 2003 for full details). Tree cover on the banks of the channel is recorded on a six-point ordinal scale in RHS: none, isolated/scattered, regularly-spaced single, occasional clumps, semi-continuous and continuous. For simplicity these were merged to give none/isolated, occasional clumps, semi-continuous or continuous cover. Using only sites in the altitudinal range covered in this study (150 - 420 m), the frequency of the different tree cover categories was compared between sites with 'extensive' improved pasture and 'extensive' rough pasture within 50 m of the channel, where 'extensive' is defined as ≥33% of the reach length (Environment Agency, 2003). Any sites that also had the other category of agricultural land use or extensive woodland within 50 m of the channel were excluded.

There were clear differences in the frequency of bankside tree cover categories between improved pasture (n = 28) and rough pasture (n = 156) land uses. Over half of sites in rough pasture land use (59 %) had isolated or no bankside trees, compared to 22% of sites in improved pasture (Figure S1). As in the present study, riparian trees usually occurred as single lines rather than riparian woodlands, with only 33% of sites with bankside trees, in either land use category, having woodland within 5 m of the channel.

Figure S1 – Differences between occurrences of bankside tree categories in upland River Habitat Survey sites with improved pasture and rough grazing land use.

6. The effects of pastoral intensification on the feeding interactions of two generalist stream predators

6.1. Summary

Anthropogenic effects on interactions between individual organisms, especially feeding links and competition, can produce wide-ranging consequences for ecosystem functioning and stability. Despite this, study of changes in feeding interactions along anthropogenic stress gradientshas been limited to acidity and temperature gradients.. Here, Next Generation Sequencing (NGS) is used to quantify the changing diet and feeding behaviour of two predatory stream invertebrates (*Rhyacophila dorsalis,* caddisfly and *Dinocras cephalotes* stonefly) along a gradient of agricultural intensification, across four seasons. This is the first study to use NGS to determine diets of aquatic invertebrate consumers.

Ten streams in South Wales were selected along a gradient of management intensity for livestock production, ranging from unfertilised pastures with native grasses and low stocking densities, to fertilised, re-seeded pastures with high stock densities. Predator gut contents were dissected, amplified using primers LCO-1490 and HCO-177 without blocking probes, and sequenced using Ion Torrent technology.

Ryacophila dorsalis was abundant in all streams whereas *D. cephalotes* was absent from the most intensive sites. *Ryacophila dorsalis* and *D. cephalotes* were shown to be generalist predators preferentially consuming the most abundant prey taxa. Dietary comparison showed the two predator species had preferences for similar prey species with no evidence that the absence of *D. cephalotes* from the most intensively managed catchments reflected a more specialised diet or competition with *R. dorsalis*. Instead, its absence could be explained by physico-chemical stressors associated with agriculture.

The diet and prey preferences of *R. dorsalis* did not respond significantly to agricultural intensification, despite changes in the composition of potential prey taxa. The strongest links in *R. dorsalis* diet were consistent across the food web, reflecting the resilience of

preferred prey taxa. Thus top-down effects of *R. dorsalis* feeding are likely to be stable across the intensity gradient. There was, however, a suggestion of food web simplification at the highest agricultural intensities with the loss of *D. cephalotes* and a decrease in the contribution of rarer taxa to *R. dorsalis* diet. There was no significant effect of season on the observed trends.

This study demonstrates the potential of NGS to reveal freshwater food webs in unprecedented detail, providing new insights into the structure and function of stream communities subject to anthropogenic stressors.

6.2. Introduction

Globally, anthropogenic activities are altering biodiversity and species composition at an unprecedented rate (Sala *et al.*, 2000). There is substantial evidence linking these changes in community composition to altered ecosystem stability and functioning rates (Tilman *et al.*, 2014), but predicting exactly how these emergent ecosystem properties will be affected is difficult because of the complexity of inter-specific interactions within communities (Kremen, 2005; Layer *et al.* 2010). The complex associations between individuals, including feeding links, competition, intraguild predation and mutualism, mean that perturbations affecting one part of the community can produce unexpected changes at the ecosystem scale (Holling, 1973; Pimm, 1984; McCann, 2000). Predicting and mitigating anthropogenic effects on ecosystems requires an improved understanding of these interactions and their responses to stressors.

Trophic links are the most commonly described ecological interactions and, by governing the transfer of energy and nutrients, are fundamental to many ecosystem functions (Memmott *et al.*, 2005; Carreon-Martinez and Heath, 2010). Predator-prey dynamics account for the majority of feeding interactions (Carreon-Martinez and Heath, 2010) and, as such, changes to predator abundance or feeding behaviour can result in major restructuring of the food web, with wide-ranging direct and indirect consequences for ecosystem processes and stability (Cohen *et al.*, 1993; McCann, 2000). A reduction in predators is a common result of anthropogenic stressors, as their large body size, low population density and slow reproductive rates can make them particularly vulnerable to perturbations (Purvis *et al.*, 2000). Rather than just a simple alleviation of top-down control, reduction in predator populations can have complex effects on community structure due to high interconnectivity, intraguild predation and competition between predators (Finke and Denno, 2005; Petchey *et al.,* 2004). Concomitantly, perturbation may alter the feeding behaviour and prey choice of generalist consumers by changing prey abundance, the availability of refugia for prey and the competitive abilities of predators (Symondson, 2002; Evans, 2004). Commonly, generalist predators preferentially forage on the most abundant resource and so a switch in prey identity may be expected along stress gradients (Gentleman *et al.*, 2003). Identifying where predators change their foraging behaviour in response to increasing stress could reveal thresholds at which ecosystem functioning may be disrupted (Woodward, 2009). Thus, consideration of predator-prey and predator-predator interactions, alongside prey choice, is essential when assessing the effects of stressors on communities (Woodward, 2009; Gray *et al.*, 2014).

Stream ecosystems are especially vulnerable to anthropogenic disturbance, being affected by all activities within their catchments. Intensification of catchment land use is the most widespread driver of biodiversity loss in aquatic systems (Dudgeon *et al.*, 2006). Much of the current understanding of land use impacts has come from studies describing the benthic macroinvertebrate community, which encompasses taxa with a broad range of sensitivity to stressors, has key roles in a wide range of ecosystem processes and tends to dominate the food web in terms of individual abundance and number of interactions (Covich *et al.*, 1999). Despite changes in community structure, including predator populations, being widely reported as a consequence of land use intensification (eg. Harding *et al.*, 1999; Yuan and Norton, 2003), the associated losses of and modifications to trophic interactions in stream food webs have received little attention (Grey *et al.*, 2014). Several experimental studies have confirmed that changing predator densities produces complex effects on stream ecosystems (eg. Soluk, 1993; Woodward *et al.*, 2008; Rodríguez-Lozano *et al.*, 2015) but studies of changes in predator populations and trophic interactions across stress gradients have been limited to acidity (Layer *et al.* 2010) and temperature (O'Gorman *et al.*, 2012).

Stream food web studies have been limited by the difficulties of identifying trophic interactions in systems where predator-prey interactions cannot be observed directly. Studies typically rely on visual identification of invertebrate gut contents, which is very time consuming, may be biased by such factors as differences between observers and prey size or digestibility, and is often unable to identify remains to species level (Woodward and Hildrew, 2002). Recent advances in molecular ecology have made rapid and accurate determination of predator diets possible and offer great potential for assessing anthropogenic effects on food web structure (Symondson, 2002; Clare *et al.*, 2014). Currently the cost of this technology prohibits exploration of entire food-webs, but valuable insights can be gained by studying the feeding behaviours of dominant predators. This approach has proved very successful with a range of vertebrate predators (eg. Vesterinen *et al.*, 2013) and, recently, with terrestrial invertebrates (eg. Lundgren and Fergen, 2014).

The goal of this study was to use NGS to quantify the diet and prey selectivity of two invertebrate generalist predators (*Rhyacophila dorsalis,* Caddisfly and *Dinocras cephalotes* stonefly), and assess how these properties changed along a gradient of agricultural intensification and over four seasons. This is the first study to use NGS to determine diets of aquatic macroinvertebrates. Previous work across the study locations revealed a change in the potential food sources with increasing agricultural intensity: reduced prey species richness and a shift from communities dominated by Ephemoptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies) and Elmidae (riffle beetles) to a predominance of dipteran larvae, molluscs and oligochaetes (Chapter 4). The two study predators showed a contrasting response to intensification: *D. cephalotes* was only present where agricultural intensity was low, whereas *R. dorsalis* did not change in abundance across the agricultural gradient, suggesting greater resistance to the effects of intensification (Chapter 4). The main aims in this study were to identify how pastoral intensification affected the diet breadth and prey taxa consumed by *D. cephalotes* and *R. dorsalis*, the potential interactions between them and how trophic interactions varied among seasons. It was predicted that: i) being generalists, both predators would consume a wide range of prey taxa in proportion to their availability, resulting in changes to *R. dorsalis* diet along the agricultural intensity gradient that reflect the changes in potential prey species (Chapter 4); ii) seasonal variability in the structure of feeding interactions would increase with agricultural intensity due to lack of compensatory feeding paths in depauperate, stressed communities, iii) the lower resilience of *D. cephalotes* to agricultural stressors compared to *R. dorsalis* was reflected in narrower diet breadth and less flexible feeding behaviour, and iv) the absence of *D. cephalotes* at the highest intensities would result in an wider feeding niche and number of feeding interactions by *R. dorsalis*, due to an alleviation of competition

6.3. Methods

6.3.1. Sample collection and preparation

Ten study sites on 2nd and 3rd order upland streams in South Wales were selected to span a range of pastoral land use intensities. These were the same ten sites used for seasonal analysis in Chapter 5 and a subset of the 29 sites sampled across this gradient in 2012 (see Chapter 4). Pastoral agriculture was the dominant land cover in all catchments (> 75 %) but catchments differed in their proportions of unimproved pasture (unfertilised native grass species supporting low densities of livestock; 0 – 100 %) and improved pasture (fertilised and reseeded with high stocking densities; 0 – 86%; Jackson, 2000).

Agricultural intensity is a multi-faceted concept, influenced by factors including stocking density and fertiliser applications for which high resolution data are difficult to obtain. Therefore, an index of in-stream physico-chemical conditions was used as a surrogate for agricultural intensity. The index (hereafter ‘intensity score’), was derived from a principal component analysis on habitat variables recorded at each site, which included water chemistry, channel morphology, bankside vegetation, erosion extent, flow velocity and sedimentation (see Section 4.3.4.1 for full details of the intensity score). Larger intensity scores equated to higher nutrient concentrations, greater poaching of the banks and fine sediment cover of the stream bed. In addition to the intensity score, nitrate concentrations and the abundance of fine sediment in the channel were used in the current study to link directly to predator abundance. Nitrate concentrations were determined in the laboratory using ion chromatography (Dionex DX-80 ion analyser; Thermo Fisher Scientific). Fine sediment was measured in three random locations at each site by pushing an open drum (25 cm diameter, 0.0625 m^2) into the substrate, disturbing the sediment to 2 cm depth for 15 seconds and capturing a 300 ml sample of the re-suspended fine sediment. The three replicate samples were combined, filtered through a filter paper with 0.025 μm pore size and ash-free dry mass determined to get a weight of inorganic sediment.

Rhyacophila dorsalis and *Dinocras cephalotes* were selected as the target predators for this study based on the results of preliminary sampling conducted in May 2012 (Chapter 4). Both species are large, active, generalist predators. *Ryacophila dorsalis* was abundant across all sites whereas *D. cephalotes* was the most abundant predator in the six sites with the lowest agricultural intensity, but was absent from the most intensive sites. Sampling was conducted in February, June, September and December 2013 to capture seasonal variation in abiotic conditions and prey populations. On each sampling occasion, three one-minute kick samples were conducted, using a 1 mm mesh size D-frame net, covering all microhabitats in proportion to their abundance, and samples were preserved in 70% ethanol. Further kick samples were then performed to obtain *R. dorsalis* and *D. cephalotes* for molecular analysis. The first ten individuals of each species, or as many as were found in one hour searching time, were immediately preserved in 100% ethanol in individual centrifuge tubes, giving a total of 497 individuals across all sampling periods.

In the laboratory, kick samples were rinsed through a 500 μm sieve and macroinvertebrates were removed from the sample, identified to genus, or a lower taxonomic resolution where this was not practicable, and counted (See Table S4.1, Taxonomic list). The foregut of each predator was dissected into a sterile Eppendorf, excluding as much of the predator's own tissue as possible.

6.3.2. DNA extraction

DNA was extracted from the dissected gut contents using the Quiagen blood and tissue kit according to the manufacturer's instructions for animal tissue. Additionally, DNA was extracted from the legs of a wide range of potential prey and both predator species using the less costly 'Salting out' method (Miller *et al.*, 1988) (Table 6.1). Negative controls were included alongside each batch of extractions to monitor for contamination (King *et al.*, 2008). Extracted DNA was stored at −20°C prior to amplifications.

6.3.3. Primer selection

A single pair of general invertebrate primers was selected for amplification of predator gut contents; LCO-1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) (Folmer *et al.* 1994) and HCO-1777 (5’-ACTTATATTGTTTATACGAGGGAA-3’) (Brown *et al.*, 2012; King *et al.*, 2015), which target a 287 bp fragment of invertebrate CO1 genes. Blocking probes were not used as the phylogenetic proximity of predator and prey made it likely that a blocking probe would prevent amplification of many prey species (Piñol *et al.*, 2015). These primers were tested for their ability to amplify DNA from 18 invertebrate taxa (Table 6.1). Temperature gradient Polymerase Chain Reactions (PCRs) were performed to determine the optimal annealing temperature at which most taxa would amplify. PCRs were run on a Peltier Thermal Cycler in 25 µl reaction volumes with conditions as follows: 1 X buffer, 4 mM $MgCl_2$, 0.05 mM dNTPs (Promega), 0.1 mM of each primer, 0.625 U Taq polymerase (Promega) and 2.5 µl of template DNA with an initial denaturation at 95°C for 2 min, 35 cycles of 30 secs at 94°C, 30 secs at 46°C, 45 secs at 72°C and a final extension of 10 minutes at 72 °C. Amplification success was visualized by gel electrophoresis. This primer pair was found to amplify all 18 of the tested taxa and was therefore used in further analysis.

Table 6.1 – Genera used to test generality of LCO-1490 and HCO-1777 primer pair for potential prey species.

Family/Genera tested	**Group represented**
Dinocras *Rhyacophila*	Target Predators
Hydropsyche *Potamophylax*	Trichoptera
Isoperla	Plecoptera
Baetis	Ephemoptera
Ancylus Physidae	Gastropod
Pisidium	Bivalvia
Asellus	Amphipods
Gammarus	Isopods
Chironomidae Limoniidae	Diptera
Planaria	Tricladia
Limnius	Elmidae
Eropobdella	Hirudinaeta
Lumbricidae	Oligochaetes
Scritidae	Aquatic Coleoptera

6.3.4. Ion torrent sequencing

Predator gut content DNA samples were prepared for Ion Torrent sequencing following recommendations for unidirectional sequencing (Ion Amplicon Library Preparation, Fusion Method). Samples were processed and sequenced in two batches, samples collected in June and December (n = 218) in May- July 2014 and samples collected in February and September (n = 176) in March-May 2015 (Table 6.2). Three individuals were included in both sequencing runs to determine whether there were differences in sequencing outputs between the two sequencing runs.

Table 6.2 – Number of individual predator gut contents successfully sequenced from each survey site with sites labelled 1 to 10 from lowest agricultural intensity to highest agricultural intensity.

	Dinocras cephalotes				*Rhyacophila dorsalis.*				Total
Site	Feb	June	Sep	Dec	Feb	June	Sep	Dec	
1	8	8	8	10	3	10	2	6	55
2	2	4	6	5	4	2	3	9	35
3	6	8	13	10	3	4	0	3	47
4	9	6	7	10	7	2	1	8	50
5	0	0	5	8	14	4	12	8	51
6	4	6	6	8	6	10	0	9	25
7	0	0	0	0	7	8	10	1	50
8	0	0	0	0	11	9	7	11	38
9	0	0	0	0	6	8	2	9	25
10	0	0	0	0	2	7	2	7	18
Total	29	32	45	51	63	64	39	71	394

Sixteen forward primers were designed, each consisting of ion torrent primer A, LCO-1490 primer and a unique ten base pair multiplex identifier sequence (MID). Fifteen reverse primers, each with the ion torrent primer B linked to the HCO-1777 primer and a unique MID were also designed. This gave 240 unique combinations of forward and reverse primer pairs, allowing each individual to be identified from the pooled data from each of two sequencing plates.

The DNA from predator gut extracts was amplified in 20 µl reactions containing 2 µl of template DNA, 10 µl of Quiagen multiplex master mix, 6 µl of water and 1 µl of the specific forward and reverse primers (at 10 µM). The PCR was run for 35 cycles of 94°C for 30 s, 46°C for 30 s and 72°C for 45 s following an initial denaturation step at 95°C for 15 min and before a final extension step at 72°C for 10 min. Amplification success was determined by running 2 µl of each PCR product on a 2% aragose gel stained with EtBr. The intensity of each band, as visualised on UVP VisionWorks® LS Analysis Software was compared with the intensity of the 500 bp ladder band. The ladder was used as a standard, corresponding to 1 µl in final pool, allowing all amplicons to be pooled into an equimolar library according to their intensity relative to the ladder. A gel extraction was performed on each pool to remove 'primer dimer' that could reduce the efficiency of ion torrent sequencing. Because of the high concentration of DNA in the pooled sample for the first round of sequencing (June and December), the sample was diluted 1:5 with purified water before running 20 µl in each of four lanes on a 1.5% aragose gel. In the second sequencing batch (February and September samples), 20 µl of the undiluted pooled sample was run in three gel lanes. The specific bands were dissected from the gel and processed using the QIAquick Gel Extraction kit (Qiagen) with a final elution volume of 40 µl. High-throughput sequencing was conducted on an Ion Personal Genome Machine (IPM) using 400 bp chemistry at the Centre de Recerca en Agrigenòmica, Barcelona. In an attempt to account for the different number of individuals in the two sequencing runs and standardise the number of sequences per individual, a 318 chip (> 3 million reads) was used for the first sequencing round and a 316 chip (> 1.5 million reads) for the second.

6.3.5. Sequence Analysis

Sequence processing was performed in Galaxy (usegalaxy.org, Giardine *et al.* 2005; Blankenberg *et al.*, 2010; Goecks *et al.*, 2010). Sequences were split by forward and reverse MIDs and adaptors, primers and MIDs were removed before filtering sequences by length (260-300 bp). Sequences from each individual were collapsed into unique haplotypes, and rare haplotypes (< 2 copies) were excluded. The remaining sequences from all individuals were combined and clustered into molecular operational taxonomic units (MOTU) using the *usearch* algorithm in Qiime (usearch61; Edgar, 2010). MOTU clustering was repeated with similarity thresholds decreasing in increments of 0.01 from 0.97 to 0.87. For each similarity value, representative sequences were selected from the resultant MOTUs and 'BLASTed' directly at the NCBI website (http://blast.ncbi.nlm.nih.gov/blast) using nucleotide BLAST (Zhang *et al.*, 2000) optimised for very similar sequences (megablast) on the nucleotide collection (nr/nt) using default parameters. The output from the BLAST alignment was imported into MEGAN (MEtaGenomics ANalyzer; Huson *et al.*, 2007), which assigns taxonomy to each MOTU at the lowest level that encompasses the top BLAST hits (Figure 6.1). The optimal similarity threshold was the value that resulted in the lowest number of species with multiple MOTUs allocated to them, whilst retaining the majority of species assignments.

6.3.6. Assigning Taxonomy

The representative sequences from the optimal MOTU clustering were compared to the BOLD database (www.barcodinglife.org). Sequences were initially queried against the 'species level barcode records' database (barcodes with species-level identification). If a match was not found, then the sequence was queried against the 'all barcode records' database. This search returns a list of the nearest matches to the query sequence and includes barcodes that do not have species-level identification. A sequence was assigned at the highest taxonomic resolution to which it had a > 98% similarity (Clare *et al*., 2011; King *et al.*, 2015). MOTUs producing no match (with > 98% similarity) or matching to contaminants (eg. bacteria, humans and algae) were removed from further analysis. The presence of each assigned MOTU was determined for each individual predator.

6.3.7. Comparing sequencing runs

There were differences between the sequencing results from June/December and February/September that did not appear to reflect seasonal changes in prey abundances. In each run, four species were identified in multiple samples that were not present in the other run. Further, there were some inconsistencies in the prey taxa identified in the three individuals that were amplified in both sequencing runs. Therefore, potential confounding effects of sequencing run were considered in all further analyses, as described below.

6.3.8. Data Analysis

6.3.8.1. Comparison of D. cephalotes *and* R. dorsalis *diets.*

The diet of the two predators was visualised using Non-metric Multidimensional Scaling (NMDS) in two dimensions with R's *vegan* library using the proportion of individuals consuming each prey species in each site/season (Oksanen *et al.,* 2013). Bray-Curtis similarities were used due to their ability to deal with zero-skewed data (Bray and Curtis, 1957). Diet breadth and the dietary overlap between the predators were estimated for: i) the full data set (40 site-season combinations) to provide an overview of the diets, and

ii) separately for the 21 site-season combinations where both predators were present (Table 6.2) to test for differences between them. Diet breadth was assessed using the mean number of prey taxa detected per individual and Levins' standardized measure of niche breadth (B_A; equation 1 in Razgour *et al.*, 2011). Smaller values of B_A indicate greater dietary specialisation, and by controlling for the number of potential prey at a location, B_A permits comparisons among locations with different prey communities. Differences in prey richness and dietary specialisation were tested between the predators using linear mixed effects models in the *nlme* package in *R* v.3.1, with site as a random term (Pinheiro *et al.*, 2015). These models were run for the whole dataset and separately for results from each of the sequencing runs. Dietary overlap between the predators was then assessed using Pianka's (1973) measure of resource sharing. Observed diets were compared to null models to test whether niche overlap was greater than expected by chance. Using *EcoSim* in *R* v. 3.1 10,000 Monte Carlo simulations were performed to generate randomised utilisation matrices for the two predators (Gotelli and Ellison, 2013). Pianka's measure was applied to these random matrices and the results compared to the observed diet matrix. The proportion of stimulated matrices exceeded by the observed data gave the probability that the overlap was greater than was expected at random (Gotelli and Ellison, 2013).

The differences in prey selection between *R. dorsalis* and *D. cephalotes* were investigated by comparing prey taxa in the diet to prey availability in each site. Consumption data for each of the two predator species, combined for all sites/seasons in which both predators were present, were compared to expected consumption rates based on prey abundances, combined from all relevant kick samples, to show whether predators were exercising prey selectivity. A Monte Carlo approach was employed in which the structure of the consumption data was maintained (number of predators and number of feeding interactions) but the identity of prey species is assigned at random in proportion to their densities in kick samples (Agustì *et al.*, 2003; Davey *et al*,. 2013). The model produces frequency distributions of expected consumption rates on each prey item after 10,000 iterations. Comparing observed consumption rates to these distributions reveals whether predators were foraging as expected at random: any observations falling outside the central 95% of simulated values were interpreted as significant deviations from random foraging and hence prey selectivity or avoidance

(Davey *et al.*, 2013). The overall strength of prey selection was summarised by calculating the absolute differences between the observed and expected consumption of each taxon, as a proportion of the total number of prey consumed, and then summing the differences across all taxa in the diet. The resulting measure equals zero when observed and expected values are identical. Values increase above zero as the difference between observed and expected consumption rates increases, and reaches one when there is no overlap between observed and expected pattern of consumption.

To determine whether the feeding behaviour of *R. dorsalis* was altered by reduced competition in the absence of *D. cephalotes* their diet breadth (B_A) was compared between sites, with and without *D. cephalotes*, using a linear mixed model with site as a random term to account for the four seasonal samples from the same location. The influence of *D. cephalotes* on *R. dorsalis* prey selectivity was assessed by comparing observed and expected consumption rates, calculated from Monte Carlo simulations, and overall *R. dorsalis* prey selection strength for sites/seasons with and without *D. cephalotes*. It was expected that in the absence of *D. cephalotes*, the reduced competition would allow *R. dorsalis* to increase its diet breadth and become less selective.

Changes in habitat were investigated as alternative explanations for the non-random distribution of *D. cephalotes* and differences in abundance of *R. dorsalis* between sites. The total abundance of predators in each site from all four seasonal samples was regressed on sedimentation and water chemistry measures using generalised linear models (GLMs) with Poisson error distributions.

6.3.8.2. Effect of agricultural intensity

Changes in the composition of potential prey communities across the intensity gradient were visualised using NMDS in two dimensions with R's *vegan* library using Bray-Curtis similarities (Bray and Curtis, 1957; Oksanen *et al.,* 2013). Data from all kick samples within each site were combined and fourth-root transformed prior to analysis to down-weight the influence of the most abundant taxa (Clarke and Warwick, 2001). Changes in abundance, richness, rarefied richness and predator representation (the proportion of the community classified as predators using data from Schmidt-Kloiber and Hering, 2012), across the intensity gradient were investigated to provide context for the molecular results. Data from the 40 site-season samples were modelled against agricultural intensity using linear mixed effects models in R's *nlme* package (Pinheiro *et al.*, 2015). Site was included as a random term to account for the non-independence of four seasonal samples taken from the same location.

The effect of increasing agricultural intensity on predator diet focused upon *R. dorsalis* as it occurred across the complete intensity gradient. To visualise differences in *R. dorsalis* feeding behaviour along the intensity gradient, a bipartitie plot was drawn, using the '*bipartite*' package in *R* (Dormann *et al.*, 2008) for the combined results of the two sites with the lowest intensity scores (*R. dorsalis* predators only), and the two sites with the highest intensity scores. Because there was no systematic bias in the sites and predators included in each of the two sequencing runs, all data were combined in these plots.

The mean number of prey taxa and dietary specialisation (B_A) were modelled across the 40 site-season samples as a function of agricultural intensity and season using linear mixed effects models, in R's *nlme* package (Pinheiro *et al.*, 2015). Site was included as a random term to account for the non-independence of four seasonal samples taken from the same location and the interaction between season and agricultural intensity score was included to determine whether land use effects varied by season. The optimal model structure was determined by selecting the model with the lowest AIC value from amongst the four models representing every possible combination of predictor variables (Burnham and Anderson, 2004). When both sequencing rounds were combined for analysis, mixed models showed February and September to be significantly different from June and December, reflecting possible differences between the two sequencing batches. Therefore, sequencing runs were separated for analysis, allowing comparisons only between the two seasons in each run (Run 1 = June and December, Run 2 = February and September).

Changes in prey availability, *R. dorsalis* diet composition and prey selectivity across the agricultural gradient were visualised as heatmaps using the '*gplot' R* package (Warnes *et al.*, 2012). Prey availability was calculated as the total number of individuals of each prey taxon from the four seasonal kick samples taken at each site. The diet composition was measured as the proportion of individuals across the four seasons that consumed each prey item. Selectivity was determined for each of the 10 sites (all seasons combined) by comparing observed consumption rates to 10,000 Monte Carlo simulations of expected random consumption based on prey availability as described above. Overall prey selection strength by *R. dorsalis* in each site was calculated from the Monte Carlo results (described above). This measure of selection strength was regressed against both intensity score and *R. dorsalis* abundance.

Selection for prey in different functional feeding guilds was also assessed using the Monte Carlo approach to investigate how changes in prey selection by *R. dorsalis* across the intensity gradient may affect wider ecosystem properties. Each prey item was assigned to its predominant functional feeding guild of predator, filter feeder, grazer, shredder, gatherer or generalist (equal affinity to two feeding guilds) using data from Schmidt-Kloiber and Hering (2012). Observed and expected consumption rates of each guild were compared for each site, as described above, to give selectivity for different guilds and an overall FFG selection strength in each site.

6.4. Results

6.4.1. Sequences analysis

DNA was successfully sequenced from the gut contents of 394 individuals (79 %); 237 *R. dorsalis* and 157 *D. cephalotes* (Table 6.2). The two sampling rounds recovered 5.3 and 3.2 million sequences, respectively, of which 1.13 and 1.08 million remained after sequence processing. Using a similarity cut-off of 0.89, sequences were assigned to 73 MOTUs in the first sequencing batch (June and December samples) and 78 MOTUs in the second (February and September samples). After removal of contaminants (nearest similarity was identified as a non-prey item eg. human, bacterium or freshwater mould) and MOTUs without a match at 98% similarity, 43 MOTU remained from the first sequencing round and 48 from the second. Where necessary, MOTUs were combined to the taxonomic level identified in the kick samples to ensure consistency across analyses (Figure 6.1). Of the sequences assigned to MOTUs, predator DNA accounted for 3.14% (3.50% in *R. dorsalis* and 0.32% in *D. cephalotes*) with similar frequencies in the two sequencing rounds. There was also occurrence of intra-guild predation with 10% of *R. dorsalis* individuals consuming *D. cephalotes* and 27% of *D. cephalotes* consuming *R. dorsalis*.

6.4.2. Overall diet and prey preference

Where both predators were present, their diets were very similar with high overlap in prey taxa consumed (Figures 6.2 and 6.3). The mean number of prey taxa consumed by individuals of both species was 4.8 (± 0.2 S.E.) and the overall niche breadths were 0.17 for *R. dorsalis* and 0.19 for *D. cephalotes*. Neither measure differed significantly between *D. cephalotes* and *R. dorsalis* across the 21 site-season combinations where they were both present (Table 6.3).

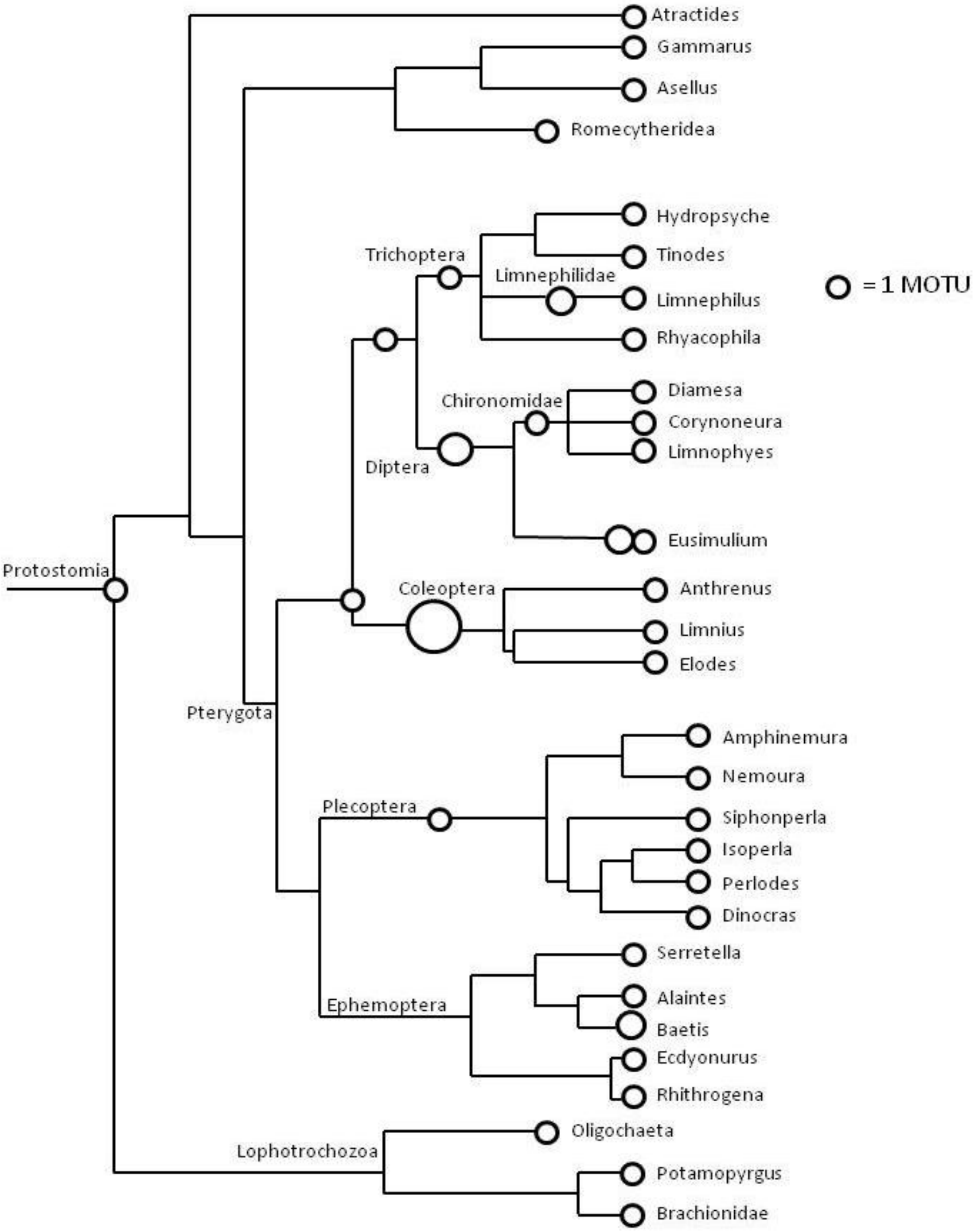

Figure 6.1 – Phylogenetic tree of all operational taxonomic units (OTUs) that were identified as prey species (eg. not contaminants or plant species). The size of the circle represents the number of OTUs assigned at each taxonomic level.

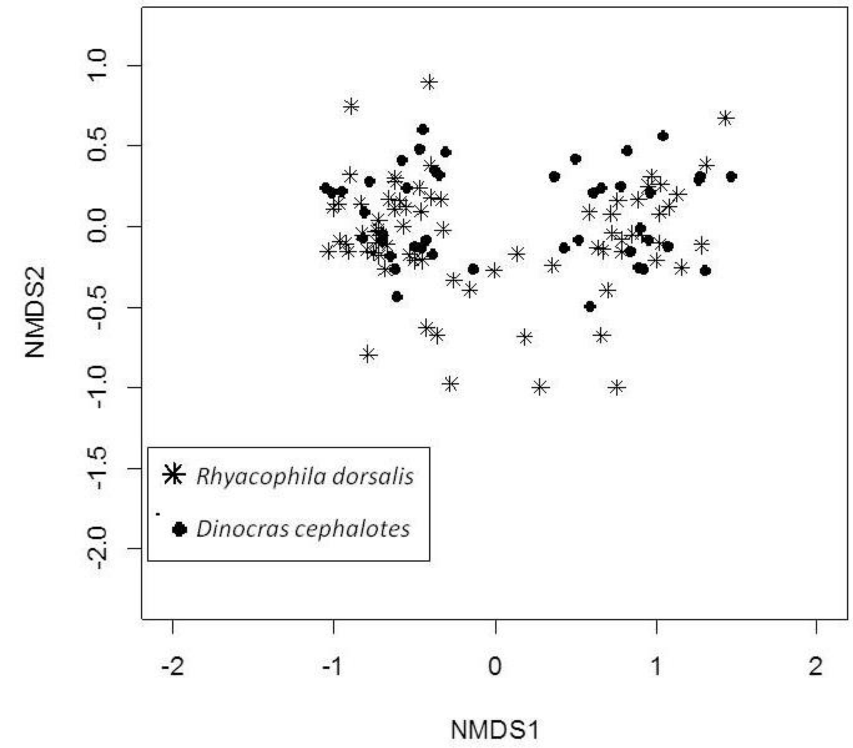

Figure 6.2 – Results of Non-metric Multidimensional Scaling analysis of diet composition for predators Rhyacophila dorsalis *and* Dinocras cephalotes *in ten sites across four seasons, based on the number of predator individuals consuming each prey taxa.*

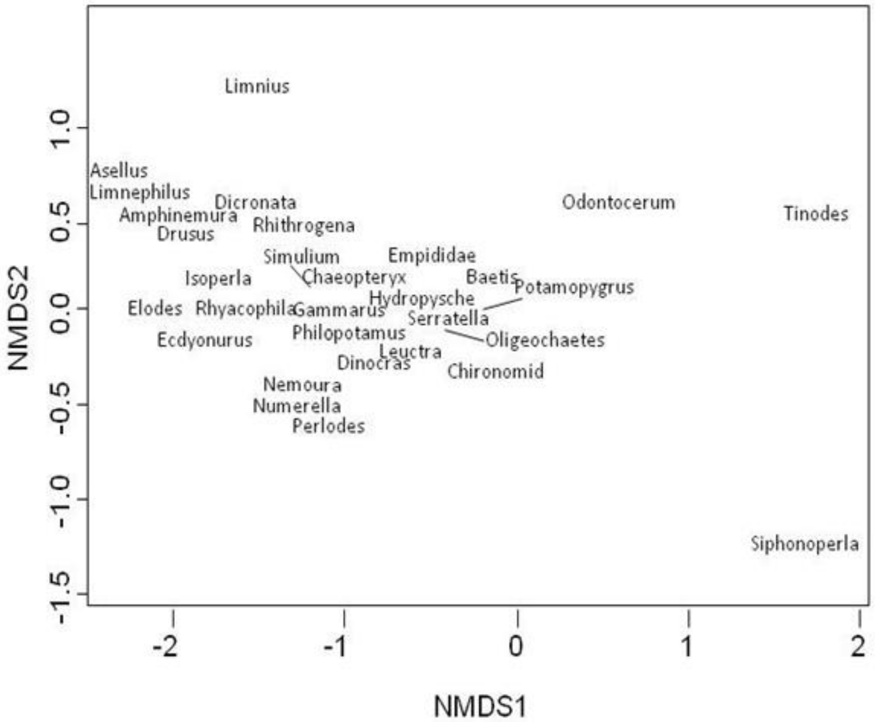

Figure 6.3 – Taxa loadings from Non-metric Multidimensional Scaling analysis of diet composition for predators Rhyacophila dorsalis *and* Dinocras cephalotes*. in ten sites across four seasons, based on the number of predator individuals consuming each prey taxa.*

Both predators showed similar, non-random prey selection (Figure 6.2 and 6.4) and overall selection strength (0.29 for *R. dorsalis* and 0.33 for *D. cephalotes*). As a result, they had high niche overlap, which was significantly greater than expected by chance, both for the whole data set (Pianka's measure O_{jk} = 0.95, $p < 0.001$) and for the 21 site/seasons with both predators present (O_{jk} = 0.40 - 0.91, all tests $p < 0.05$). The two species consumed each other with a greater frequency than expected at random, with *D. cephalotes* showing a very strong preference for *R. dorsalis*. Both predators selectively consumed *Baetis, Nemoura, Philopotamus* and Simuliidae, and avoided Heptageniidae (*Rhithrogena* and *Ecdyonurus*), *Gammarus, Leuctra* and *Limnius* (Figure 6.3 and 6.4) The most notable difference between the feeding behaviour of the two predators was an avoidance of Chironomidae by *D. cephalotes* but a preference by *R. dorsalis* (Figure 6.4).

Table 6.3 – The observed mean values of niche breadth and number of prey taxa, and results of linear mixed models comparing these diet metrics, for Rhyacophila dorsalis *and* Dinocras cephalotes *across all sampling periods and separately for sampling periods within each of the two sequencing batches (samples from June and December were run together on one ion torrent chip and samples from February and September run together on a separate chip).*

Diet metric	All sites/seasons					Sequencing Round 1					Sequencing Round 2				
	Mean ± S.E. *R. dorsalis*	Mean ± S.E. *D. cephalotes*	t value	*d.f.*	*p* value	Mean ± S.E. *R. dorsalis*	Mean ± S.E. *D. cephalotes*	t value	*d.f.*	*p* value	Mean ± S.E. *R. dorsalis*	Mean ± S.E. *D. cephalotes*	t value	*d.f.*	*p* value
Niche breadth	0.17 ± 0.01	0.19 ± 0.02	1.29	49	0.202	0.15 ± 0.02	0.19 ± 0.03	1.223	20	0.239	0.19 ± 0.01	0.19 ± 0.02	0.264	18	0.792
Number of prey taxa	4.5 ± 0.017	4.5 ± 0.024	0.25	49	0.803	3.79 ± 0.18	4.83 ± 0.26	1.34	20	0.195	5.1 ± 0.33	4.2 ± 0.35	1.74	18	0.10

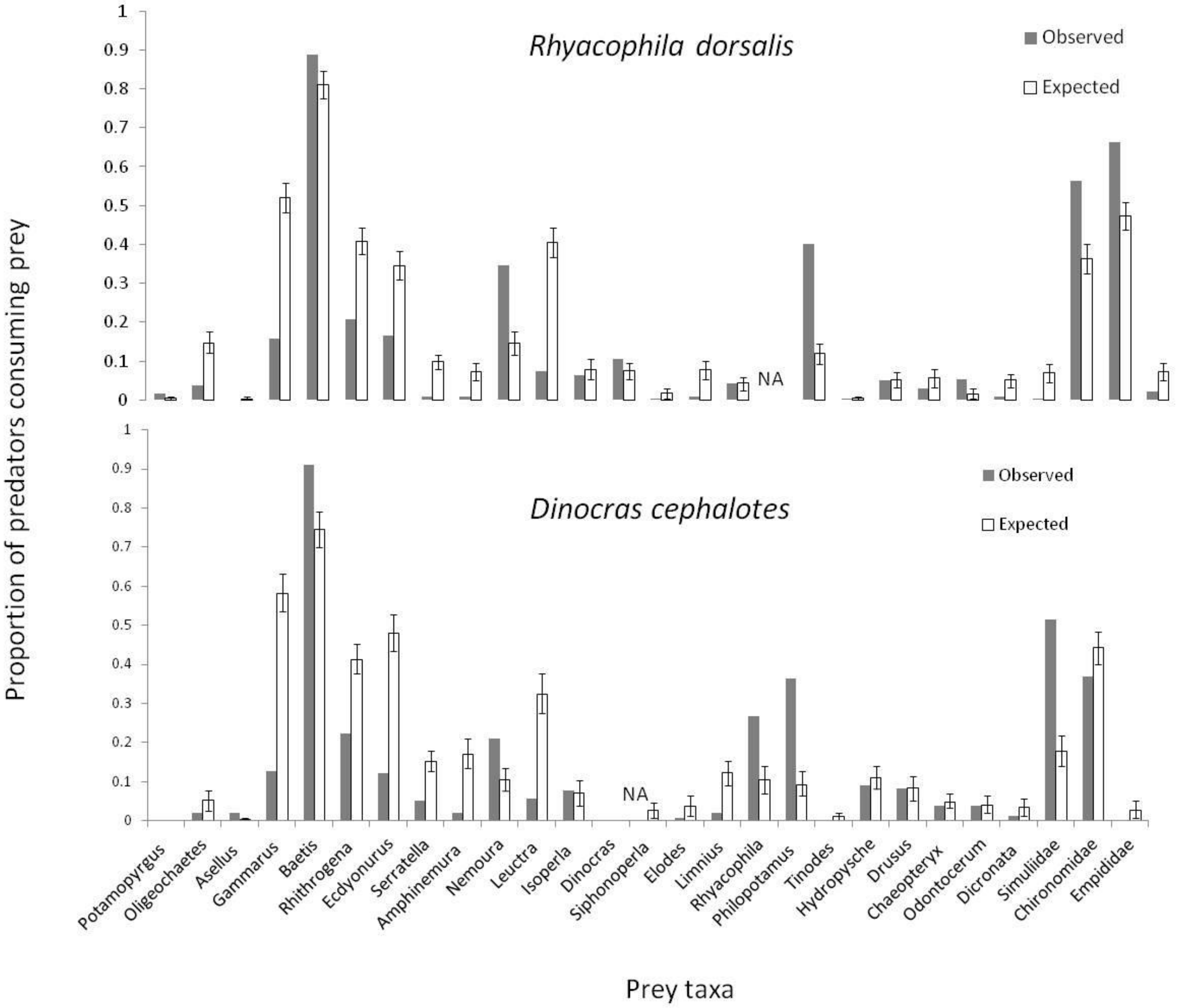

Figure 6.4 – Proportion of predators consuming different prey taxa, compared to random expectation calculated from prey availability. Error bars show 95% confidence limits of expected random consumption. Observed values exceeding the upper limit of this 95 % confidence interval show significant preference for a given prey taxa. Observed values *below the lower limit of this 95 % confidence interval show significant avoidance of a given prey taxa. Observed values within the confidence interval show no significant deviation from random foraging.*

In contrast to the similarity in diet, the two predators showed a marked contrast in response to the physico-chemical environment. There was strong evidence of *D. cephalotes* responding to physical habitat with significant non-linear declines in abundance with increasing sedimentation and nitrate concentrations (z = 31.03, $p < 0.001$ and z = 19.06, $p < 0.001$, Figure 6.5). *Dinocras cephalotes* was absent from sites with > 13 mg L^{-1} inorganic sediment and sites with > 8 mg L^{-1} nitrate but as these conditions co-occurred their effects on *D. cephalotes* could not be separated. The abundance of *R. dorsalis* did not show any significant response to sediment, nitrate or overall intensity score (Figure 6.5).

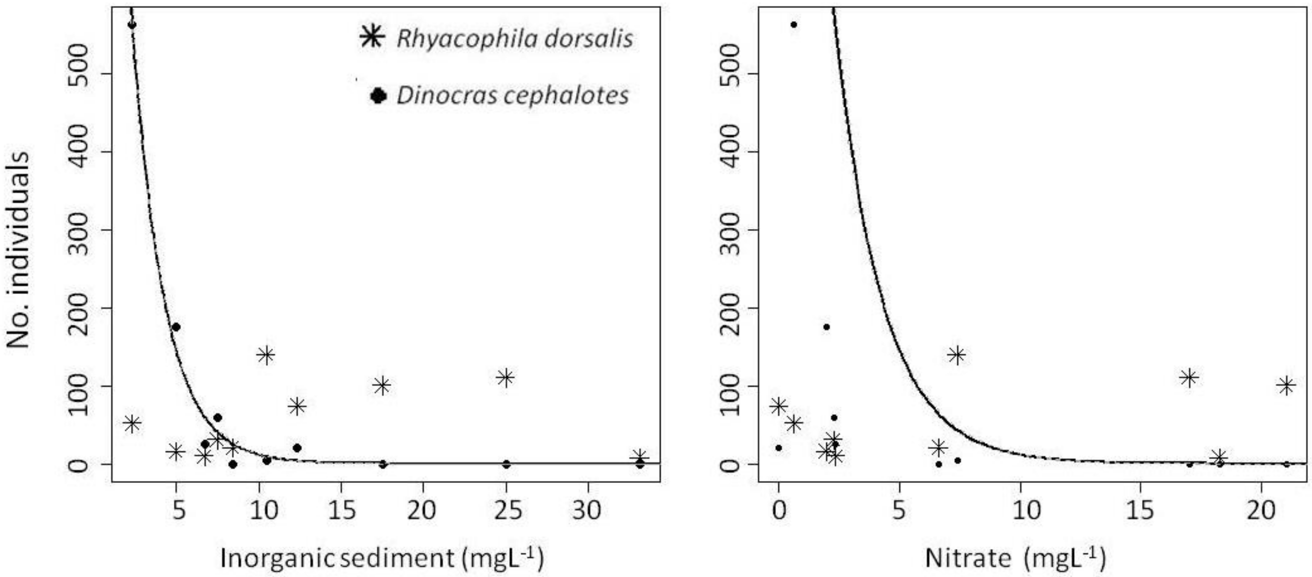

Figure 6.5 – Relationship between abundance of Dinocras cephalotes *and* Rhyacophila dorsalis *and inorganic sediment and nitrate concentration. Black lines show modelled Poisson relationships for* D. cephalotes*.*

The feeding niche of *R. dorsalis* in sites with and without *D. cephalotes* present almost entirely overlapped and this overlap was much greater than expected by chance ($O_{jk} = 0.973$, $p < 0.0001$). There was no significant difference in dietary specialisation ($t = 1.402$, $p = 0.199$) in sites with and without *D. cephalotes*, and the pattern of prey selection was near identical ($t = 0.19$, $p = 0.85$, Figure 6.6).

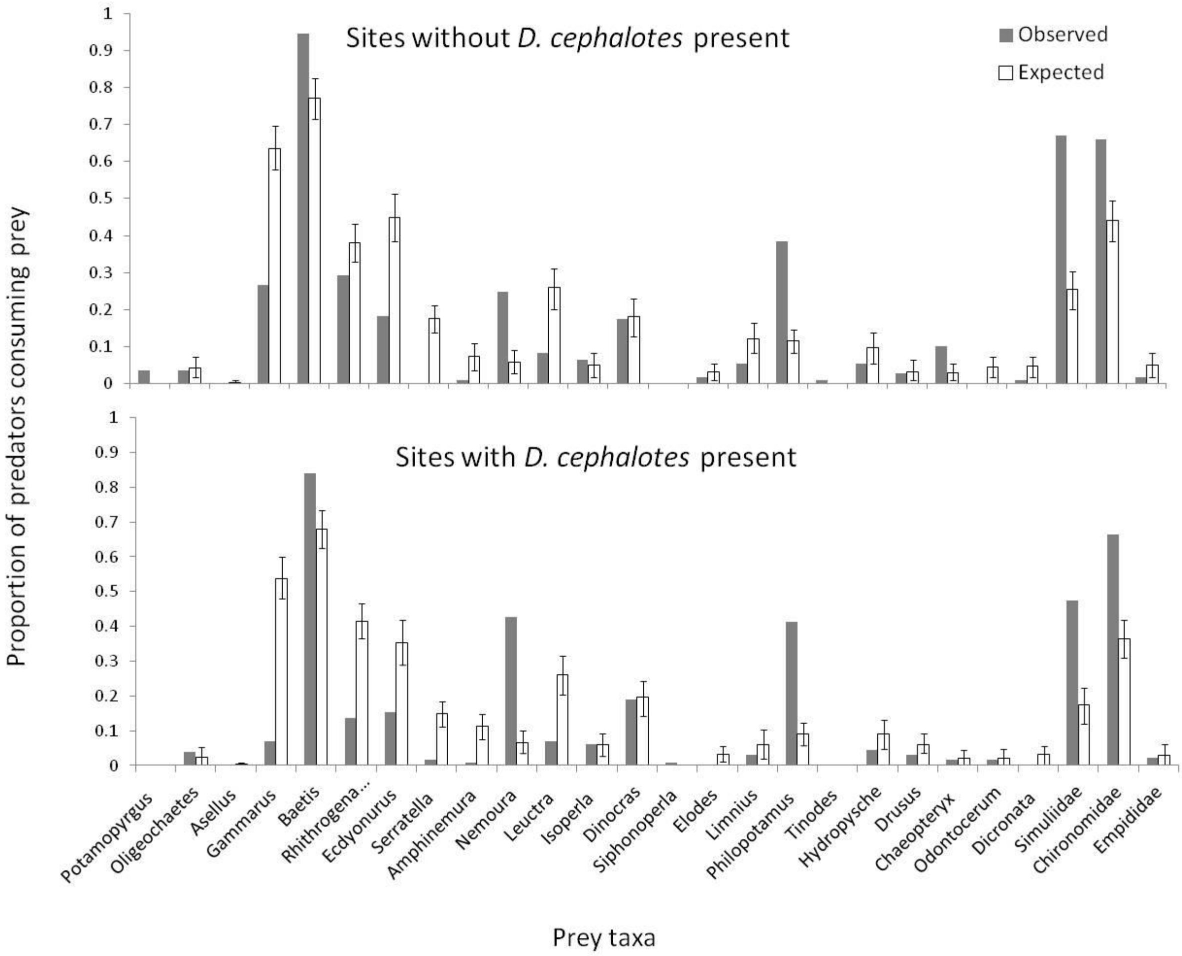

Figure 6.6 – Proportion of Rhyacophila dorsalis *consuming different prey taxa, compared to random expectation, based on prey availability in sites with and without competitors* Dinocras cephalotes *present. Error bars show 95% confidence limits of expected random consumption. Observed values exceeding the upper limit of this 95 % confidence interval show significant preference for a given prey taxa. Observed values below the lower limit of this 95 % confidence interval show significant avoidance of a given prey taxa. Observed values within the confidence interval show no significant deviation from random foraging.*

6.4.3. Effects of land use and season on R. dorsalis diet and foraging behaviour

The overall taxonomic composition of the macroinvertebrate community changed with land pastoral intensity. Intensity score had a positive relationship with NMDS Axis 1 values (Pearson's r = 0.85 *p* = 0.002) but was not significantly related to Axis 2 values (Pearson's r = 0.24, *p* = 0.50; Figure 6.7). This indicates a switch from communities dominated by Ephemoptera, Plecoptera, Trichoptera and Elmidae to communities dominated by molluscs and dipteran larvae along the intensity gradient

(Figure 6.8). Changes in other invertebrate community metrics across the agricultural intensity gradient were not significant in 2013 (Figure 6.9; *cf.* 2012; Chapter 4). There was a significant increase in the representation of predators, however, which was largely attributable to an increase in Tanypodinae dipteran larvae. There was high variability in the abundance of *R. dorsalis* in a site but this did not show a trend across the intensity gradient.

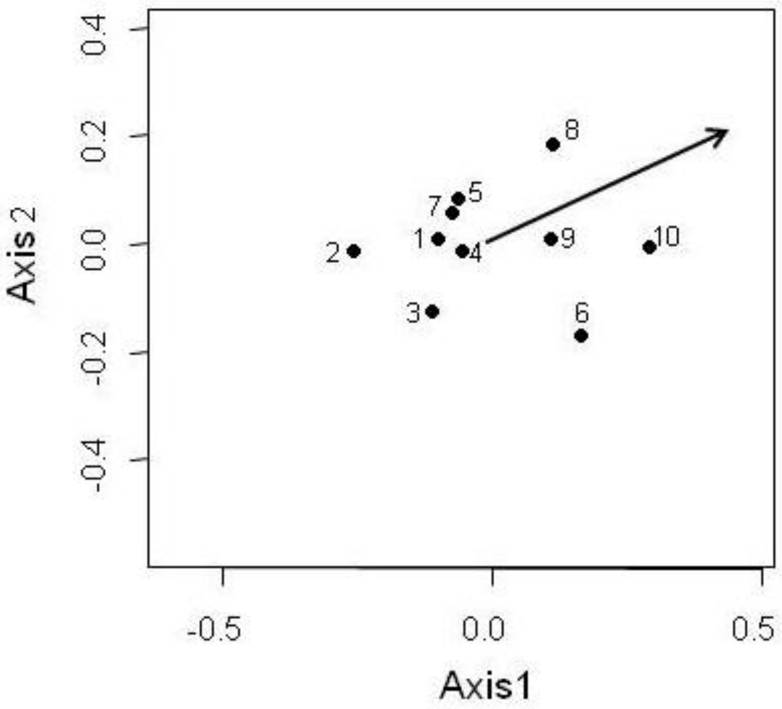

Figure 6.7 - Site loadings from Non-metric Multidimensional Scaling Analysis of invertebrate taxonomic composition. Sites are numbered based on rank of pastoral intensity (1 lowest, 10 highest). Arrow shows vector of increasing intensity score.

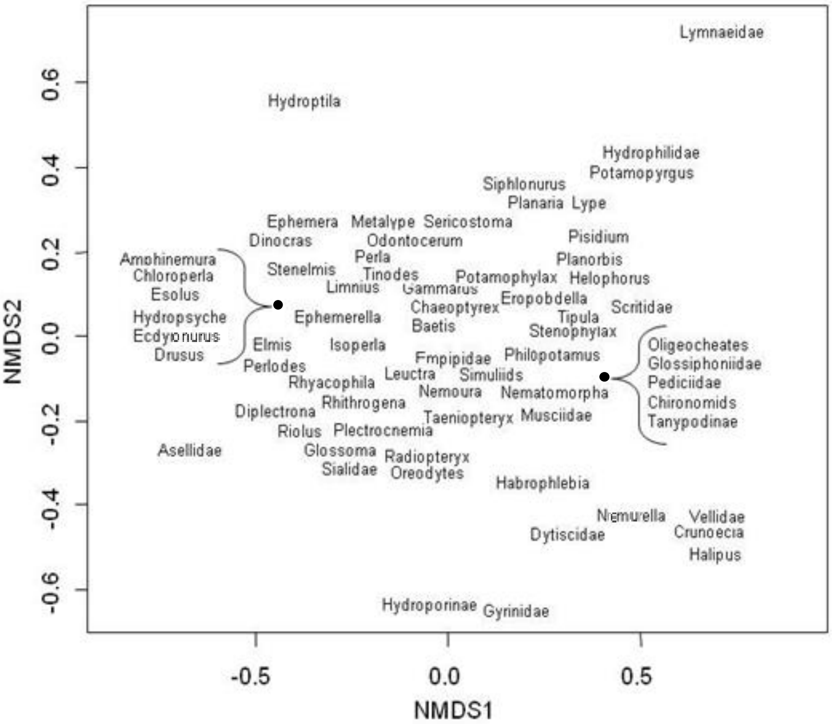

Figure 6.8 – Results of Non-metric Multi-Dimensional Scaling Analysis of invertebrate taxonomic composition.

There was little evidence of changes in the diet or foraging behaviour of *R. dorsalis* across the agricultural gradient (Figures 6.10 and 6.11). The number of prey species and the strength and identity of trophic interactions were similar between the extremes of the intensity gradient (Figure 6.11). Generally, the contribution of each prey taxon to *R. dorsalis* diet reflected its abundance in the environment, with *Baetis*, Chironomidae and Simuliidae accounting for the largest proportions of prey taxa consumed. There was a turnover of rarer prey taxa, with sensitive species (eg. *D. cephalotes*, *Siphonoperla* and *Amphinemura*) absent from high intensity sites and some taxa (eg. *Potamopygrus*) absent from the lowest intensity sites, which resulted in some changes to prey identity with increasing intensity (Figure 6.10). The overall strength of *R. dorsalis* prey selectivity was not significantly related to land use intensity or intraspecific competition (*R. dorsalis* abundance) ($t = 1.75$, *d.f.* = 8, $p = 0.118$ and $= 1.82$, *d.f.* = 8, $p = 0.107$). Whilst diet breadth did not vary significantly across the land use gradient, it did vary between seasons (Table 6.4). The number of prey taxa per individual was significantly greater in June than December and September than February (Table 6.4).

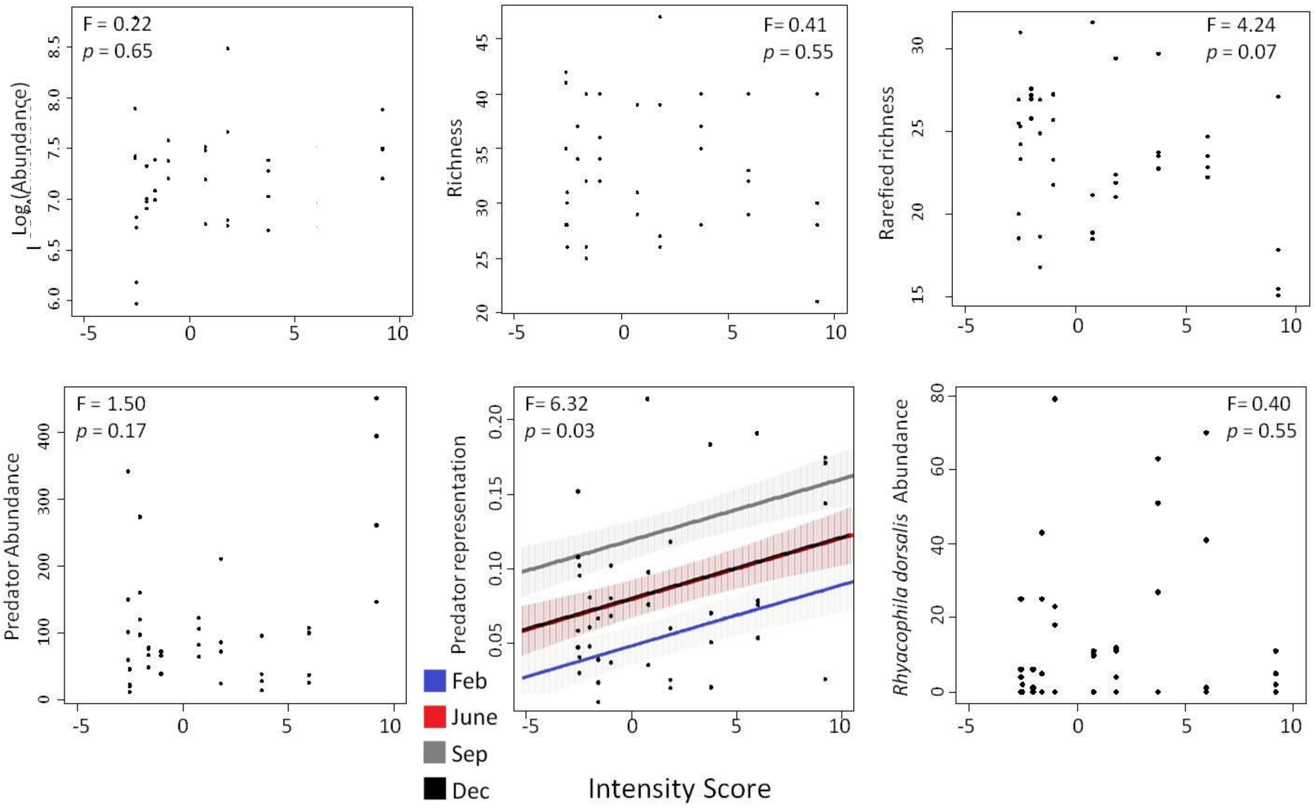

Figure 6.9 – Changes in metrics of invertebrate community composition across a gradient of agricultural intensity. Results of linear mixed models of the relationship between intensity score and each metric are given and displayed where significant, with different colours for the four seasons.

Table 6.4 – Results of linear mixed models of the effects of season and land use intensity on metrics of predator feeding behaviour. Values are displayed from the optimal models, which only contained season.

Response variable	Sequencing Round 1			Sequencing Round 2		
	$F_{1,9}$	*p*	Result	$F_{1.7}$	*p*	Result
Prey taxa per individual	4.225	0.040	June > December	8.076	0.025	September > February
Niche breadth	3.007	0.117		2.171	0.184	

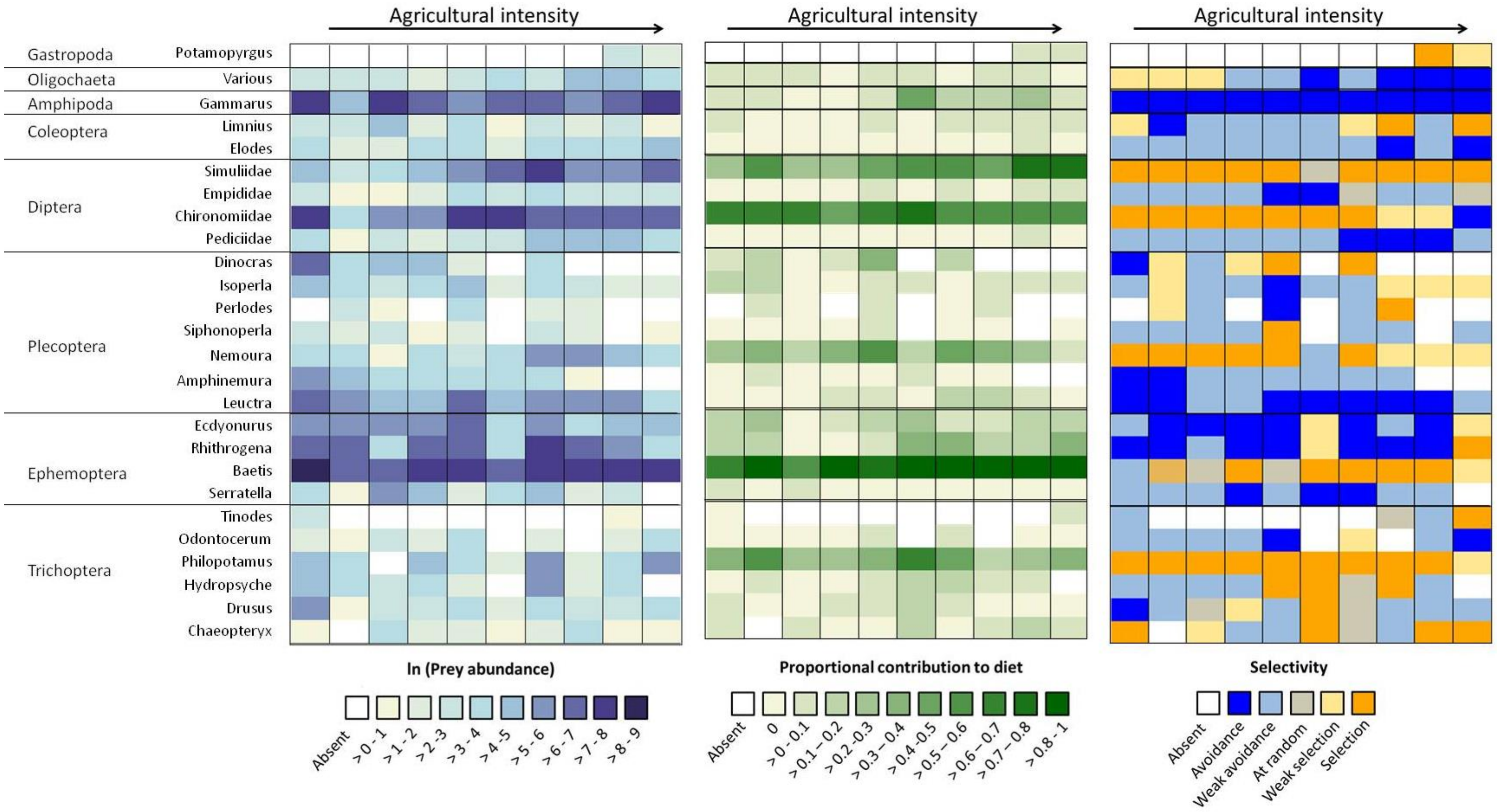

Figure 6.10 – Changes in prey availability and the diet and foraging behaviour of Rhyacophila dorsalis *in ten sites across a gradient of agricultural intensity. Prey abundance was calculated as the total number of individuals in four seasonal kick samples. Contribution to the diet is the proportion of* R. dorsalis *individuals in each site observed to have consumed each prey item. Selection strength was determined from comparing observed consumption rates to 10,000 Monte Carlo simulations of expected random consumption based on prey availability. Consumption rates that were above or below the central 95% of simulated values were interpreted as 'Selection' and 'Avoidance' respectively, and observed consumptions that were above or below the average estimated value interpreted as 'weak selection' and 'weak avoidance'.*

1. Potamopyrgus
2. Oligeochaetes
3. Asellus
4. Gammarus
5. Baetis
6. Rhithrogena
7. Ecdyonurus
8. Serratella
9. Amphinemura
10. Nemoura
11. Leuctra
12. Isoperla
13. Perlodes
14. Dinocras
15.Siphonoperla
16. Elodes
17. Limnius
18. Rhyacophila
19. Philopotamus
20. Tinodes
21. Hydropysche
22. Drusus
23. Limnephilus
24. Chaeopteryx
25. Odontocerum
26. Dicronata
27. Simuliidae
28. Chironomidae
29. Empididae

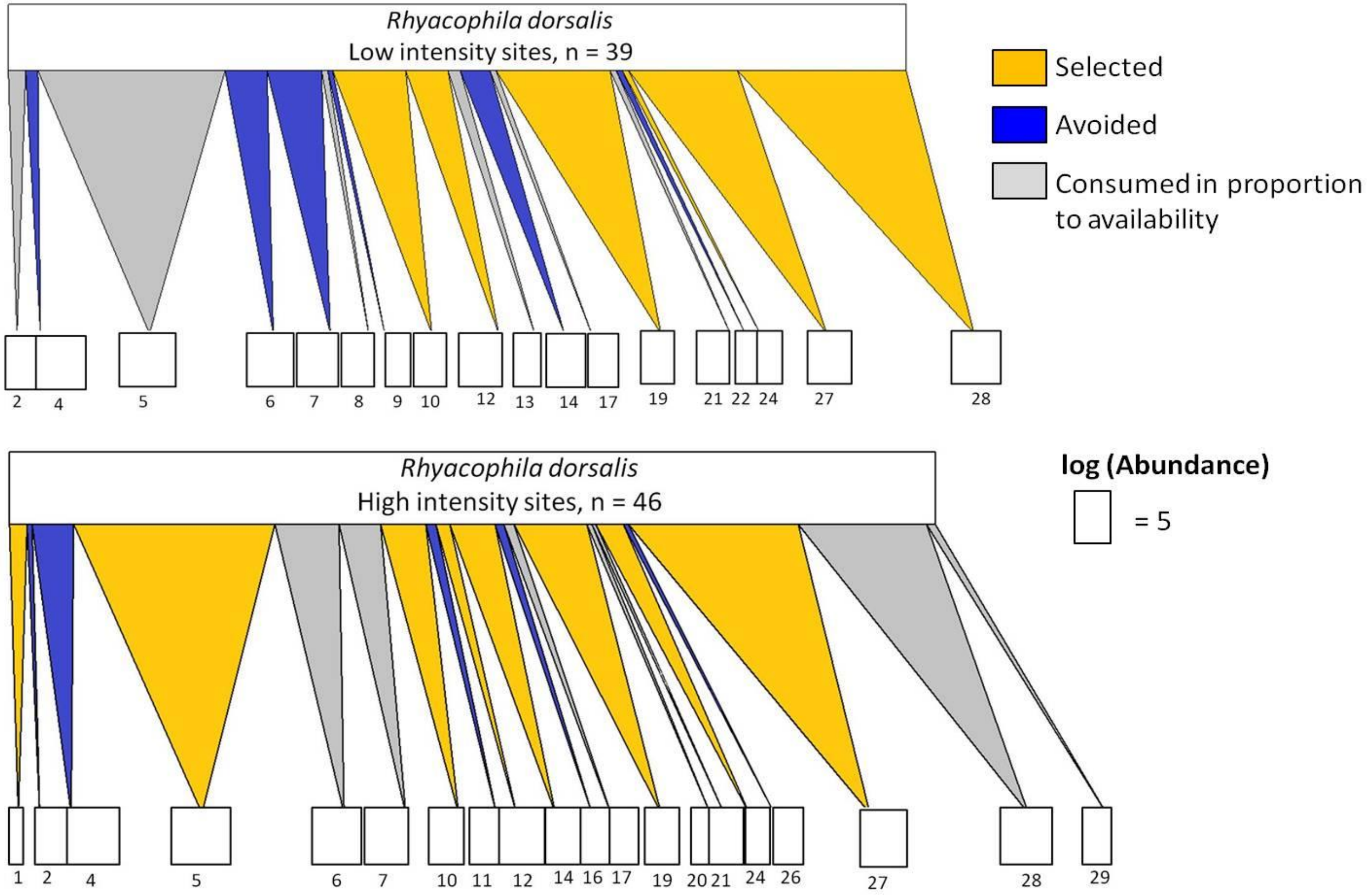

Figure 6.11 – Observed feeding interactions of Rhyacophila dorsalis *combined from the two sites with the lowest intensity land use (top) and the two sites with the highest intensity land use (bottom). Interactions are scaled according to the number of predator individuals observed to consume each prey taxon and colour coded as to whether this is greater, less or equal to random expectation.*

The proportions of different feeding guilds in the diet of *R. dorsalis*, and the pattern of selection for different guilds, varied among guilds and sampling sites, but did not show a clear trend across the intensity gradient (Figure 6.12). Gatherers only contributed to the diet of a very small number of predator individuals, which was consistently lower than expected at random. Conversely filter feeders were consumed by a higher proportion of predators than expected at random across all sites but the strength of this selection was not related to intensity score (Figure 6.12). Selection for grazers and shredders was very variable between sites. There was suggestion of increased selection for predatory taxa with increasing intensity score, with lower than expected consumption in low intensity sites and higher than expected consumption in several of the highest intensity sites (Figure 6.12). The overall strength of *R. dorsalis* prey FFG selectivity was not significantly related to land use intensity (t = 0.06, *d.f.* = 8, *p* = 0.955).

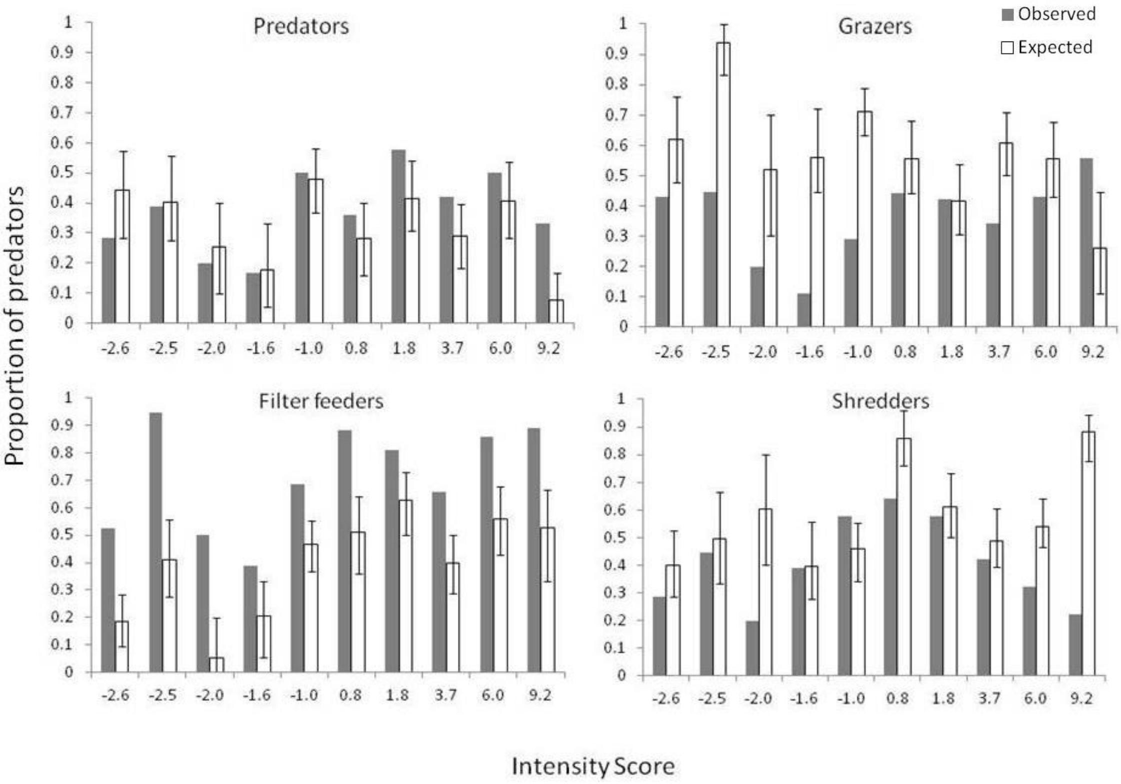

Figure 6.12 – Proportion of Rhyacophila dorsalis predators consuming prey taxa from each functional feeding guilds compared to random expectation, based on prey availability. Error bars show 95% confidence limits of expected random consumption. Observed values exceeding the upper limit of this 95 % confidence interval show significant preference for a given prey taxa. Observed values below the lower limit of this 95 % confidence interval show significant avoidance of a given prey taxa. Observed values within the confidence interval show no significant deviation from random foraging.

6.5. Discussion

6.5.1. Overview

In small fishless streams, large invertebrate predators may exert top-down control on communities such that their feeding habits can influence ecosystem functioning (Wipfli and Gregovich, 2002). Despite this, changes in predator feeding behaviour along stress gradients have received little attention. Here, in one of the first studies to apply molecular techniques to improve the resolution and accuracy of stream food web characterisation, *R. dorsalis* and *D. cephalotes* were shown to be very generalist predators which preferentially consumed the most abundant prey taxa. Agricultural intensification did not significantly change predator foraging behaviour or diet, as preferred prey taxa were resilient to agricultural stressors and were abundant across the intensity gradient. There was, however, a suggestion of food web simplification at the highest intensities with the loss of *D. cephalotes*. Although the diet varied among seasons, the effect of agricultural intensification was consistent across them.

6.5.2 Evaluating the ion torrent sequencing approach for invertebrate diet analysis

Molecular techniques provide valuable tools for constructing empirical food webs, improving upon traditional techniques by increasing the detection of rare prey taxa and confidence in prey identification, and reducing processing time, allowing larger sample sizes (Clare *et al.*, 2014). Sequencing results are, however, still subject to many of the same uncertainties present in morphological gut content analysis, such as the inability to identify secondary predation or scavenging (Symondson, 2002). Further, molecular sequencing has its own unique sources of error. The degree to which the technology used affects sequencing results remains uncertain, with MID choice, sequencing platform and MOTU clustering algorithm all affecting results (Deagle *et al.*, 2009, 2013). Biases affecting numbers of prey sequences, and ways of calibrating these, are explored by Thomas *et al.* (2014) and Thomas (2015) and, in light the differences between results from the two sequencing runs in the present study, further work to quantify these uncertainties across a wider range of study systems is recommended. As there is no attempt made in this work to quantify predation on the basis on numbers of sequences, using instead the numbers of predators testing positive as a more conservative quantitative measure, the effect of sequencing run differences should be minimal.

Nevertheless, it was necessary to account for the different sequencing runs in all of the analyses.

In many systems, including the present study, the phylogentic proximity of predator and prey prevents the use of predator blocking probes, presenting the risk that the majority of sequences will belong to the predator. In an analysis of spider diets, sequenced without blocking probes, over 75% of the sequences obtained belonged to the predator (Piñol *et al.*, 2015). Although there were sufficient remaining sequences to resolve prey species, this reduction in the number of useable sequences is likely to reduce the ability to detect rare prey items. Here, only 3% of useable sequences were the predator's own DNA, which is attributed to the ease of gut dissection in the relatively large predators used in this study. This technique of sequencing without blocking probes is recommend for species where gut dissection is possible to ensure no loss of prey species.

Despite the risks and uncertainties associated with sequencing of gut contents, the technology affords great potential to resolve food web interactions and, as price per read falls, investigation of entire food webs is likely to become feasible. Even restricted to resolving feeding links for individual predators, the utility of the molecular approach is evident from comparisons of the present results to previous studies of the feeding behaviour of *R. dorsalis* and *D. cephalotes* based on visual gut content analysis. The most commonly observed prey taxa in the present study closely reflect results from morphological gut content analysis studies, with Muotka (1993) showing *R. dorsalis* diets to be dominated by simuliids, Baetidae and chironomids, and Dudgeon (2000) and Bo *et al.* (2007) finding predatory stonefly diets to be dominated by chironomids, *Philopotamus* and Ephemoptera. There was, however, a much lower ability to detect rare prey items in previous studies based on visual identification compared to the present study using molecular techniques. Due to the difficulties of identifying body fragments of soft bodied taxa, such as oligeochaetes and several dipteran larvae, in visual gut content analysis, taxa are usually identified at a lower taxonomic resolution (eg. order or family level) than in molecular studies. At a family or order level, only very crude traits can be assigned, masking the consequences of changes in food web structure for functional diversity and wider ecosystem functioning. Molecular techniques are able to resolve these prey taxa for the entire duration of their passage through the gut and therefore identify a greater range of prey items. These limitations may explain Bo *et al.*'s (2007) finding that *D. cephalotes* had only 1.13 (± 1.15) prey taxa per predator, from 15 taxonomic groups, compared to 4.5 prey taxa from 24 groups identified here. The total in the present study was restricted by the taxonomic resolution used in the identification of community composition samples: molecular results identified the majority (71 %) of taxa to species level, which may give greater insight into food web structure and is relevant where species have unique functional roles or are of conservation importance. A disadvantage of the technique applied in this study is the inability to identify vegetative material which makes an important contribution to the diet of both predators, even at larger instars (Bo *et al.*, 2007; Céréghino, 2002, 2006). DNA could not be amplified from around 20% of collected predators and suggest this may be because their guts contained only plant material. Future studies could screen predators with generic plant primers to determine the level of herbivory by these predators as well.

6.5.3. Feeding behaviour and niche overlap between R. dorsalis and D. cephalotes

As predicted, and as observed by Dudgeon (2000) for predatory stoneflies, both predators appeared to consume prey roughly in proportion to their availability. Some prey choice was evident, but was relatively modest and mainly reflected avoidance of larger prey taxa that were abundant in the community (eg. Heptageniids and *Gammarus*). These results suggest that several prey species offered nutritional equivalence and that encounter rate is likely to be the biggest determinant of prey choice for these predators, although capture success, handling efficiency and nutritional quality are also likely to contribute to predator foraging choices (Symondson, 2002).

Not only did the most abundant species make the greatest contributions to predator diets, but they were consumed in greater proportions than expected. This preferential consumption of the most common prey types is predicted under optimal foraging theory which postulates that predators form a search image for the most common prey and increase the efficiency with which they capture and handle it, resulting in the most common prey becoming the most profitable for the predator (Krebs, 1978; Elliot, 2004).

Contrary to predictions, there was no significant change in *R. dorsalis* diets with increasing agricultural intensity. Despite significant changes in the species composition of the invertebrate community across the intensity gradient, *R. dorsalis* preferentially consumed prey taxa that were resilient to agricultural stressors and were present across the whole land use gradient (Baetidae, Chironomidae, Simuliidae and *Philopotamus*). There was, however, a change in the contribution of rarer taxa to the diet of *R. dorsalis*, reflecting the turnover of prey species. Species sensitive to agricultural stressors (eg. *Siphonoperla*, *Amphinemura*) were only present and contributing to *R. dorsalis* diets in low intensity sites, whereas taxa that were not present in low intensity sites, such as *Potamopyrgus*, became part of *R. dorsalis* diet in high intensity sites. Despite these changes in the identity of prey taxa, there was no significant effect of agricultural intensity on the representation of different functional feeding guilds in predator diets. Across all sites filter feeders were consumed by a larger number of predator than expected at random and grazers, gatherers and shredders were generally consumed less than expected at random. This is likely to reflect the difference in motility between these feeding guilds, with filter feeders being more sedentary and therefore easier to predate.

The most significant effect of agricultural intensification for the present study was the loss of *D. cephalotes* in the highest intensity sites. The very high overlap in dietary niche and similar strength of selectivity between the two predators suggests that *D. cephalotes* is not a more specialised predator than *R. dorsalis*, and therefore their lower resilience to agricultural stressors was unlikely to be a result of feeding behaviour. Several of the prey taxa that were most heavily selected for by *D. cephalotes* (eg. Baetidae, Simuliidae and *Philopotamus*) were present across the agricultural gradient, such that declines in prey availability could not explain the loss of *D. cephalotes* at high agricultural intensities. Instead, the negative relationship between *D. cephalotes* abundance, and sedimentation and nitrate concentrations suggests the absence of *D. cephalotes* at high intensity sites was due to a greater sensitivity to physico-chemical stressors associated with agriculture.

Understanding the circumstances under which the loss of a predator results in changes to food web structure is critical for understanding the functional consequences of biodiversity loss (Worsfold *et al.*, 2009), but can be very context dependent. Here, the loss of *D. cephalotes* did not affect the feeding niche, dietary specialization or prey selection strengths of *R. dorsalis* diet but the high niche overlap between these predators suggests they would be competitors. There was no effect of intra-specific competition observed for *R. dorsalis*, with prey selection strengths unrelated to abundance. Determining the effects of competition on feeding behaviour requires investigation into the interactions of all competing predators.

There was, of course, a loss of feeding interactions associated with absence of *D. cephalotes,* including the preferential consumption of *R. dorsalis* by *D. cephalotes*, and vice versa. The generality of *D. cephalotes* feeding behaviour makes it unlikely that its loss would result in major changes to community structure or secondary extinctions (Worsfold *et al.*, 2009) but it is likely to correspond with a decline in food web size and connectivity. These properties are positively related to basal energy availability and habitat heterogeneity through their effects on taxon richness (Townsend *et al.*, 1998). Mild nutrient enrichment from agriculture has therefore been widely associated with increases in food chain length and connectance (Townsend *et al.*, 1998; Jaarsma *et al.*, 1998; Riley *et al.*, 2003) but the effects of intensive agriculture have not been assessed. In the present system, taxon richness declined at high agricultural intensities, probably in response to reduced habitat heterogeneity (Chapter 4), suggesting that the loss of *D. cephalotes* feeding links is part of an overall simplification of food web structure.

Simplification of stream food webs has also been observed in response to stressors of acidification (Layer *et al.*, 2010) and drought (Ledger *et al.*, 2013). Both studies showed streams under stress to have smaller food webs with fewer trophic interactions. In theory, simplification may make food webs more stable if interaction strengths remained constant (McCann, 2000) but if the number and positioning of strong links is altered the consequences for ecosystem stability and functioning could be far-reaching (McCann, 2000; Ledger *et al.*, 2013). Quantifying interaction strengths alongside site-specific foraging behaviour is therefore a priority for food web studies. The present results provide a first indication of changes in trophic interactions over a wide stressor gradient but further work is required including more predator taxa to determine the consequences for stability and ecosystem functioning.

In line with the positive association between algal productivity and food web size and connectance described by Townsend *et al.* (1998), the number of prey taxa consumed was higher in June and September than December and February. A similar result was also observed by Woodward *et al.* (2005) and attributed to a higher abundance of rare prey items in summer months when in stream production was highest. Woodward *et al.* (2005) also found variability in prey identity between seasons reflecting ontogenic changes in the prey taxa size. This effect was not observed in the present study (results not shown), which may be due to the focus on large predators that are likely to be able to handle a wide range of prey sizes. Contrary to predictions, there was no difference in the degree of seasonal variability in predator feeding behaviour across the intensity gradient due to the generality of these predators and abundance of preferred prey taxa across the intensity gradient in all seasons.

Overall, this study demonstrates that sequencing without blocking probes on dissected predator guts is a successful method for determining stream invertebrate diets, with many advantages over traditional visual techniques. Enhanced resolution of trophic interactions will improve understanding of the complex direct and indirect effects of anthropogenic stressors on ecosystem functioning (Gray *et al.*, 2014). The consistency of predator feeding behaviour with increasing agricultural intensity observed here is a first step towards understanding the thresholds at which land use change may disrupt stream ecosystem functioning.

7. General Discussion

7.1. Overview

The overarching aim of this study was to assess the impacts of intensification of pastoral agriculture on the community composition, functional attributes and feeding interactions of stream macroinvertebrates. The studies in this thesis attempted to fill some of the wider knowledge gaps identified by the literature review (Chapter 2) which included: i) the responses of macroinvertebrate community metrics to a broad gradient of physico-chemical conditions associated with increasing agricultural intensity (Chapters 3 and 4); ii) the consequences of altered community composition for functional diversity (Chapter 4); iii) the effects of pastoral intensification on macroinvertebrate feeding behaviour (Chapters 5 and 6); and iv) the degree of seasonal variability in these effects (Chapters 5 and 6).

In combination the results supported the hypothesis that pastoral intensification alters stream physico-chemical characteristics with consequences for taxonomic composition, trait diversity and feeding behaviour. As predicted, the responses of taxonomic and functional diversity to increasing pastoral intensity were non-linear, declining once a threshold intensity had been passed. Only relatively minor changes were evident in predator-prey interactions across the pastoral intensity gradient, whilst there was some suggestion that methane derived carbon became an increasingly important energy source. These findings expand on current knowledge of agricultural stressor effects on stream ecosystems, and provide valuable insights to guide land management efforts that aim to conserve stream ecosystem integrity in the face of changing agricultural practices.

7.2. Synthesis of results

7.2.1. Physico-chemical effects of agricultural intensification

National scale monitoring and field data both showed that concentrations of fine sediment and nutrients in streams increased with the percent cover of intensive pasture in the catchment. These results support a wealth of previous literature that has demonstrated the effects of fertiliser application, livestock poaching and soil compaction on stream ecosystems (eg. Allan, 2004). Most previous studies, particularly experimental ones, focussed only on sediment and nutrient effects, but this study showed that a broad range of physico-chemical changes are associated with agricultural intensification, including increases in trace metals and salts. As stressors are known to interact in complex ways (Townsend *et al.*, 2008; Matthaei, *et al.*, 2010), these results highlight the need to assess the combined effects of all land use stressors: the development of the agricultural intensity score (Chapter 4) was one way of doing this.

7.2.2. Taxonomic and functional diversity

Taxonomic richness and functional diversity showed threshold responses to increasing agricultural intensity, with weak support for the predicted subsidy-stress response. Both metrics showed non-significant increases with pastoral intensification until a threshold intensity level beyond which they declined. Further work would be required to confirm the presence of this possible subsidy effect. Subsidy effects of low levels of intensification can be attributed to mild nutrient enrichment increasing the quality and quantity of algal food resources, which has been previously observed (eg. Liess *et al.*, 2012) and was demonstrated in Chapter 5. For the upland catchments in this study, the threshold at which these metrics began to decline equated to around 40% catchment land cover under improved pasture, 4 mg L^{-1} nitrate concentrations or 8% fine sediment cover (Chapter 4). Analysis of national scale monitoring data suggested that intensive pasture streams have higher richness and sensitive species representation than the background mix of other land uses (Chapter 3). This result indicates that, on average, the current level of pastoral intensification in England and Wales falls below the intensity level at which effects become negative, although these thresholds may vary between stream types.

There are many instances, however, both within the U.K. and globally, where threshold intensity levels are exceeded such that intensive pastoral agriculture leads to declines in taxonomic and functional diversity of macroinvertebrate communities. This occurs as a result of environmental filtering along gradients of agricultural stressors with non-random loss of traits poorly adapted to the conditions. Grazing invertebrates were particularly vulnerable to sedimentation effects, as were taxa with large sizes, gill respiration and crawling behaviour (Chapters 4 and 5). Intensively farmed sites had a pool of functionally similar species (Poff, 1997; Cornwell *et al.*, 2006). These changes in the way species feed and move, and in their size distribution, are likely to produce changes in their interactions and their contribution to ecosystem functioning (Tilman *et al.*, 1997).

7.2.3. Basal resource use and predator-prey interactions

The suite of physico-chemical changes associated with pastoral intensification had direct impacts on ecosystem processes, including primary productivity and nutrient cycling. In turn, this altered the availability and quality of basal energetic resources for invertebrate primary consumers. Contrary to original predictions, the invertebrate community appeared to become increasingly reliant on terrestrial energy resources with pastoral intensification, as a result of the association between pastoral intensity and riparian tree cover. Further, isotopic data indicated a contribution of methane-derived carbon to the food web which has not previously been observed in upland streams and constitutes a significant change in basal energy sources. The differences in nutritional quality between algae, which supported communities in low intensity streams, and detrital material and methane derived carbon, which supported communities in high intensity streams, indicates that pastoral intensification is likely to have implications for secondary production and nutrient budgets (Kominoski and Rosemond, 2012).

There was evidence that methane derived carbon was transferred up the food chain to predatory invertebrates, demonstrating the potential for bottom-up controls on food web properties. There was little indication of top-down control from the two focal predators, both of which were generalist consumers with weak preferences for the most abundant prey species.

Contrary to predictions, there was very little effect of season on predator feeding behaviour, although this was somewhat obscured by differences between the DNA sequencing runs, which were divided by seasons. Similarly, the predicted change in the feeding behaviour of *Rhyacophila dorsalis* with increasing agricultural intensity was not observed. However, the decline in overall species richness, coupled with the potential decline in habitat heterogeneity (Chapter 4), the latter known to be positively correlated with food web size and connectivity (Townsend *et al.*, 1998), both point to a simplification of the food web with increasing agricultural intensity.

7.3. Implications and management recommendations

In sum, the results from this study demonstrate the importance of conducting investigations into land use change over a broad range of intensities (Vaughan and Ormerod, 2003). The non-linearity in response patterns means that results will depend on the portion of the intensity gradient considered. This is demonstrated by the difference in the observed effects of intensification on invertebrate richness by Riley *et al.* (2003), who showed increases in richness over a nitrate gradient from 0.0035 to 0.34 mg L^{-1}, Niyogi *et al.* (2007), who found no significant change in richness over a nitrate gradient from 0.005 to 1.8 mg L^{-1}, and the present study which showed a non-linear change in richness over a much wider nutrient gradient from 0.1 to 25.9 mg L^{-1}.

The results presented in this study demonstrate that impairment of ecosystem functioning by pastoral intensification may be greater than predicted from traditional structural measures such as richness, highlighting the need to incorporate measures of functional diversity into routine biomonitoring of stream ecosystems (Tilman, 2001; Mouchet *et al.*, 2010). Although this would require data at a higher taxonomic resolution (genus/species) than currently recorded in routine monitoring (family level), the improved ability to identify impaired ecosystem functioning, and hence target mitigation measures, would likely offset the additional cost.

Overall, the results presented here demonstrate that the effects of intensive pastoral agriculture on stream macroinvertebrate communities are a concern for both biodiversity conservation and ecosystem integrity at intensity levels that are often exceeded worldwide. The challenge for sustainable land management is to maintain agricultural production, as necessary to meet a growing demand for food, whilst ensuring associated stressors remain below the level at which the condition of neighbouring ecosystems and the ecosystem services they provide is compromised (Sutherland *et al.*, 2006).

The threshold relationship between agricultural stressors and biological responses observed in this study provides evidence of the levels to which sediment, nutrients and related stressors need to be reduced to maintain stream ecological condition. These results will allow management efforts to be focused on streams close to these thresholds, where mitigation measures will be most cost-effective and provide greatest benefits (Chuffney, 2000). Although reductions in sediment and nutrient inputs are highlighted as priorities for both arable and pastoral systems (*c.f.* Matthaei *et al.*, 2010; Wagnehoff *et al.*, 2011), the results demonstrate the importance of taking a holistic approach to reduce the multiple, interacting stressors associated with agriculture (Wagenhoff *et al.*, 2011). This does not necessarily require a reduction in overall intensity, but could involve reducing the connectivity of these stressors to the stream by implementing water friendly farming techniques such as riparian tree planting, field margins or silt traps (Osborne and Kovacic, 1993).

7.4. Strengths and Caveats

This study benefited from using a diverse range of techniques to assess the effects of pastoral land use on stream ecosystems. Not only did this improve the results, it provided insight into the potential utility of such techniques for assessment of stressor effects in stream ecosystems.

An observational approach was used to collect the field data used in chapters 4-6, comparing catchments with differing levels of pastoral intensity. This approach is potentially liable to confounds but care was taken to minimise differences between the catchments; streams were generally a similar size, spanned a relatively narrow altitudinal range and did not have other land uses next to the channel. The homogeneous pedology and geology resulted in relatively constant background water chemistry across all sites, limiting the potential for background differences in acidity or nutrients to confound the study (Larsen *et al.*, 2009). The observational approach allowed hypotheses to be tested on a catchment scale, integrating the combined effects of all agricultural stressors. Studies using catchment scale replication are rarely performed but have greater relevance for management practices than mesocosm studies which are necessarily restricted to a subset of stressors (Allan, 2004; Wagenhoff *et al.*, 2011).

Due to a lack of data on stocking densities and land management practices an intensity index was derived from stream physico-chemical characteristics to represent the combined effects of a given level of pastoral intensity. The non-linear relationship between this index and the proportion of the catchment under intensive pasture demonstrates that proportional cover is a poor measure of land use intensity (*cf.* Harding *et al.*, 1999). Although use of this intensity index limits comparison with other studies and direct application to land use management, it affords a more representative measure for evaluating conceptual models of pastoral land use effects. The similarity between the pattern of results from data collected in 2012 for this thesis and data collected in 2006 in the same region (Larsen *et al.*, 2009) lends greater credibility to the observed trends.

The propensity modelling approach also assessed the combined effects of agriculture and removed the effects of potential confounds, providing insight into the effects of agriculture at the national scale which complemented the results of field data. Streams in intensive agriculture were compared to the background of contemporary land use, not, like many studies, from some defined ‘reference’ condition which is largely absent in the highly modified landscapes of England and Wales (Stoddard *et al.*, 2006). Despite the limitations of the dataset, including low taxonomic resolution and lack of abundance data, the results had high generality across different regions and are relevant for guiding national-scale environmental policy on changing land cover.

The molecular approach employed in this study was successful in determining stream invertebrate diets, with many advantages over traditional visual techniques. Ion torrent sequencing without blocking probes on dissected predator guts amplified a large number of prey species with very low representation of the predator’s own DNA and is recommended for future studies. The use of isotopic analysis was less successful in this study. The change in generalist consumer diets over the agricultural gradient could not be resolved using this technique because consumers were assimilating carbon from unmeasured basal resources and there was very high spatio-temporal variability in basal resource isotopic signatures (Deins *et al.*, 2009). Greater sampling effort over a wider range of possible sources may help to overcome this issue. Isotopic data from this study, did, however, reveal a contribution of methane derived carbon to upland stream foodwebs. This novel finding could have significant consequences for understanding of biogeochemical cycling in upland streams (Shelley *et al.*, 2014).

7.5. Future directions

The findings of this study have highlighted several research gaps that warrant further investigation. First and foremost, future studies should assess the general applicability of the thresholds observed here to other regions and stream types. Lowland streams, for example, may show different responses to increasing pastoral intensity or have a different threshold level due to naturally higher nutrient loading. A similar study for arable land use would also be instructive given the results from Chapter 3, which suggest that at current intensity levels arable land use has a more detrimental effect on stream ecosystems than pastoral.

The lack of seasonality in the magnitude of agricultural effects observed here is surprising and may reflect the relatively small sample size of ten sites. Further studies into seasonal variability in anthropogenic stressor effects, covering a greater number of sites, are recommended. Additionally, conducting studies over multiple years would allow exploration of inter-annual variation and the effect of agricultural intensity on resilience to longer term climatic events such as floods and low flows (Collier, 2008).

The novelty, cost and time involved in molecular techniques limited the present study to an analysis of two predators. Future work should seek to expand the number of species and characterise more of the food web, and this will become possible as the cost per sequencing run decreases. It was postulated that some of the predators dissected in this study were also consuming plant material. Investigating this herbivory using plant specific primers would be a valuable addition to this work as omnivory is important in stabilising food webs (McCann and Hastings, 1997). Quantifying changes in interaction strengths remains a priority for food webs studies as the number and positioning of strong links determines food web stability (McCann, 2000).

As the relative contribution of algal and detrital resources to consumer diets could not be resolved using stable isotopes in the present study, repeating the study with more frequent sampling of a greater array of basal resources is recommended. The contribution of methane to upland stream food webs warrants further investigation to determine the prevalence of this energy source. This could be achieved by analysis of hydrogen stable isotopes across a range of consumers which provides a more direct measure of the contribution of methane derived carbon to consumer tissues (Deines *et al.*, 2009).

7.6. Conclusion

In concert, the data presented in this thesis demonstrated significant changes in macroinvertebrate taxonomic composition, functional diversity and food web structure in upland streams in response to pastoral intensification. Both taxonomic richness and functional diversity showed a threshold response to increasing intensity of pasture whilst methane derived carbon was increasingly used to fuel stream food webs. Together these changes could radically alter emergent ecosystem properties such as secondary production and nutrient processing. This work furthers current understanding on the effects of land use for ecosystem structure and functioning, but more research is required to determine how food web structure and stability change across anthropogenic stress gradients. It is important to anticipate how human-induced global change processes will affect ecosystem functioning in headwater streams, given their importance for ecosystem service provision. The data gathered highlight the management priority of preventing physico-chemical conditions reaching the identified threshold levels at which they begin to deleteriously affect stream ecosystems, although further work is required to determine whether these thresholds are applicable across a range of ecosystems.

8. References

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