Confirming the status of Lancashire's endemic freshwater Nemertean – *Prostoma jenningsi*

by

Siobhan Marie Quigg

A thesis submitted in partial fulfilment of the requirements for the degree of MSc (by Research) at the University of Central Lancashire

May 2017

Student Declaration

Concurrent registration for two or more academic awards

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

Material submitted for another award

I declare that no material contained in the thesis has been used in any other submissions for an academic award and is solely my own work.

Signature of Candidate:

Type of Award: *MSc (Research)*

School: Forensic and Applied Sciences

Abstract

The aim of this study was to confirm the status of Lancashire's endemic freshwater Nemertean – *Prostoma jenningsi.*

Due to human induced environmental change and the degradation of habitats, a vast decline in biodiversity has been witnessed on a global scale, with losses occurring among many native UK species. *P. jenningsi* (known locally as the 'Croston Worm'), is considered to be Lancashire's only endemic species, thought to exist solely in the Clay 'Ole, Bretherton; however, its existence at the site has not been confirmed since 1999. Endemic species are considered to be of intrinsic value to the biodiversity of the UK and *P. jenningsi* was designated a UK Biodiversity Action Plan (BAP) species in 2007 and a Species of Principal Importance to England through the Natural Environment and Rural Communities Act from 2008, but is currently listed in the British Red Data Book as Insufficiently Known.

Nemertea are a diverse group of free-living, benthic, simple soft-bodied acoelomate animals. An eversible proboscis, used primarily in prey capture, is a shared characteristic of the taxon. Nemertea have very few morphological characteristics that can be used for diagnosis of species, genera or even family, thus making taxonomy difficult. Therefore, doubt surrounds existing species descriptions and their relationships.

In 2011, researchers at the University of Central Lancashire (UCLAN) (including the author) initiated a project in conjunction with Natural England and the Wildlife Trust for Lancashire, Manchester and North Merseyside (LWT), to confirm the existence of the 'Croston Worm' at the Clay 'Ole. The initial project was unable to locate a population of *P. jenningsi*; however, subsequent research revealed two populations of *Prostoma* spp at alternative pond locations in Lancashire. Initial DNA analysis revealed the populations to be *Prostoma eilhardi*, a species with known worldwide distribution. A putative, type specimen of *P. jenngsi*, was obtained from the Natural History Museum to allow comparisons with collected specimens; however, due to the age and preservation methods associated with the sample, it was not possible to extract

DNA for analysis. Results from the study raised questions regarding the validity of the designation of *P. jenningsi* as a unique species.

This Masters by research project sought to build upon this preliminary (unpublished) work to confirm the current status of *P. jenningsi,* through the extensive re-sampling of the Clay 'Ole site and expanding sampling to further selected locations across Lancashire.

A population of *Prostoma* spp was located at the Clay 'Ole site and three additional populations recorded at locations in Lancashire. Comparisons of 18s (nuclear) gene and COI (mitochondrial) gene sequences, made with those stored on global databases (GenBank and BOLD), found recovered specimens to be identical to both *P. graecense* and *P. eilhardi.* This questioned the validity of information supplied by the online databases and confirmed the miss-identifications of *P. jenningsi* as a separate species made through traditional histological methods. DNA barcoding, using the COI gene, is considered to be an effective tool in resolving species identity in Nemerteans; however, in the case of *Prostoma* spp, a larger data set may be required to distinguish whether *P. graecense* and *P. eilhardi* are two distinct species. In addition, the limited genetic diversity displayed between samples from Lancashire and locations in both Europe and USA raised further questions related to species dispersal and mode of reproduction. Further DNA research is required in order to address these questions.

In the case of *P. jenningsi,* it is proposed that the current listing in the British Red Data Book and designation under the Natural Environment and Rural Communities Act are no longer valid.

Acknowledgements

I would like to thank my family, friends and supervisory team for their advice, support and patience throughout this research project. It has all been very much appreciated.

Table of Contents

List of Figures

List of Tables

Chapter 1 – An Introduction

1.0 Introduction

Nemertea, often considered a minor phylum, are a diverse group of free-living, benthic, simple soft-bodied acoelomate animals. An eversible proboscis used primarily in prey capture is a shared characteristic of the taxon (Gibson,1972; Sundberg & Gibson, 2008). Due to their predatory nature, Nemertea are considered to play an important role within ecosystems; despite this, they remain a relatively understudied group. Nemertea display very few morphological characteristics that can be used for the identification of species, genera or even families, thus making taxonomy difficult. Therefore, many aspects of their biology and ecology remain essentially, if not completely, unknown and doubt surrounds existing species descriptions and their relationships (Gibson, 1982; 1998; Andrade *et al.,* 2012). In recent years, the use of DNA taxonomy and DNA barcoding have been introduced to aid identification of Nemertea and 'disentangle' relationships (Sundberg*,* 2015).

Nemertea are predominately found in marine and estuarine environments, with a small number known to inhabit terrestrial and freshwater habitats. There are considered to be approximately 22 freshwater Nemertean species, 11 of which belong to the genus *Prostoma*, a group of hoplonemertea that exhibit a worldwide distribution. Despite their worldwide range, *Prostoma*, like all freshwater Nemertea have only ever been found in single localities (Sundberg & Gibson, 2008).

Prostoma jenningsi (known locally as the 'Croston Worm') is thought to be found solely at the Clay 'Ole, Bretherton, Lancashire, UK. The species was first recovered from the pond in 1969 by J. O. Young, when researching freshwater triclads in Lancashire ponds (Gibson, pers comm., 2011a). *P. jenningsi* was recognised to be of the phylum

Nemertea and identification of this ‘new’ species was officially confirmed by Professor Ray Gibson in 1971 (Gibson & Young, 1971). As with many Nemertean species, research focused largely on species description, using aspects of internal morphology; however, some ecological observations were made, relating to seasonal changes in abundance, size-structure and sexual maturation of the population.

Due to human induced environmental change and degradation of habitats, a decline in biodiversity on a worldwide scale has occurred. Such decline and loss have been witnessed among many native UK species (Natural England, 2010). Having yet to be discovered at any other location worldwide, *P. jenningsi* is considered to be the only species endemic to Lancashire (LWT, 2001); however, the existence of *P. jenningsi* has not been confirmed at the Clay ‘Ole since 1999. Such endemic species are considered to be of intrinsic value to the biodiversity of the UK (Natural England, 2010). *P. jenningsi* is currently listed in the British Red Data Book as Insufficiently Known and was designated a UK Biodiversity Action Plan (BAP) species (2007) and a Species of Principal Importance to England through the Natural Environment and Rural Communities Act from 2008 (JNCC, 2015).

In 2011, researchers at the University of Central Lancashire (UCLan) (including the author) initiated a project in conjunction with Natural England and the Wildlife Trust for Lancashire, Manchester and North Merseyside (LWT), to confirm the existence of the ‘Croston Worm’ at the Clay ‘Ole Site. Following the work undertaken in 2011, research expanded to determine the distribution of *Prostoma* species at other pond locations in Lancashire. The author played a lead role in this initial research and this Masters by research project seeks to build upon this initial (unpublished) work to confirm the current status of *P. jenningsi.*

1.1 Aims and Objectives

The main aim of this research project was to locate and confirm the status of *P. jenningsi* with a focus on the Clay 'Ole Site in Bretherton.

The aim of the study was achieved by addressing the following objectives:

1. Undertaking a review of relevant literature, to gain a greater understanding of Nemertean ecology
2. Consolidating previous research / information specifically related to the 'Croston Worm'
3. Sampling for Nemertea at the Clay 'Ole site, where *P. jenningsi* was originally recorded
4. Expanding sampling for Nemertea to other selected locations across Lancashire (site selection based on information obtained in (1) and (2)).
5. Undertaking DNA analysis on collected *Prostoma* spp. specimens, to determine whether *Prostoma* from the Clay 'Ole site (if discovered) are distinct to those found both in other locations in Lancashire and worldwide (by comparing DNA sequences with those recorded on validated genetic databases (e.g. GenBank; BOLD).

1.2 Structure of Thesis

This thesis begins with a review of relevant literature, in order to place all aspects of the study in context (Chapter 2). Chapter 3 then details preliminary research that has contributed towards, and formed a basis for, the current study. Chapter 4 provides a description of the methods used in the collection and laboratory analysis of Nemertea, followed by associated results. Chapter 5 focusses on DNA analysis of collected specimens. Results from the study and associated wider implications related to

identification of *Prostoma* spp are discussed in Chapter 6 before recommendations for further study are provided and a conclusion summarising the research findings.

Chapter 2 – Literature Review

2.0 Literature Review: Introduction

The literature review below sets the context of the research. Firstly, it explores the general 'idea' of conservation and its importance to *P. jenningsi*, discussing its current conservation status. It then introduces the phylum Nemertea and discusses its classification, along with aspects of ecology, before focussing on freshwater Nemertea, the *Prostoma genus* and specifically *P. jenningsi.* The complexities surrounding *Prostoma* taxonomy, are explored, before the review focusses on species identification through histology and DNA barcoding. Finally, suitable sampling methods for the collection of Nemertea are reviewed.

2.1 Conservation

Nature Conservation is the process by which things of 'value' are protected and managed. These values are thought to have emerged through ecological science. Through its relationship with science, conservation is able to 'act' for nature in a complex, modern and industrial society. Conservation is an active process, surrounding beliefs and ideas supported by both science and law, that embody nature as being both threatened and good (Hinchcliffe, 2007). Conservation is carried out by a diverse range of organisations and through a wide variety of activities, depending on the 'specific' conservation need. The main focus of conservation work aims to maintain and increase healthy species and habitat conservation status, to prevent, protect and restore habitat loss and degradation, and to educate society towards a more sustainable use of natural resources, in order to prevent further loss of biodiversity (O'Connell & Yallop, 2002).

2.1.1 International Conservation

The International Union for Conservation of Nature (IUCN), established in 1948, is the oldest and largest of the world's global environmental organisations, with biodiversity conservation being central to its mission. It acts as an impartial body to governments, NGOs, business, scientists and local communities, seeking to find practical solutions to conservation and development challenges (IUCN, 2016).

Established in 1964, the IUCN's Red List of Threatened Species is the most comprehensive resource, encompassing the global conservation status of plants and animals. It highlights species threatened with extinction and supports and encourages their conservation (Rodrigues *et al.,* 2006). The Red List was created in an attempt to improve the knowledge base of global biological resources (IUCN, 2004) and has become an influential instrument for conservation planning, management, monitoring and decision making (Rodrigues *et al.,* 2006). The IUCN (2012) considers over 50% of animal species to be vulnerable to extinction, endangered or critically endangered, and expects that over the next few decades, a significant proportion of the planet's taxa will be threatened with extinction. Using IUCN Red Data Book criteria and categories, conservation status was assigned to certain British flora and fauna (JNCC, 2013).

2.1.2 UK Biodiversity Action Plan

Published in 1994, the UK Biodiversity Action Plan (BAP) was the response of the UK Government to the signing of the Convention on Biological Diversity in Rio de Janeiro, 1992. The UK BAP described the biological resources of the UK and provided in-depth plans for the conservation of these resources, with the aim of reducing biodiversity loss within the UK over a 20-year period (JNCC, 2015). Drawing on data and

information gathered from various organisations throughout the UK, the UK BAP listed species and habitats with priority for conservation, for which individual action plans were drawn up (O'Connell & Yallop, 2002; Anderson *et al.,* 2009). Consolidating species information in the UK BAP, allowed for a broader level of knowledge concerning individual species and/or habitats and a multidisciplinary approach to conservation and biodiversity, with focus remaining on priority species within the UK (O'Connell & Yallop, 2002).

The UK BAP is no longer in operation, superseded in 2012 by the 'UK Post-2010 Biodiversity Framework', which follows the new strategic guidelines in the UK towards biodiversity, with a focus on managing the environment as a whole (JNCC, 2015).

2.1.3 The Conservation Status of *P. jenningsi*

Having yet to be discovered at any other location worldwide, *P. jenningsi* is considered to be the only species endemic to Lancashire (LWT, 2001). Such endemic species are of intrinsic value to the biodiversity of the UK (Natural England, 2010); as a consequence, *P. jenningsi* was listed as a priority species under the UK BAP scientific criteria of 'international threat'. The species is currently listed in the British Red Data Book as Insufficiently Known (JNCC, 2015), and since 2008 it has been designated a Species of Principal Importance to England through the Natural Environment and Rural Communities Act (Natural England, 2010).

Such recognition is important, as, at the time of discovery, the single pond location in which the species had been found did not fall under any legal protection; therefore, the population could potentially have declined, or been wiped out, should any adverse actions have been taken on the pond, such as the addition of chemical herbicides (JNCC, 2010).

2.2 Nemertea

The phylum Nemertea, found predominately in marine or estuarine environments, (Gibson, 1972; Turbeville, 2002) is made up of approximately 1,150 nominal species, distributed between 250 genera (Gibson, 1995). Nemertea are unsegmented, bilaterally symmetrical, acoelomate animals with a gut, possessing separate mouth and anus and a blood vascular system. They range in length from a few millimetres to about 30 metres, with a width that rarely exceeds a few millimetres (Turbeville, 2002). All Nemertea possess a characteristic eversible proboscis, situated dorsal to the gut in an enclosed tubular cavity, the rhynchocoel. The proboscis is used predominately for prey-capture (Gibson, 1972), with the majority of Nemertea considered to be active carnivores (Caplins *et al.,* 2012).

2.3 Classification

Nemertea have been recognised as a distinct taxon for more than 150 years and as a phylum for circa 60 years, distinguished by the presence of the aforementioned eversible proboscis encased within a rhynchocoel. It is, however, suggested that relationships and systematics are uncertain, due to often vague and incomplete published species descriptions, based largely around external characteristics (Strand & Sundburg, 2005; Sundberg *et al.*, 2010). Although descriptions have advanced to include morphological characteristics, in many cases this is still deemed inadequate, due to the low number of these (particularly within the smaller species). In addition, there are problems associated with histological analysis, particularly during fixation, due to the soft-bodied and contractile nature of Nemertea (Strand & Sundberg, 2005; Andrade *et al.*, 2011) – see also section 2.10.

This traditional classification system, developed by Stasny-Wijnhoff (1930), divided Nemertea into two classes: Anlopa, which were made up of Nemertea that possessed a proboscis with no armament (stylet), and Enlopa, which possessed a proboscis armed with one or more stylets. Anlopa were further divided into two orders – Paleonemertea and Heteronemertea; and Enlopa into Hoplonemertea and Bdellonemertea. Hoplonemertea were further subdivided into Monostilifera and Polystilifera (Gibson, 1982; Andrade *et al.*, 2012; 2014).

2.4 Nemertean Ecology

Nemertea have been studied for more than a century, with records indicating that species are dispersed over a wide range of habitat types; however, many aspects of their biology and ecology remain essentially, if not completely, unknown (Gibson, 1982; 1998). With the exception of the true pelagic species, that float inertly or swim slowly, Nemertea are considered to be benthic in habitat, living beneath embedded boulders and rocks, within algae, burrowing into sands, mud and gravels, with a few forms living in tubes or inhabiting empty burrows of polychaetes or amphipods (Gibson, 1972). There are a number of known terrestrial forms and a small number recorded in freshwater environments (Turbeville, 2002).

Ecological studies relating to Nemertea have largely focused on their feeding behaviour, or prey interactions, supplemented by laboratory experimentation (McDermott & Roe, 1985), with research primarily focused on marine and estuarine species. Little is known about the tolerance of Nemertea to environmental factors; any insights into this area have often accumulated as a result of investigations with different objectives (e.g. Gibson, 1972; Zhao & Sun, 2006). Research has been carried out on the effects of salinity, temperature and pH (e.g. Zhao & Sun, 2006). In addition,

community studies (marine/estuarine) have been undertaken concerning the roles of Nemertea (McDermott & Roe, 1985; Wilson 1991). It is not clear how transferable results from these studies are to freshwater species. Laboratory experiments have suggested that suctoral hoploNemertea can exercise a potentially significant effect on benthic communities. It is thought that Nemertea may not be important prey for epi-benthic predators and thus their populations may be regulated by other factors (McDermott, 1993). A study by Cook & Herrmann (1997), regarding the feeding behaviour and habitat of a population of *Prostoma graecense,* however, only recorded *P. graecense* in locations where they appeared to be at the top of the food chain. Populations were not recorded in locations where potential predators, such as fish and dragon-fly larvae, were present.

2.5 Freshwater Nemertea

Only 22 freshwater Nemertean species having been identified, representing less than 2% of the total number recorded (Turbeville, 2002). Most freshwater species belong to a monotypic genus with their distribution being fairly sporadic and, in most cases, species are known only from single localities (Gibson, 1982; Gibson & Moore, 1976; Sundberg & Gibson, 2008). The freshwater genus *Prostoma*, currently thought to contain 11 species, is an exception to the freshwater form, as it is known to be widely distributed on a global basis, although locally sporadic in occurrence (it is important to note, however, that this known occurrence may be largely reflective of sampling efforts, rather than actual species distribution). Two particular species, *P. eilhardi* and *P. graecense* have been reported to range from Europe to Africa, with *P. eilhardi* also occurring in Brazil and possibly Argentina and Uruguay, and *P. graecense's* range extending to the British Isles, Japan, America, Australia and Tasmania. The origins of

these species are not known, but possible causes that have led to their widespread distribution include the importation and exportation of freshwater vegetation or being carried on the feet of water birds (Gibson, 1972; Sundberg & Gibson, 2008).

There are currently two hypotheses surrounding the evolutionary routes taken by Nemertea to colonise freshwater (and terrestrial) habitats. One suggests that marine Nemertea colonised the land before making a transition to freshwater habitats (Moore & Gibson, 1985); the other proposes marine Nemertea moved from fully marine to the spray zone, where freshwater saturation may occur, before invading the land (Smith, 2001). It is, however, more likely that Nemertea have come to be found in both freshwater and terrestrial environments, having followed a number of different routes of transition (Gibson, 1988), an idea supported by recent molecular analysis (Andrade *et al.,* 2012). Moore & Gibson (1973) suggest that *Prostoma* may have evolved from marine ancestors, their origins being suggested from the known habitats of near relatives (see *figure 1*).

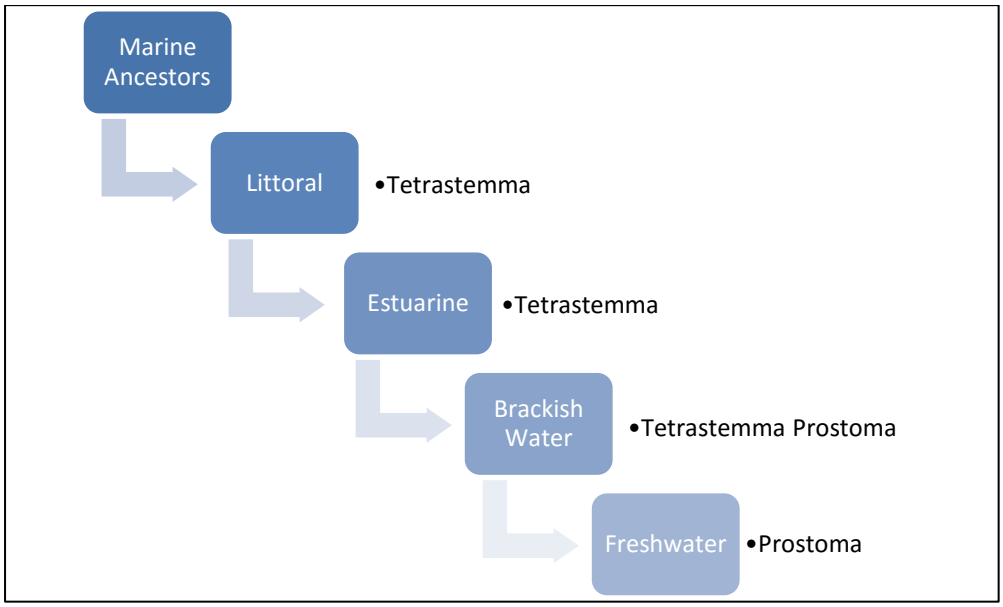


Figure 1 – Transitional Route of Prostoma (adapted from Moore & Gibson, 1973)

Evidence of this deviation can be seen in the occurrence of *P. graecense* in brackish regions of the Gulf of Finland and the tolerance to seawater immersion shown by terrestrial Nemertea; *Geonemertes agricola* (Moore & Gibson, 1985) and *G. nightingaleensis* (Gibson, 1972).

Freshwater species are thought to be predatory, 10-40 mm in length and of pink colouration, although differences in colour from sandy-yellow to red, and even green, have been reported; and in most cases colouration tends to vary according to the maturity of the animal. *Prostoma* have been recorded feeding on oligochaete worms, but are also known to feed on crustaceans, nematodes, tubellaria, midge larvae and other small invertebrates. Feeding is thought to be more intense at night (McDermott & Roe, 1985; Thorp 1991; Cook & Herrmann, 1997).

Prostoma, like other freshwater and terrestrial species, are able to secrete a sticky mucus that may be used for both adhesion and lubrication. Moore & Gibson (1985) consider that it may be multi-functional; it may be used as a form of defence against predators; it is considered to aid cross-fertilisation and may also aid dispersal.

Habitat requirements for freshwater Nemertea are poorly understood (Cook & Herrmann, 1997). Species are considered to be free-living benthic animals confined to small permanent, weed-dominated ponds and sluggish backwaters of streams and rivers. Thorp (1991) suggests that *Prostoma* are clearly associated with littoral habitats and found a significant number of *Prostoma* species to be associated with filamentous algae in lakes. Furthermore, Laumer (2012, pers comm.) suggested that *Prostoma* seem to have slightly restricted thermal preferences. Throughout his study, he found them to occur most often in springs and seeps (see also Cook & Herrmann, 1997), in cool, well-oxygenated, flowing reaches of creeks, where leaves

or aquatic plants have accumulated, and in standing water, mainly in the early spring and autumn, when it grows quite cool (Laumer, 2012, pers comm.).

Freshwater species often exhibit limited local distribution, located in one small habitat, when nearby areas appear to be virtually identical; this is evident in the case of *P. jenningsi,* where the species is believed to have only been found in one particular bed of *Phragmites* at the Clay 'Ole site in Lancashire, UK (see section 3.2.1). When present, freshwater species may usually be found amongst submerged vegetation and particularly on the lower surface of floating leaves (Williams, 1980; Thorp, 1991; Cook & Herrmann, 1997).

2.6 *Prostoma eilhardi*

Figure 2 - Prostoma eilhardi (adapted from Andrade *et al*., 2012)

As with all freshwater Nemertea, research relating to *P. eilhardi (*see *figure 2)* is limited, with most of that available focusing on taxonomic description; however, certain aspects of the species' autecology have been established.

P. eilhardi was originally described by Montgomery (1894) as *Stichostemma eilhardi.* In external morphology, *P. eilhardi* resembles both *P. jenningsi* and *P. graecense;* it rarely exceeds 20 mm in length, with a diameter of 0.3-0.4 mm. It is characteristically reddish-brown in colour but has also been reported as yellowish, greenish-brown and bright orange. The number of eyes is thought to vary with age; younger individuals often have only four and there are more commonly six in adults. Identification of the species relies on aspects of its internal morphology and must be confirmed through histological and DNA analysis (Young & Gibson, 1975b; Strand & Sundberg, 2005).

P. eilhardi has been reported to range from Europe to Africa, with reported occurrences in South America (Brazil and possibly Argentina and Uruguay); it is thought to be the most widely distributed of the *Prostoma* species.

Research undertaken on two populations in Kenya suggests that when animals have attained a length of 6 mm, they are sexually mature. Like all freshwater Nemertea, *P. eilhardi* is hermaphroditic, an adaptation thought to be significant to the successful widespread distribution of all *Prostoma, w*hich are often found in isolated habitats (Gibson & Moore, 1985). Reproduction in *P. eilhardi* is considered to be sexual, with (in favourable conditions) up to 210 eggs being produced per reproductive cycle throughout the year (Young & Gibson, 1975). It is thought that reproductive activity may be associated with water temperature, being most intense during temperature peaks (Young, 1975). Despite this, evidence suggests that reproduction continues throughout the year, due to the continued presence of small animals (1-3 mm in length). It is important to note that such observations may only be reflective of conditions experienced in the Kenyan populations. Despite observations made regarding the reproduction of the species, its lifespan remains unknown (Young & Gibson, 1975).

2.7 *Prostoma graecense*

Figure 3 - Prostoma graecense (adapted from Spacek, 2017).

Although little is known about freshwater Nemertea, some details have been recorded for the North American species *Prostoma rubrum* (now reclassified as *P. graecense* (see *figure 3),* due to vague species description (see Gibson & Moore, 1976; Poluhowich 1968). This species was found

commonly amongst filamentous algae but also occurred regularly upon other aquatic plants. Individuals appeared to be restricted to the pond marsh interface, where an abundance of organic matter was noted. Water temperature was reported to vary from 2-19 ˚C, with a reasonably constant acidity (pH 5). The species was reported to be abundant within the pond's margins throughout the year; however, evidence of seasonal migration to deeper waters was also noted (Gibson, 1972).

P. graecense possesses a worldwide, though sporadic, distribution (as mentioned above), reported to be found in freshwater streams, rivers and ponds (Gibson, 1982; 1995). Specimens recovered in Japan were found in rice fields and ponds, especially mineral-rich water (Iwata, 1954). In Tasmania, populations were found on decaying leaves and stems of reeds and rushes growing in what were reported to be somewhat stagnant areas of a freshwater creek; they were also reported to have been found in empty cells in plant tissue, where eggs had been deposited (Gibson & Moore, 1976). In addition, Cook & Herrmann (1997) recorded populations within spring seepage near Bogg's Creek, Colorado, where they reported populations to be sparse, with some sample sites lacking any Nemertea. A single Nemertean was also recovered from the Arkansas River. Specimens were found within very small habitats and thought to exist within patches of moss. No specimens were recovered during the summer months; it was considered that elevated summer temperatures may have caused the Nemertea to retreat into crevices.

2.8 *Prostoma jenningsi*

P. jenningsi has only ever been recorded at the Clay 'Ole, Bretherton, Lancashire, UK; however, its existence has not been confirmed at this location since 1999. *P. jenningsi* was first recovered from samples collected at the study site during July 1969 as part of a study concerning freshwater triclads in Lancashire ponds by J. O. Young (Gibson,

pers comm., 2011). *P. jenningsi* was recognised to be of the phylum Nemertea; identification was later officially confirmed with the help of Gibson in 1971 and type specimens are held at the Natural History Museum, London. The species, as with all other freshwater Nemertea, is not considered to hold any economic or medical value (Sundberg & Gibson, 2008).

P. jenningsi is a small slender hoplonemertean of elliptical body section. The species possess four to eight eyes (most commonly six) with the anterior two pairs being most well developed and generally larger than the others. Body colouration is thought to be dependent upon age and size, with no specimens below 6 mm in length displaying the full adult colouration. Young worms, approximately 0.5 mm in length at hatching, are an opaque-translucent white. From about 4 to 6 mm in length, they gradually assume a yellowish hue, sometimes with a nervous system tinged pink or red, which generally deepens with age to the adult colour of darkish-yellow to pale reddish-brown. The maximum recorded length of the species is 18 mm, as measured in normal locomotory extension (Gibson & Young, 1976). Exact locomotory movement of the species is unclear; however, amongst the terrestrial and freshwater genera, it is considered that adult Nemertea can only crawl, while small juveniles also swim, and the use of the proboscis for rapid forward movement has been reported (Moore & Gibson, 1973).

P. jenningsi is a true hermaphrodite and thought capable of breeding throughout the year; however, reproduction is considered to be more intensive between late autumn and early spring, previous studies having indicated that greater numbers of the species are found at this time, with fewer numbers evident within the summer months. It is also considered that a migratory event into deeper waters may occur during this period, thus accounting for fluctuations in numbers at this time. Despite this, Gibson & Young, (1976) stated that testes could be found throughout the year, and, on occasions, the

presence of sperm on the outer egg membrane, or in the ovarian chambers, was observed. They remained unsure, however, as to whether cross or self-fertilisation was the normal means of reproduction. As yet, neither the rate of reproduction nor the lifespan of the species is known.

On internal examination, it is the presence of eleven proboscidial nerves alone that was used to distinguish *P. jenningsi* from all other species within the genus – external characteristics, appear identical (Gibson & Young, 1971). *P. jenningsi* is thought to be predominately associated with marginal vegetation, particularly beds of *Phragmites* within the Middle Bay of the Clay 'Ole site (see *figure 4* on Pg. 28), where it feeds on Oligochaeta, particularly Naididae (Gibson & Young, 1976).

With the exception of research, outlined in Chapter 2, the only other instances of *Prostoma* having been reported within the UK have been in Cambridgeshire, with *P. graecense*, collected from the River Cam, in 1944 and *Prostoma* spp reported from the River Ouse in 1972 (Gibson & Moore, 1976).

2.9 Taxonomy of the genus *Prostoma*

Taxonomy is the biological discipline that identifies, describes, classifies and names species and other taxa, both extant and extinct (Padial *et al*., 2010). Taxon identification plays a fundamental role in taxonomy, systematics, ecology, and biodiversity conservation; without identification, organisms cannot be conserved, nor can stability or change of animal and plant relationships be monitored (Tautz *et al.,* 2003; Mace, 2004; Sundberg *et.al.,*2016a). Existing taxonomy dates back to the introduction of the binomial naming system by Linnaeus in the 1750s and now encompasses work collected over the last circa 250 years (Padial *et al.,* 2010).

Species taxonomy is faced with the task of fully integrating new methods, data and theory from disciplines that study the origin, limits and evolution of species (Padial *et al*., 2010). Sundberg *et al*., (2016a) suggest that confusion surrounds many species descriptions and to ensure scientists are discussing the same individual species, the correct and accurate identification, the use of correct taxonomic name of formerly named species, and description and naming of new species are essential. If generic processes are not followed, the value of data (i.e. molecular, morphological and ecological) will become diminished (Sundberg *et al.*, 2016a).

Traditionally, identification of Nemertea is based on description of external characteristics, followed by details of internal anatomy (achieved through histological sectioning). Sketches or pictures accompany such descriptions highlighting the shapes and positions of such internal characteristics (Roe, *et al.,* 2007; Strand & Sundberg, 2011). More recently, however, DNA-based species identification, in particular DNA 'barcoding', is becoming a valuable tool, not only in species identification and classification, but also in answering questions surrounding the ecology and evolution of natural systems (Kress *et al.,* 2015). See section 2.11, for further discussion.

2.10 Histology

Histology is the general term for the study of tissue, viewed using light microscopy. It is the process by which cellular components are artificially stained, following their preservation (in life-like condition), in order to reveal the morphology of internal structures (Sundberg & Strand, 2010). Nemertea are considered to be problematic to identify through histological investigation, due to their contractile nature during the fixation process; thus, anatomical characteristics are thought to become difficult and

time-consuming to interpret (Sundberg *et al.,* 2010; Andrade *et al.*, 2011). It is considered that, even if carefully fixed, variation (or interpreted variation) in internal characters within a species can be high and that such variation can be mistaken for 'new' species where few samples are available (Sundberg & Strand 2010; Sundberg *et al.,* 2016b).

In terms of *P. jenningsi*, Gibson & Young (1971) experienced difficulty, when examining sections, in identifying the species' distinguishing 11 proboscidial nerves. They stated that many of the sections were 'far from clear, even when under oil immersion' and that there was 'variable cross-sectional appearance', related to the contraction of the proboscis.

2.11 DNA barcoding

A DNA barcode is essentially one gene sequence, or a few short gene sequences, (648 base-pairs) of DNA, taken from a standardised portion of the genome that can be rapidly sequenced after PCR (polymerase chain reaction) amplification to identify animal species (Waterton *et al.*, 2013; Kress *et al.*, 2015). DNA barcoding, using the mitochondrial gene cytochrome *c* oxidase I (COI), was first proposed by Hebert *et al.,* (2003a), who considered barcoding to be a reliable, inexpensive and relatively easy process that would both eliminate issues surrounding animal species identification and produce significant insights into molecular evolution and the diversification of life (Hebert, *et al.,* 2003a). COI regions were found to be ineffective when used for the identification of plants and fungi, as, in evolutionary terms, they possess a slower rate of change, thus exhibiting a lower rate of nucleotide substitution within the mitochondrial genome. Genes taken from the chloroplast region (matK and rbcL) are,

however, effective and used together in plant identification through DNA (Waterton *et al*., 2013).

2.12 The 'Barcoding Gap'

The DNA barcoding gap is the term used to describe genetic difference within, and between, species. Hebert *et al*., (2003b; 2004) consider that inter-specific genetic variation exceeds intra-specific to such a degree (a minimum of 10 times greater) that a clear gap exists, thus enabling the identification of individuals to species with an insignificant error rate (Hebert *et al*., 2003b; Hebert *et al*., 2004). Therefore, species identification using DNA barcodes may only be reliable if a significant difference between the average intra-specific and the average inter-specific genetic distance can be consistently detected (Candek & Kuntner, 2015). Kvist (2013) argues that, for true species identification through DNA barcodes to occur, prior knowledge of inter-specific and intra-specific genetic variation within a target group must be obtained, as well as the application of a 'well sampled genetic data base'. Kvist suggests that, for Nemertea, DNA barcoding may work, as a distinct barcoding gap may exist; however, as yet there is insufficient reliable data, partly due to poor species level identification (Kvist, 2013).

2.13 DNA Databases

With the increase in use of DNA barcodes for species identification, online databases have been developed, which enable such information to be analysed, monitored, stored, shared and accessed. The Barcode of Life Database (BOLD, Ratnasingham & Hebert, 2007) stores specimen data and images, as well as sequence data, specific to DNA barcoding (Waterton *et al*., 2013). BOLD offers reliable information, in which users can have confidence, due to database checking mechanisms; an automated

Barcode Index Number (BIN), based on sequence divergence of the COI barcode region, is assigned to data sets analysed directly in BOLD. Analysis can be carried out to determine whether specimens assigned to the same species can be found within the same BIN and the taxonomic reliability of each BIN can be evaluated (Ratnasingham & Hebert, 2013). It is important to note, however, that Ratnasingham & Hebert (2007) state that 'barcode records that have not been through full validation will derive from misidentified specimens or will reflect analytical errors.' GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez) is a publicly available genetic sequence database, comprised of all available DNA sequences (including those in BOLD), run through the USA's National Centre for Biotechnology Information (NCBI) (Waterton *et al*., 2013). Submissions are made individually from laboratories; however, although comprehensive, not all sample data uploaded to the site have been run through external verification processes (as would be associated with voucher specimens); thus, it is considered that data error may occur (Shen *et al.,* 2013).

2.14 DNA and Nemertea

Kvist *et al.* (2014) suggested that, in terms of Nemertea, DNA barcoding is an instrument that can quickly and accurately identify specimens which, as a phylum, are morphologically challenging. Recent studies of Nemertea have utilised DNA sequence information to aid research surrounding their identification and classification (Sundberg *et al.,* 2010). Acquired DNA sequences have been placed on the online databases, GenBank and BOLD, each with a unique accession number (Andrade *et al.,* 2011). Such work has emphasised the poor taxonomy within the phylum Nemertea and highlighted that morphological differences are not consistent with evolutionary lineages. It is thought that such morphological differences may be simply due to intra-

specific variation, or the changes that occur as the animals mature - for example, variation in shape and colour.

To date, Nemertean studies have used various DNA regions: nuclear 18s rRNA and 28s rRNA genes and histones, H3 and H4 genes, in addition to mitochondrial 16s rRNA and COI genes, when looking at relationships within the phylum (Andrade *et al.,* 2011). Mitochondrial DNA is inherited exclusively from the maternal lineage and exhibits a rapid pace of evolution, whereas nuclear genes are inherited equally from each parent; thus, both nuclear and mitochondrial genes are selected in order to gain a full understanding of the evolutionary history (Avise, 2009). DNA data has a significant role to play in the future identification of Nemertea, particularly in those that do not possess unique and distinct external characteristics (Andrade *et al.*, 2011).

2.15 Sampling Techniques for freshwater invertebrates

There are a number of methods that can be used to sample macro-invertebrates in freshwater habitats; however, through a review of the literature, it is apparent that there is no standardised procedure for the sampling of freshwater invertebrates in lentic conditions; nor is there a standard procedure for sampling within marginal vegetation, reed beds and substrata. The Freshwater Biological Association (FBA) suggests that the most appropriate method depends on the purpose of the sampling and that recorders tend to develop their own techniques, tailored towards the specific organisms in which they are interested (FBA, 2016). O'Connor *et al.,* (2004) suggest that, within the literature, there has been a disproportionate focus placed on lotic macro-invertebrate sampling techniques in comparison with lentic methods, due to the use of aquatic invertebrates for biological monitoring of stream and river quality, such as RIVPACs adopted by Great Britain (Cox *et al*, 1997).

2.15.1. Net sampling

The pond net is possibly the most popular device employed in freshwater sampling; however, while procedures have been outlined for pond net sampling of lotic systems, the same is not true of lentic systems. A number of varying techniques are thus described in the literature. O'Connor *et al.* (2004) suggest that pond nets may be swept or 'shuffled' (in a modification of the lotic kick-sampling method) and the size of the sample determined by time, distance, area or number of sweeps, with sweeping being best suited to macrophyte beds and soft substrata and 'shuffling' best when dealing with stony or gravelly substrata (see also Mackley *et al.*, 2010; Bilton *et al.,* 2006). Although a widely used method, the data obtained cannot be considered quantitative and questions can be raised about its effectiveness, when sampling for slower-moving and bottom-dwelling animals (O'Connor *et al.*, 2004; Sychra & Adamek, 2010).

When investigating *P. jenningsi,* Gibson & Young (1971) collected specimens by means of a standard FBA zooplankton net, mounted on a square frame. The net was used to sweep through the marginal vegetation, with which the Nemertea were predominately associated (particularly the beds of *Phragmites*), and to scoop up the substrata found in the locality of the plants (Gibson & Young, 1971). The procedure was carried out for a five-minute period in each of the reed beds sampled. Collections were placed in 3-litre narrow-necked glass containers, which were then returned to the laboratory and left to stand overnight, at room temperature, allowing the oxygen in the water to become depleted. Animals present within the containers would move up, on, or near to the surface of the film due to the depletion in oxygen, enabling specimens to be easily seen and removed. Samples were then subjected to a flotation technique (see Gibson & Young, 1971) for the separation of any individuals previously missed. Gibson & Young acknowledged that, due to difficulties of sampling among reed roots,

the data they obtained could not be considered as quantitative; however, they stated that it was hoped that any changes in relative abundance might be discernible (Gibson & Young, 1971).

2.15.2 Sediment corers

Sediment corers have been used when sampling for macro-invertebrates in aquatic environments, particularly those associated with substrata and submerged roots. Corers can be used to both extract samples, including plant stems and submerged roots, and take stand-alone sediment samples. The advantage of coring devices is their ability to allow sampling to occur at different levels: in water, at the water-mud interface and along a vertical profile within sediments, which should, theoretically, remain undisturbed. Various sediment corers are available, such as gravity corers, piston corers and box corers (Jones *et al.,* 2000). Soumille & Thiery (1997) developed a sediment corer specifically for sampling invertebrates at different levels of rice plants within a shallow rice field, allowing stratified sampling to occur at varying vertical depths. Disadvantages of sediment coring techniques include the narrowness of the cores being extracted and the need for many replicates to be taken, in order to gain a true representation of a given study area and provide comprehensive data for a thorough site survey; such methods are often time-consuming.

2.15.3 Grab Samplers

Grab samplers, such as Ekman grab, van Veen grab and Surber samplers, are considered a good technique for sampling sediments that accumulate under water, working best in fine sediments, such as muds and sands. Grabs collect bulk sediment using a scoop or bucket lowered to the bed with varying mechanisms, depending on the design. Such samplers allow sampling to be rapid and efficient; as large quantities are able to be collected per sampling attempt (sample size is dependent on size of

grab being used). The use of such samplers is, however, not best suited to areas of dense vegetation, or where samples are required to be taken between plants. Although they may result in the capture of sediment-dwelling organisms, any animals present within the water column, and on submerged reeds, are likely to be missed (Jones *et al.*, 2000). Pauw & Vanhooren (1983) found that the diversity of species in hand net samples was often greater than in samples taken with grab sampling devices. Although such devices can be effective, they are often more suited to areas of open water at greater depths and where techniques such as net sampling cannot be applied.

2.15.4 Box Samplers

Box sampling methods can be used to provide quantitative data when sampling for aquatic invertebrates, as they provide a known area and volume for each sample collected; they also allow for the inclusion of vegetation within the sample area. Like net sampling, various box sampling techniques exist, with no standard method apparent. Storey (2007) outlines a technique in which a round open-bottomed barrel was placed around plants and into the water. Plant parts above the top of the barrel were removed and discarded, while the remaining plants were cut at ground level and retained for examination as part of the sample. Water within the barrel was then poured through a sieve into a pail, in order to separate any animals present. Samples were then returned to the laboratory for further investigation. O'Connor *et al.* (2004) adopted a slightly different approach. A box frame was created by cutting out the bottom of a plastic storage box, which was lowered into the water and held firmly against the substrata; any organisms within the area were then removed, using an aquarium-style fish net. Samples were then returned to the laboratory. Although both of these methods allow for quantitative data to be obtained, they also present similar problems to net sampling, in that organisms may be missed. They can also be difficult to use

and require more than one person to carry out sampling. Gerking (1957) developed a box sampler specifically for surveying benthic macrofauna and phyto-macrofauna within the littoral zone of lakes. The box, consisting of a metal frame with mesh sides, is placed over the vegetation and into the water. Vegetation is then cut as near to the substrata surface as possible. A sliding door is then closed across the bottom of the box and the box lifted from the water. The contents are then placed into a bucket and returned to the laboratory for further examination. This model has more recently been adapted to include poles for fixing the frame into the substratum and the sliding door replaced with a sliding cutting device. These additions allow for the plants to be cut below the surface and any organisms dwelling within the substratum to be collected (Sychra & Adamek, 2010).

Chapter 3 – Preliminary Research

3.0 Preliminary Research: Introduction

During the summer of 2011, a student internship was undertaken (by the author) at UCLan, in conjunction with LWT, investigating the 'Croston Worm' (*P. jenningsi*), which had not been confirmed at the Clay 'Ole, Bretherton, since 1999. The key objectives of this initial project were to: 1) Compile information on the ecology of freshwater Nemertea and the history of the Clay 'Ole site; 2) Systematically survey the Clay 'Ole pond and establish the presence of the species, estimating, if possible, its population density; and should *P. jenningsi* populations be located, the priority of the project was to 3) Secure populations by translocation to similar locations.

Following a number of failed sampling attempts to recover *P. jenningsi* from the Clay 'Ole, the project was developed to include the use of alternative sampling methods and the investigation of further sampling locations in the locality, as outlined in this chapter.

This chapter is intended as a summary of the preliminary stages of the research project and should be read in conjunction with detailed project reports produced for the project partners (Natural England and LWT) supplied in Appendix 1.

3.1 Stage 1: Initial project

An initial search of the literature was undertaken, to gain a greater understanding of *P. jenningsi*, freshwater Nemertea and the phylum Nemertea as a whole; this highlighted the complexities of the phylum and gaps in the research, such as limited knowledge of the species' ecology. In addition, appropriate sampling methods for the recovery of specimens were also explored and a detailed site description of the Clay 'Ole developed.

3.1.1 The Clay 'Ole

The Clay 'Ole (SD48566,19839) is a Biological Heritage site made up of a flooded brick pit, surrounded by species-rich damp grassland and scrub, amounting to approximately 8 ha (Lancashire County Council, 1999). The pond itself is made up of three interlinking basins resembling an 'E' – shape (see *figure 2*).

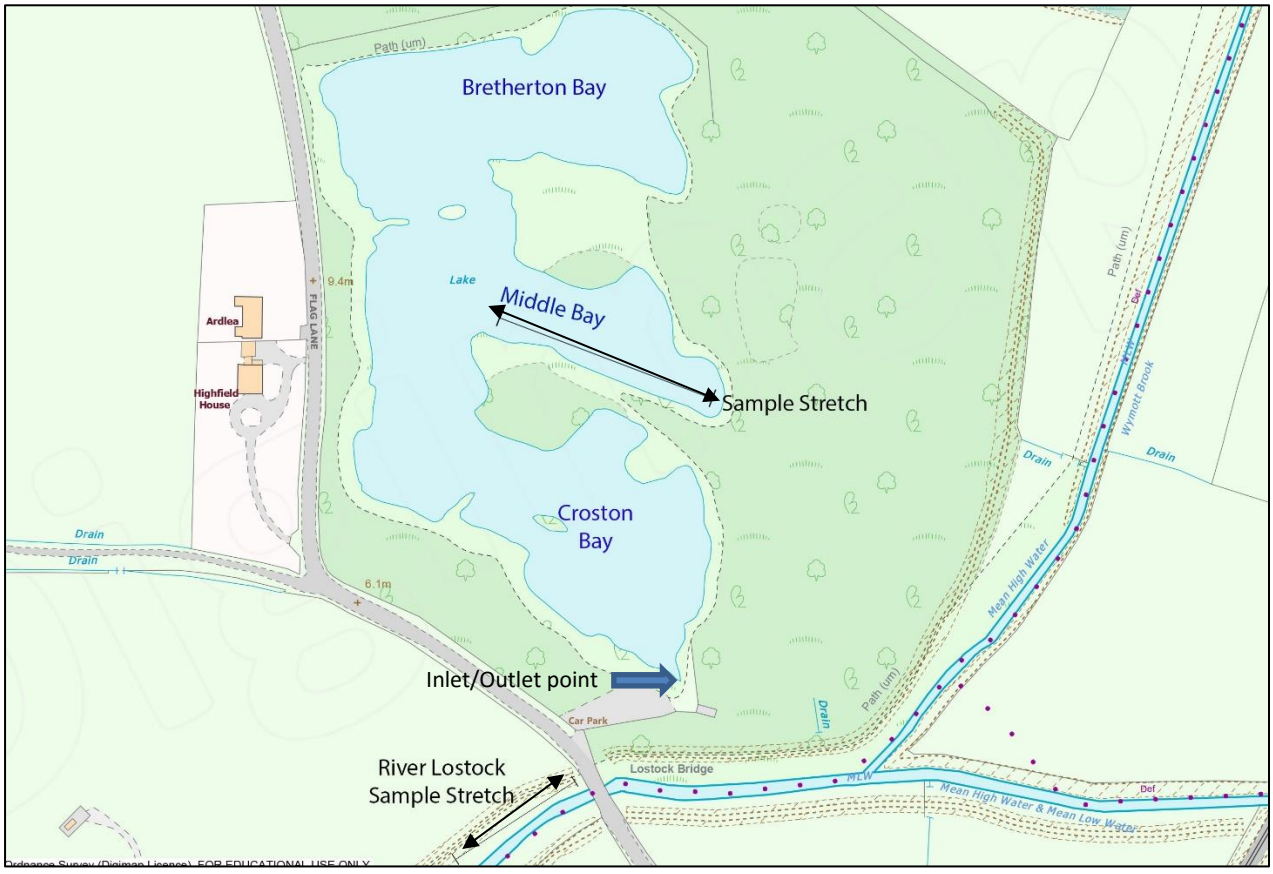


Figure 4 – Clay 'Ole and River Lostock (adapted from Edina Digimap)

The water within the littoral zones of the site is generally shallow (approx. 0.5 m); however, depths in excess of 6 m have been recorded, where clay excavation has previously taken place (Gibson & Young, 1976). The Clay 'Ole is located adjacent to the River Lostock, which is joined by a tributary, Wymott Brook, only a few hundred

metres upstream. The pond is directly linked to the river via a small inlet/outlet pipe that controls the water level of the pond – see *figure 4*.

Research of local archives and historical maps suggested that, prior to clay extraction, a number of small pits were located in areas that are presently occupied by the Clay 'Ole. The convergence of the nearby rivers was within the tidal reach, the surrounding area was marshy and saltings were present – suggesting that any water within the original pits may have been brackish. Despite historical attempts to improve flood defences, the area remained prone to flooding and may have been frequently flooded with tidal water (Quigg & Lowe, 2011a).

Clay extraction began at the site circa 1910, with the current structure of the Clay 'Ole being formed at some point between 1935 and 1955 (Quigg & Lowe, 2011a). Whilst writing on the ecology of *P. jenningsi*, Gibson & Young (1971) suggested that the site was flooded to form the pond approximately 25 years prior to their study, circa 1946. It is likely that the Clay 'Ole was flooded using water from the River Lostock, although suggestions have been made by local anglers that the site is spring-fed (there is no evidence available, however, to support these claims). Further recent attempts to improve flood defences in the area have been undertaken; to date, however, widespread flooding still occurs in the area and, although the 'normal' tidal reach has retreated further downstream, the stretch of river adjacent to the Clay 'Ole remains within the highest tidal point (Quigg & Lowe, 2011a).

The Clay 'Ole has been used as a fishery since approximately 1955 and since 1968 has been leased by Bretherton and Croston Angling Club. During this period, the site has been designated a County Biological Heritage site. The site supports a wide variety of aquatic and terrestrial plants (including several very rare native plants),

animals and bird species. The fishery has thirty-two permanent angling swims, the areas between swims having been left as natural and undisturbed wildlife areas, with the exception of clearance for pathways. Although the use of algaecide for the removal of weeds, particularly *Elodea canadiensis,* has been used in the past, it is thought that its use was limited and did not occur within the middle bay of the pond, the only stretch of the pond in which *P. jenningsi* has been previously found (see *figure 2*). A site survey, carried out by Gibson (1998), confirmed that the use of chemicals on the site had not eradicated the species and the population remained (Quigg & Lowe, 2011a). For a full site description, including historical maps, see Quigg & Lowe (2011a) – Appendix 1.

Questions were raised as to how a population of *P. jenningsi* may have become established at the Clay 'Ole, given that, in evolutionary terms, the pond is a relatively recent addition to the landscape. As discussed in Chapter 2, a number of theories exist, including the import of freshwater vegetation, or being transported on the feet of aquatic birds (Gibson, 1972; Sundberg & Gibson, 2008). Further to this, it was considered a reasonable hypothesis that *P. jenningsi* may have entered the pond, via this route, by means of the adjacent River Lostock (SD48491,19613), particularly given the local flooding events (see Quigg & Lowe, 2011a).

3.1.2 Sample Collection and Processing

Initial sampling for Nemertea at the Clay 'Ole focused on the Middle Bay and also the nearby River Lostock. Having reviewed sampling methods, it was considered a net sampling technique, similar to that used by Gibson & Young (1976), would be most appropriate for the study. If specimens of *P. jenningsi* were to be found, this would allow for comparisons with previous work. Sample sites were chosen for their ease of access and in order to cause the least amount of disturbance.

Sampling took place at the pond / river margins, within stands of *Phragmites*. A five-minute sampling period per replicate, as employed by Gibson & Young (1976), was adopted. During this time, a standard hand net (1 mm mesh) was used in a jabbing/shuffling motion throughout the littoral zone and substrata between the reeds. The contents of the net were emptied into a 3.4 litre, rectangular, air-tight plastic container filled with pond water. The sampling time included the time taken to empty the net contents into the container – this occurred approximately three to five times per replicate, with ten replicates taken per sampling site. On occasions, when the container became full before the five-minute time period had been reached, sampling ceased. Once sampling was complete, samples were returned to the laboratory for processing.

Sampling was found to be difficult, due to the density of the reed beds and the nature of the pond. There was a reasonably shallow 'shelf' within the margins of the pond, which dropped off steeply where clay excavations had occurred, as demonstrated in *figure 5*.

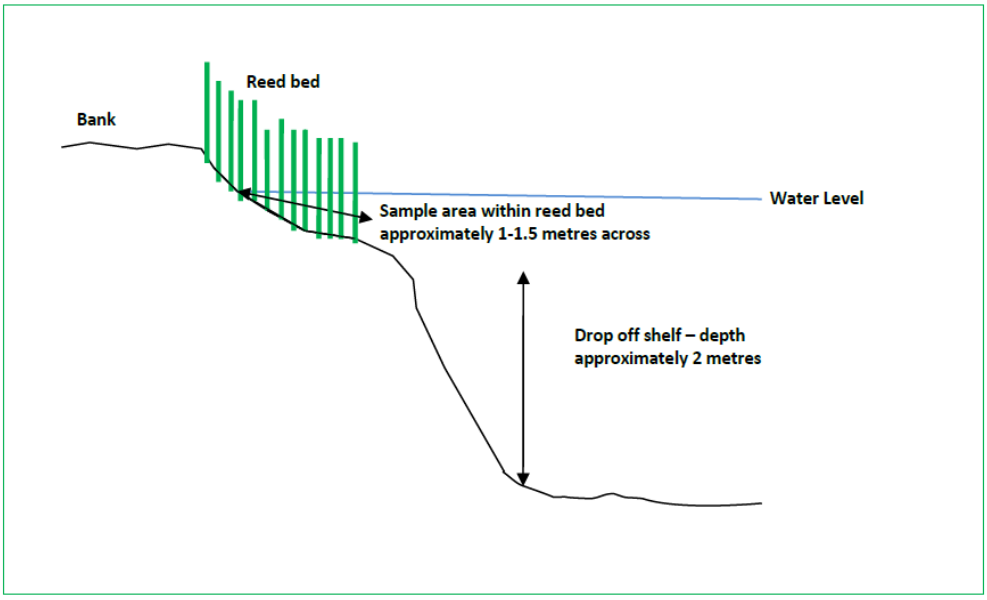


Figure 5 - Profile of the Clay 'Ole - not drawn to scale (adapted from Quigg & Lowe 2011b)

3.1.3 Laboratory analysis

Initially, samples were returned to the laboratory and left overnight for oxygen depletion to occur before the contents were examined, as described by Gibson and Young (1976) – see section 2.15.1. Due to the amount of litter and debris within each sample, this technique was extremely arduous and samples quickly perished. Flotation techniques, as described by Gibson and Young (1976), were investigated; however, the correct procedure could not be ascertained. Therefore, the laboratory technique was revised and the following technique employed throughout the research.

After transporting samples to the laboratory, larger pieces of organic debris, floating at the surface of the samples, were carefully examined and any organisms retrieved, before being removed from the containers. The sealed containers were then left to stand overnight at room temperature, allowing the oxygen in the water to become depleted and bringing organisms near, on, or up to the surface film. Due to the inexperience of the researcher and the limited knowledge surrounding *Prostoma* species, any 'worm-like' organisms were then removed from the container and preserved in 4% formalin for further microscopic investigation and identification. Sampling of replicates was limited to a one-hour period per container, as it was considered that this would give a representative sample, whilst adhering to the time constraints involved with processing samples of freshwater macro-invertebrates. Care was taken, however, not to exclude any specimens of *P. jennings*i present. Once preserved, specimens were identified under a binocular microscope, with the help of species identification guides. Any specimens considered not to be *P. jennings*i were excluded from the results.

No Nemertean specimens were recovered from either the Clay 'Ole or the River Lostock during this initial stage of the project. Despite this, the Clay 'Ole appeared to be a healthy and invertebrate species-rich pond.

3.2 Stages 2 & 3: Addition of a second location and assessment of an alternative sampling technique

As a result of the initial internship, further research (funded by Natural England) was undertaken, in which an alternative (quantitative) sampling method was explored (the modified Gerking Box Sampler) and an additional pond within the locality (Ulnes Walton) was sampled (location suggested by LWT). This pond is also a former clay excavation site, with beds of *Phragmites* present. It was considered important to explore similar pond locations in the area, for the possibility that *P. jenningsi* may have existed in other 'similar' ponds in the locality and, should *P. jenningsi* have been recovered from the Clay 'Ole, a suitable pond would be required, in which to translocate and secure further populations. Research was undertaken during winter 2011 and spring 2012, to account for variations surrounding seasonal changes and the possibility of population fluctuations (see Gibson & Young, 1971).

Net sampling and laboratory methods were used as described in section 3.2.2. For a full description of the Gerking Box sampling technique, see Quigg & Lowe (2012a), Appendix 1. Water samples were taken at both sites and fundamental water chemistry parameters tested – see Quigg & Lowe (2012a) – Appendix 1.

3.2.1 Assessment of the Gerking Box Sampler

Although effective where conditions would allow, the modified Gerking Box Sampler was considered unsuitable for the research, as it was labour-intensive and required a certain amount of force and manipulation to be used when placing into the substratum,

cutting away reeds and sliding the cutter shut. The device was less effective in cutting dense reeds than had been hoped, with difficult conditions and high water levels at the time of sampling contributing to the problems. This led to manual cutting, with shears, of the submerged reeds within and around the box frame. Researchers were increasingly exposed to the cold water and put at risk of entering into deep water, due to the nature of the pond, as described above – see *figure 5* (Note: For safety, life preservers were worn during all sampling periods).

3.2.2 Summary of Water Chemistry

Although remaining within the 'normal' range, levels of sodium and chloride were found to be noticeably higher within the Middle Bay and around the outlet of the Clay 'Ole site, compared with other areas of the pond and the pond at Ulnes Walton. As there were no previous data on concentrations of sodium and chloride levels at the Clay 'Ole, allowing for comparisons to be drawn, it was unclear as to whether such levels were a 'normal' occurrence. It was considered that such raised levels may have been due to the influx of brackish water from the nearby River Lostock, either via the outlet pipe or through local storm flooding events. Other parameters tested included temperature, pH, dissolved oxygen, total dissolved solids, nitrates, potassium and phosphates, all falling within normal expected levels for freshwater - see Quigg & Lowe (2012a) – Appendix 1.

3.2.3 Summary of Stages 2 & 3

Despite further sampling during different seasons, an alternative sampling technique and an additional location, no specimens of *P. jenningsi* were recovered. At this stage, the possibility that *P. jenningsi* had become locally extinct at the Clay 'Ole site was considered; however, due to the limited time over which the study had been executed and inadequate quantitative data, comprehensive conclusions could not be drawn.

Questions surrounding the origin of *P. jenningsi* remained unanswered; thus, further research was undertaken – see Appendix 1 for full unpublished reports (Quigg & Lowe 2011a; 2011b; 2012a).

3.3 Stage 4: Extended survey of Ponds associated with the River Lostock/Douglas

Following recommendations made in pr[pevious stages of this work, further sampling was conducted in autumn / winter, 2012. The principal objective remained to confirm the existence of *P. jenningsi* at the Clay 'Ole and, during this stage, the survey area within the Clay 'Ole pond was widened, in order to investigate the possibility that the species might be located in other areas of the pond. The search for *P. jenningsi* was also widened, to include further similar ponds in the surrounding area, in order to explore the hypothesis that the species may have entered the Clay 'Ole site through a number of different means. Of particular interest was the suggestion that the species had entered the Clay 'Ole via the network of nearby rivers (the River Lostock and the River Douglas) (Sundberg & Gibson, 2008; Quigg & Lowe, 2011). Additional ponds connected to the river system were therefore identified and sampled - Twin Lakes (in Croston), a former clay extraction works, situated downstream of the Clay 'Ole, near to the River Lostock (see full description in section 4.1.3) and Alty's Pond (Hesketh Bank), again a former clay extraction works, sitting adjacent to the River Douglas, of which the River Lostock is a tributary – see *figure 15* (Pg.52) for proximity to the Clay 'Ole and rivers.

Like the Clay 'Ole, both ponds, (at the time of the study), were used for fishing and under the management of local angling clubs and both exhibited stands of *Phragmites* - as mentioned above, considered to be the preferred habitat of *P. jenningsi* (Gibson & Young, 1976). (See Quigg & Lowe, 2012b – Appendix 1, for full site descriptions.)

As with previous stages of the study (Section 3.2.2), a net sampling technique was employed and water samples were taken, to test basic water chemistry parameters.

3.3.1 Summary of Water Chemistry

Water chemistry results showed Twin Lakes to have similar properties to those seen at the Clay 'Ole, displaying slightly raised levels of sodium and chloride. Recent widespread storm flood events had occurred in the area prior to sampling; thus, it was considered that this may have contributed to such findings, as more 'brackish' water from the river is likely to have entered the ponds. Alty's Pond, Hesketh Bank, was also found to be of a brackish nature. During high tide, tidal water was observed flowing into the pond via an inlet linked to the nearby River Douglas.

3.3.2 Recovery of *Prostoma* spp.

Despite widening the search for *P. jenningsi* within the Clay 'Ole, no specimens were recovered; however, specimens of possible *P. jenningsi* were recovered from Twin Lakes and from Alty's Pond. All specimens were recovered from within the stands of *Phragmites* and found in, on and around the littoral layer, as suggested by Gibson & Young (1971; 1976). Given the brackish nature of Alty's Pond, questions were raised concerning the tolerance of *Prostoma* to saline conditions and such a finding could support thoughts of Moore and Gibson (1973), who suggested that limnetic hoplonemertea, such as *Prostoma,* may have evolved from marine ancestors.

The recovered specimens were initially confirmed as belonging to the genus *Prostoma* through visual identification (via an emailed photograph – see *figure 6*) by Professor Gonzalo Giribet (Harvard University, USA), Professor Jon Norenburg (The Smithsonian, Washington) and Professor Ray Gibson, all experts in the field.

3.4 Species Verification

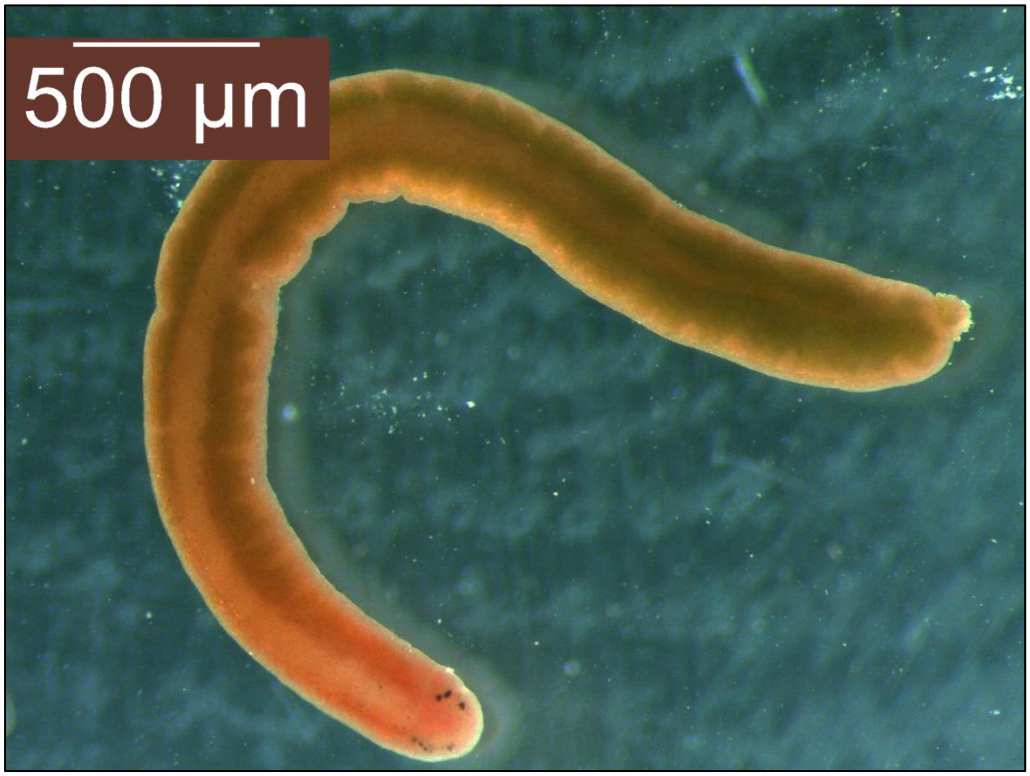


Figure 6 – P.jenningsi? (Source – Authors own)

In order to confirm the species as *P. jenningsi,* further analysis was required. As described in section 2.8, external morphology of *P. jenningsi* is identical to its close relatives, *P. graecense* and *P. eilhardi*, and it is the presence of eleven proboscidial nerves alone that have previously distinguished *P. jenningsi* from all other *Prostoma* species within the genus (Gibson & Young, 1971).

Where specimens of possible *P. jenningsi* were recovered, individuals were removed from pond samples and placed into separate containers (with holes in the lids) containing original pond water and stored in an incubator at 10 °C. This enabled live specimens to be viewed under the binocular microscope, as *Prostoma* are considered to be more easily identifiable when alive (Gibson, 2012). Once identification to genus

was confirmed, specimens were either preserved in 100% ethanol, or kept alive in the incubator for further investigation.

3.4.1 Histological Analysis

A number of specimens were fixed, sectioned and stained for histological analysis. Individuals were placed on a microscope slide and then flooded with 1% $MgCl_2$ for approximately 45-60 minutes. If specimens were still moving after this time period, they were transferred to a clean slide with pulled Pasteur's - a very small amount of the 1% $MgCl_2$ remaining. Individuals were straightened manually and 2% $MgCl_2$ was added, a few drops at a time, until the specimen stopped moving. (This occurred after approximately 10 minutes.) Bouin's fixative was then heated to 80 °C and a small quantity added - enough to flood the slide. This was then left in a Petri dish for 12 hours. The individuals were then transferred to a universal container, with 70% ethanol, where they remained until being processed for histological examination.

Samples were transferred from the ethanol to a small envelope, made from lens tissue paper, and put into a histology cassette (VWR), before being loaded into the tissue processor (Citadel 2000 Thermo) and put on a run.

Short programme:

Use of 90% ethanol for 15 mins (IMS); 100% ethanol 15 mins; 100% ethanol 15 mins; 100% ethanol 15 mins; Histoclear (national diagnostics) 15 mins; Histoclear 15mins; Histoclear 15 mins; Wax (pastillated gurr 56 vwr) 30 mins; Wax (left in hot wax until am, approx. 10 hours)

Once the process was complete, the specimens were removed from the histology cassettes and made into blocks, using metal moulds and cassettes, on an RA lamb

blockmaster3. A Leica RM2235 microtome was used to create 4 µm sections, which were placed onto slides by floating sections in a water bath.

Sections were then stained, using Mallory's trichrome, as suggested by Gibson (2012): Solution A=1% acid fuchsin; Solution B= 1% phosphomolybidic acid; Solution C= Orange G 2 g, methyl blue 0.5 g, oxalic acid 2 g, 100 ml distilled water.

In order to distinguish *P. jenningsi* from other *Prostoma* species within the genus, it was necessary to identify the presence of eleven proboscidial nerves (Gibson & Young, 1971, Gibson, 2012). Due to the expertise required to identify *P. jenningsi* from histological samples, Professor Ray Gibson analysed the prepared slides. Unfortunately, histological analysis was inconclusive as, during fixation, specimens tended to contract and, when contracted, the proboscis folded itself within the rhynchocoel, making location and identification of the proboscidial nerves impossible. A completely straight fixed specimen is required to produce a cross-section suitable for identification (Strand *et al.*, 2005). An example of a prepared histological slide can be seen in *figure 7.*

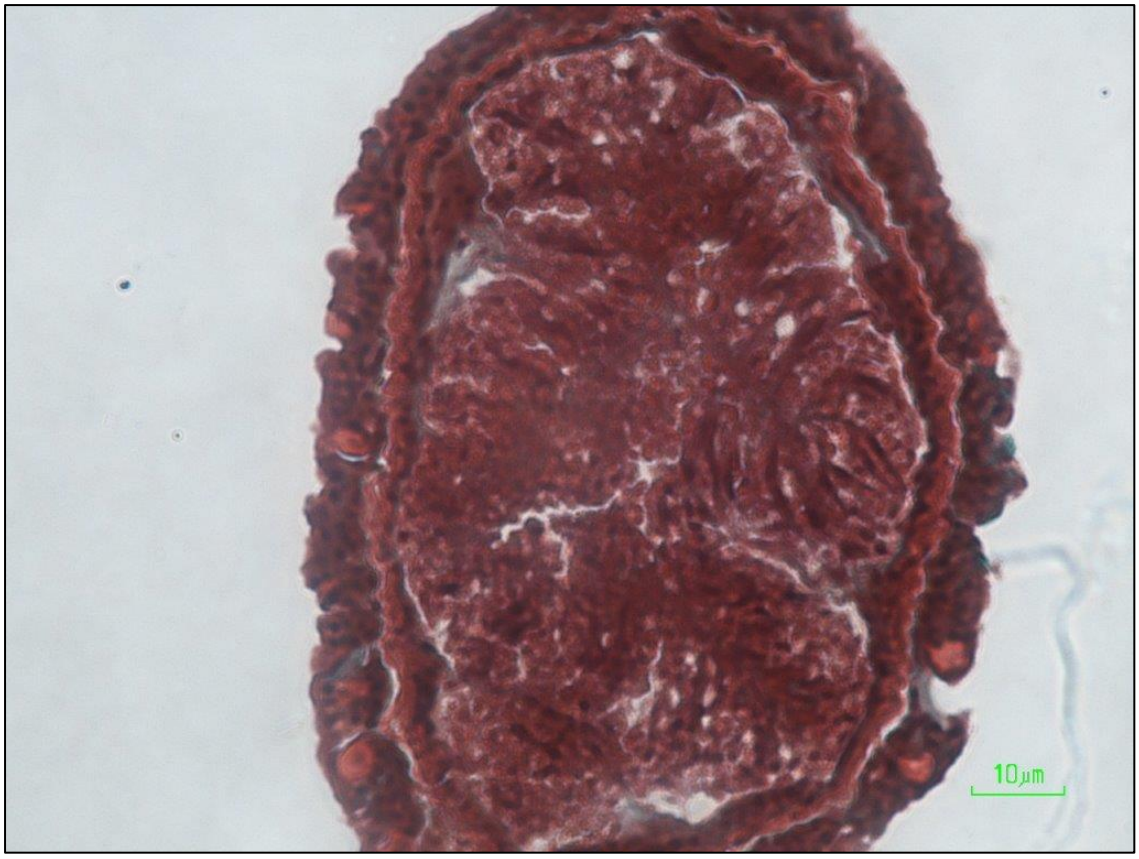


Figure 7 - Prepared histological section of *Prostoma spp* (Authors own)

3.5 DNA Analysis

Seven preserved *Prostoma* spp individuals were sent to Harvard University for DNA analysis. Sequence data was acquired from 18s and 28s rRNA and all 7 specimens were found to have a 100% match with sequences for a *Prostoma eilhardi* present on GenBank. This was the first reported occurrence of *P. eilhardi* in the British Isles.

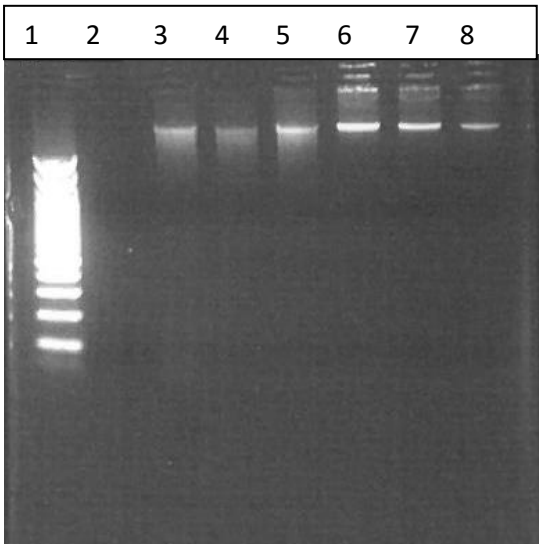
Although the presence of *P. jenningsi* could not be confirmed at the Clay 'Ole site, the genus *Prostoma* had been confirmed within the locality; this in itself was significant, as so few freshwater populations have been recorded within the UK.

3.5.1 DNA analysis – Type Specimen

Samples of the archived type specimen of *P. jenningsi,* as deposited by Gibson, were acquired from the Natural History Museum (NHM), to allow comparisons with collected specimens.

DNA was extracted using a QIAamp® DNA Micro kit for DNA extractions, with a few kit for DNA extraction from tissue, with a few modifications to the manufacturer's protocol (1uL of carrier RNA (1ug/uL) was added at step 5, and incubation was carried out for 10 minutes at step 13). PCR amplification was then undertaken and sequences from two regions, 18s (nuclear) and COI (mitochondrial), of all viable samples were obtained. These markers have been used in a number of phylogenetic analyses of Nemertea (see Giribet *et al.*, 1996; Sundberg *et al.*, 2001; Strand & Sundberg; 2005; Andrade *et al.*, 2011). Primers were designed to amplify products of 294 bp and 275 bp of the 18s ribosomal RNA (nuclear) and the Cytochrome Oxidase subunit I (COI) (mitochondrial) genes, respectively. PCR primers were designed for short amplicons, as it was likely that the specimen from the NHM would have poor quality (i.e. broken-up) DNA. Ideally, longer sequences would be obtained; however, the shorter sequences were considered suitable to give a good indication of species relationships. DNA analysis of *P. jenningsi* was run alongside three specimens of *Prostoma* spp collected from Twin Lakes (as confirmed through DNA analysis carried out by Harvard University) and three Lambda DNA standards.

3.5.2 *Prostoma jenningsi* (NHM sample)



Lane 1: Ladder

Lane 2: *Prostoma jenningsi*

Lane 3: *Prostoma eilhardi* 1

Lane 4: *Prostoma eilhardi* 2

Lane 5: *Prostoma eilhardi* 3

Lane 6: Lambda DNA standard 46 ng

Lane 7: Lambda DNA standard 23 ng

Lane 8: Lambda DNA standard 12 ng

Figure 8 - Figure 8 - Image of gel showing DNA extracts from P. jenningsi type specimen (NHM) and P. eilhardi collected from Twin Lakes

It can be seen in *figure 8* that there is no DNA in lane 2, where DNA extracts would be expected to be seen for *P. jenningsi.* Lanes 3, 4 and 5 show DNA extracts for *P. eilhardi* and lanes 6, 7 and 8 show the standards. Due to the age (circa from 1970) and preservation methods associated with the putative *P. jenningsi* sample (possibly formaldehyde or Bouin's solution), it was not possible to extract any DNA for analysis.

3.6 Summary of preliminary work and recommendations

The preliminary study was unable to confirm the existence of *P. jenningsi* at the Clay 'Ole site; however, following the expansion of the search to include additional ponds, two new populations of *Prostoma* spp were found to exist at nearby locations, associated with the same river system. Difficulties in the identification of Nemertea through histological analysis were experienced, which raised questions surrounding the validity of using histology as a definitive technique in Nemertean identification.

DNA analysis undertaken by Harvard University indicated that specimens collected from Twin Lakes and Alty's Pond were all *P. eilhardi.* As these specimens were not

collected from the Clay ‘Ole itself, it remained conceivable that *P. jenningsi* could still exist within the pond. With the failure to extract DNA from the putative type specimen supplied by NHM, this hypothesis could not be confirmed. The confirmation of the genus *Prostoma* being found within the locality of the Clay ‘Ole remained significant, as only two previous freshwater populations have been recorded in the UK (Gibson & Moore, 1976).

It was considered that further, more extensive sampling at the Clay ‘Ole might still yield specimens of *Prostoma* spp. The hypothesis that the population may have simply migrated to another area of the pond needed to be further explored. Given that *Prostoma* had been found in two other similar ponds in the locality, there appeared no valid explanation as to why they should no longer exist within the Clay ‘Ole. If acquired, DNA analysis could be undertaken on collected *Prostoma* spp. specimens, to determine whether *Prostoma* from the Clay ‘Ole site were distinct from those found, both in other locations in Lancashire and worldwide, by comparing DNA sequences with those recorded on validated genetic databases (e.g. GenBank; BOLD). Further pond locations in Lancashire could also be investigated, to gain further insights into the distribution of *Prostoma* spp, and inter and intra-specific genetic variation explored. The recommendations of this preliminary stage formed the basis of the Masters by Research.

Chapter 4 - Nemertea Collection, Processing and Results

4.0 Nemertea Collection, Processing and Results: Introduction

This chapter gives site descriptions and materials and methods used in order to fulfil objectives 3 and 4, as stated in section 1.1.

Local sampling sites were re-visited and new sites explored, to obtain Nemertean specimens for DNA analysis. Permission was gained from site managers before sampling took place at any location. Unfortunately, permission to return to Alty's Pond, at Hesketh Bank, was not granted.

If found to be present at any study site, subsequent DNA analysis of *Prostoma* would allow for comparisons to be made between specimens obtained from each pond sampled. To improve the robustness and validity of the study, and to overcome potential genetic variation within populations, a target of 50 individuals (with a minimum threshold of 30 individuals) per site was established. Once achieved, comparisons could be made between sequences obtained from each location and with other *Prostoma* sequences available from existing databases (i.e. GenBank and BOLD).

All samples were taken using the net sampling technique, as described in Chapter 3; section 3.1.2.

4.1 Study Sites

4.1.1 Clay 'Ole

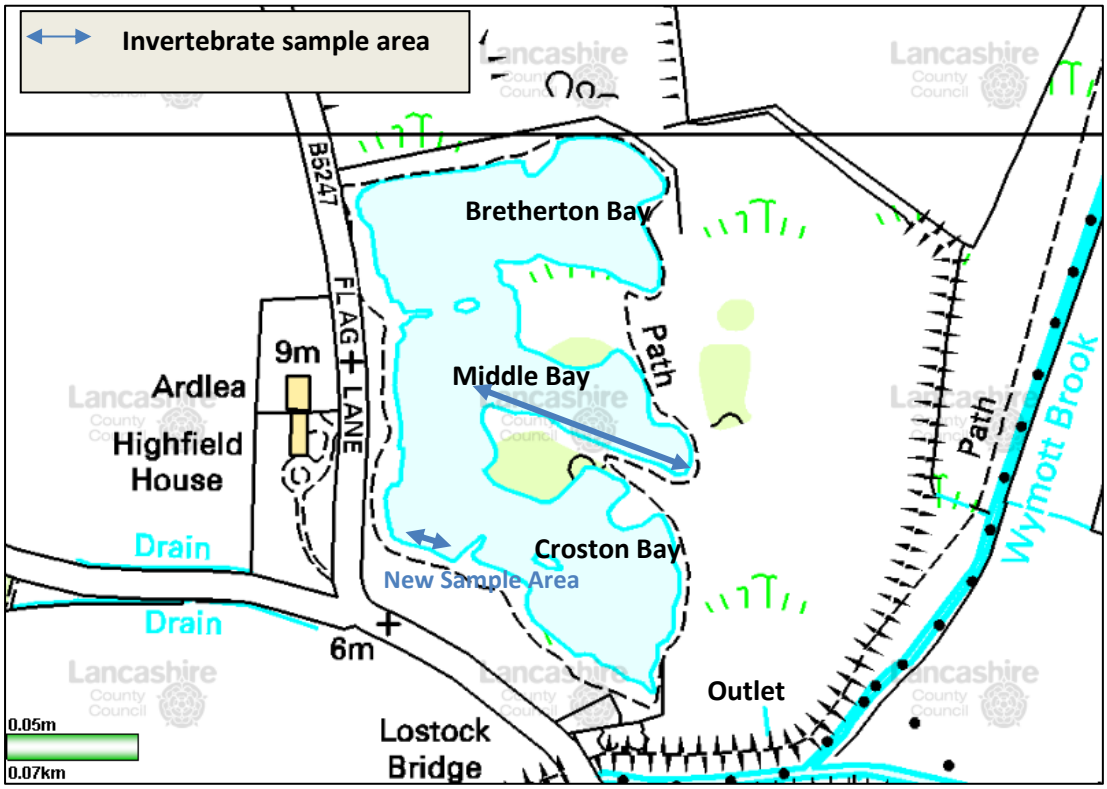


Figure 9 – Clay 'Ole Sampling Areas (adapted from MarioMap)

Following the preliminary research, where focus was placed on the stretch of pond located within the Middle Bay of the Clay 'Ole (see *figure 9*), sampling was extended to include the entire pond margins, exploring the possibility that *P. jenningsi* may have migrated to another location within the pond.

The first visit to the pond uncovered a population of *Prostoma* spp; therefore, the subsequent sampling, undertaken over two additional visits, was concentrated at this location - see *figure 9*. Sampling was also undertaken in the Middle Bay of the pond, where *P. jenningsi* were originally recorded. (A full site description can be found in section 3.2.1.)

4.1.2 Twin Lakes

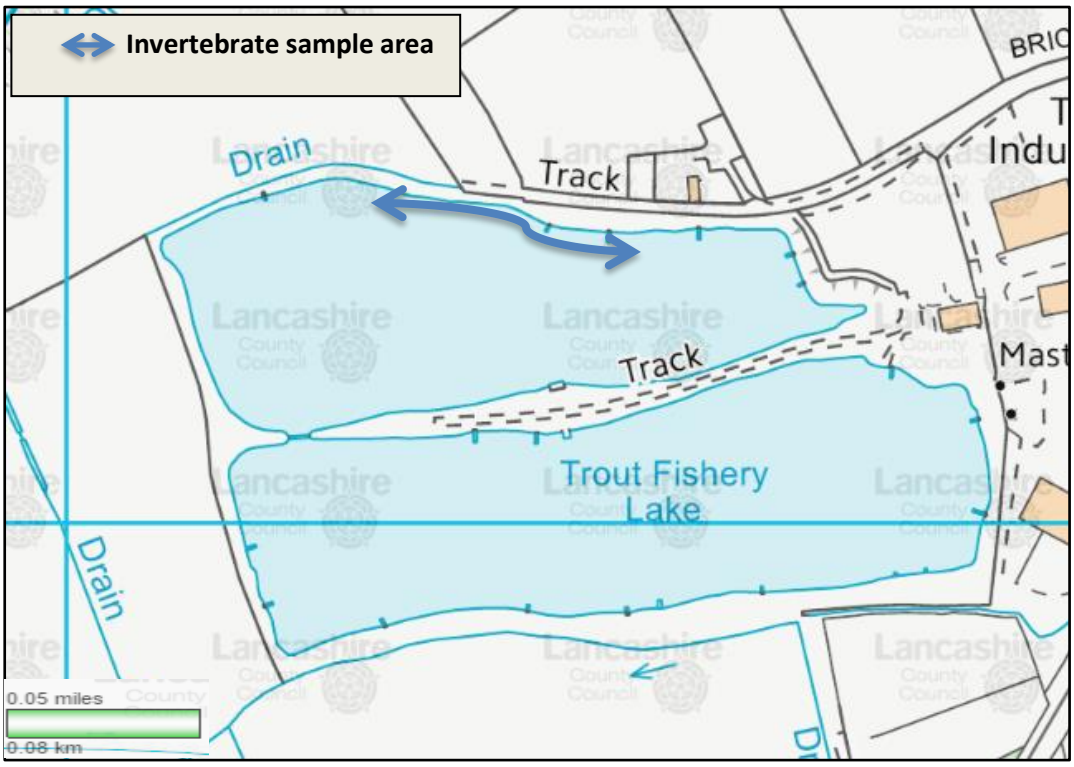


Figure 10 – Twin Lakes Sampling Area (adapted from Mariomaps)

The Twin Lakes site (SD48249,19135) is situated downstream of the Clay 'Ole, near to the River Lostock (see *figure 10*), on the site of a former clay extraction works**.** The site has been used as both a trout and course fishery for a number of years, having recently been taken on by Southport and District Angling Association. Access to the pond is gained by a path running around its perimeter and fishing pegs/small pontoons are in place. The general upkeep of the site is good. The surrounding vegetation is relatively sparse, with a number of mature trees and some scrub present. The pond shares similar characteristics to those of the Clay 'Ole, exhibiting stands of *Phragmites*, *Juncus* and other macrophytic vegetation around its margins. (These are, however, limited to small areas and much of the upper zones of the pond's margins are artificial.) The pond is very deep, dropping off almost immediately from the water's edge. Water levels within the pond are prone to fluctuation and the pond is linked by

an outlet/inlet pipe to a drainage network which, in turn, is connected to the River Lostock.

The site was re-sampled, in order to confirm that the population of *Prostoma* spp*,* (as found during the previous stage of the project) remained. Invertebrate samples were collected from stands of *Phragmites*, in which the Nemertea were found previously - see *figure 8*. Specimens collected from Twin Lakes would allow for further DNA analysis of individuals from this site and for a direct comparison with individuals from the Clay 'Ole site, should they be recovered. Sampling was carried out during two site visits.

4.1.3 River Lostock

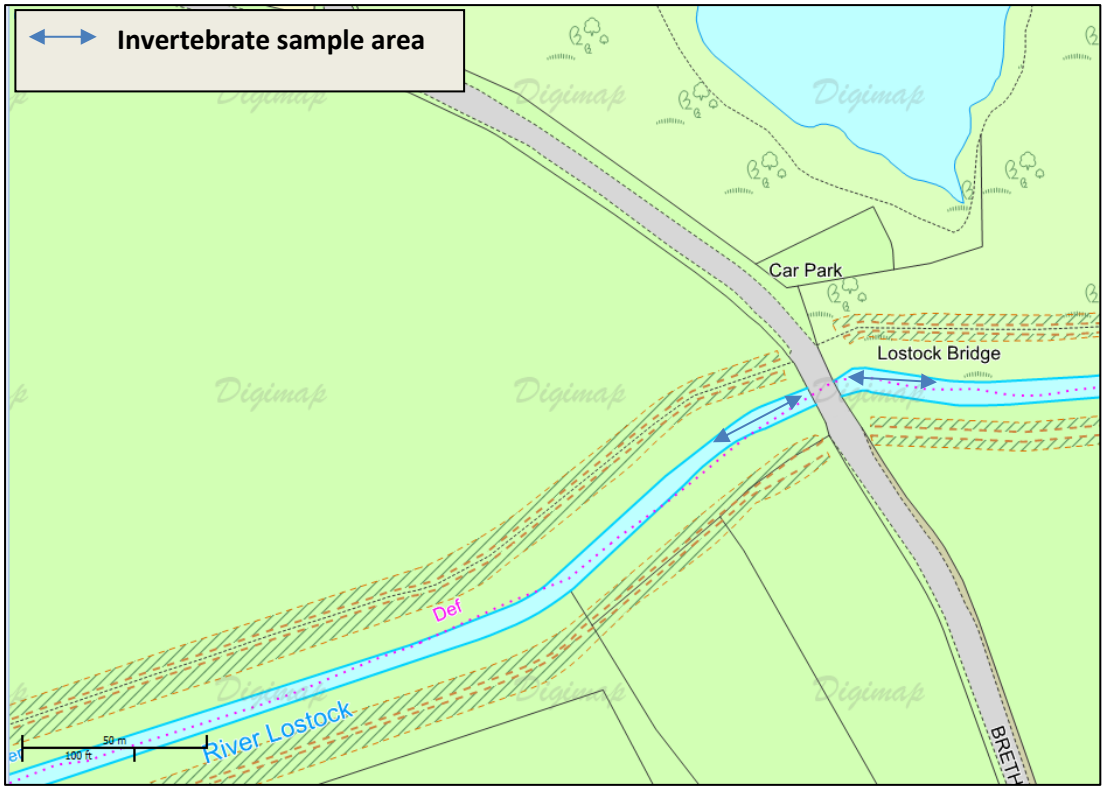


Figure 11 – River Lostock Sampling Area (adapted from Edina Digimap)

The River Lostock sits between the Clay 'Ole and Twin Lakes (see *figure 15*). The stretch downstream of Lostock Bridge was considered to be the point of the mean high

tide. The river is under the management of the Environment Agency, and, although relatively shallow when conditions are dry, it is prone to flooding following heavy rainfall events. Little aquatic vegetation is present on the river bed, which consists mostly of sandy clay. The river banks are straight and steep following improvements in an attempt to prevent the river over-topping. Grasses and reeds are present within the ponds margins. Robust sampling of the River Lostock in the vicinity of the Clay 'Ole and Twin Lakes Ponds was undertaken over three visits – see *figure 11*. This location is of interest as it has been suggested that as the river connects a number of the sampled locations where *Prostoma* spp have been found and that it may act as a reservoir for the colonisation of these sites. See *figure 15* for location in relation to other sites.

4.1.4 Mere End

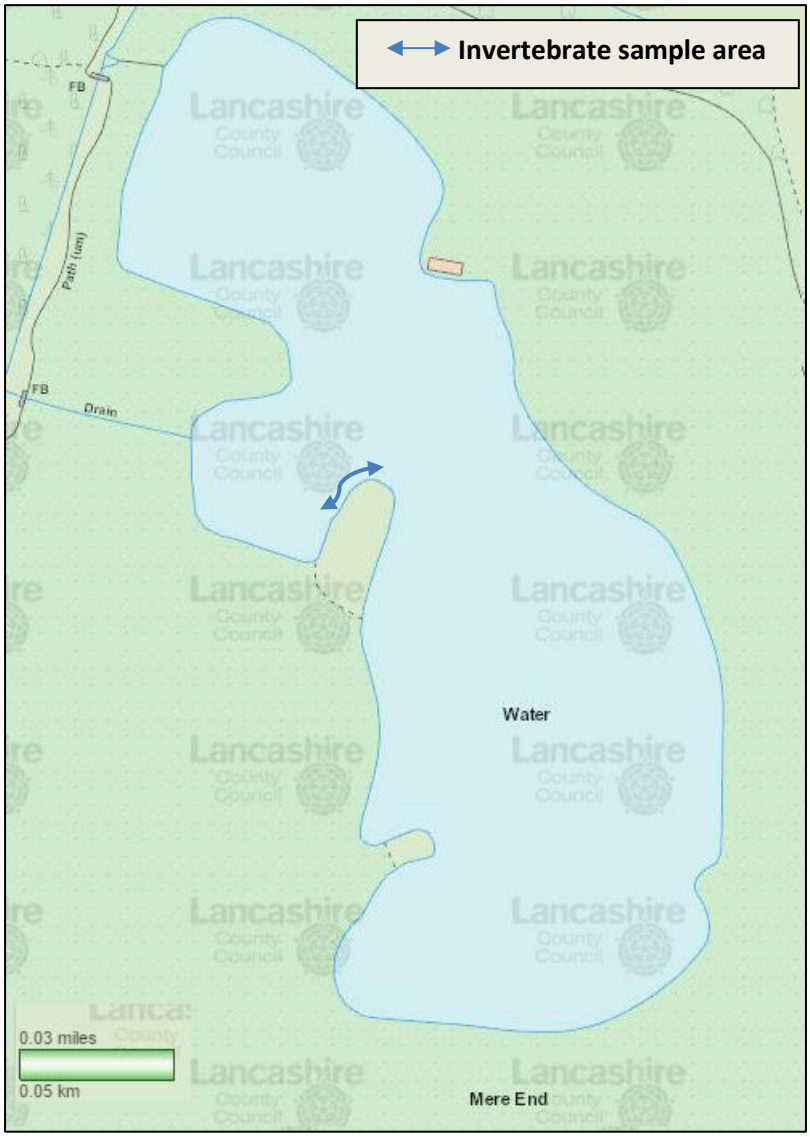


Figure 12 – Mere End Sampling Area (adapted from MarioMap)

Mere Sands Wood Nature Reserve is situated near Rufford, on the site of a former sand extraction site (SD45002,15666). The site was acquired by the Wildlife Trust for Lancashire, Manchester and North Merseyside in 1982; it has since been managed as a nature reserve. Historically the site was on the shores of 'Martin Mere', a large naturally occurring lake; however, the area was drained for agricultural use, as with large areas of surrounding peatland on the West Lancashire plain. The 42 ha reserve is a designated Site of Special Scientific Interest (SSSI). It is well maintained and made up of a number of lakes, mature broadleaf and conifer woodland, sandy, wet meadows

and heath (see LWT n.d). This site was chosen as it is isolated and has no links to the other ponds sampled (some of which are linked through river networks).

Samples were collected from Mere End over two visits. The pond is not connected to the River Lostock and therefore should *Prostoma* spp be present within the pond, it would represent an isolated population. Collection of individuals from this location for DNA analysis would provide a useful comparison with the Clay 'Ole and Twin Lakes samples and allow for further assessment to be made concerning interconnected populations and isolated populations, while also providing further information on the distribution of *Prostoma* spp within Lancashire. Samples were taken from a bed of *Phragmites* chosen for ease of access and to cause the least disturbance to the surrounding areas – see *figure 12*. Having established that *Prostoma* were present at the site – the location was visited on two more occasions. See *figure 15* for the pond in relation to other sites.

4.1.5 Brockholes Nature Reserve

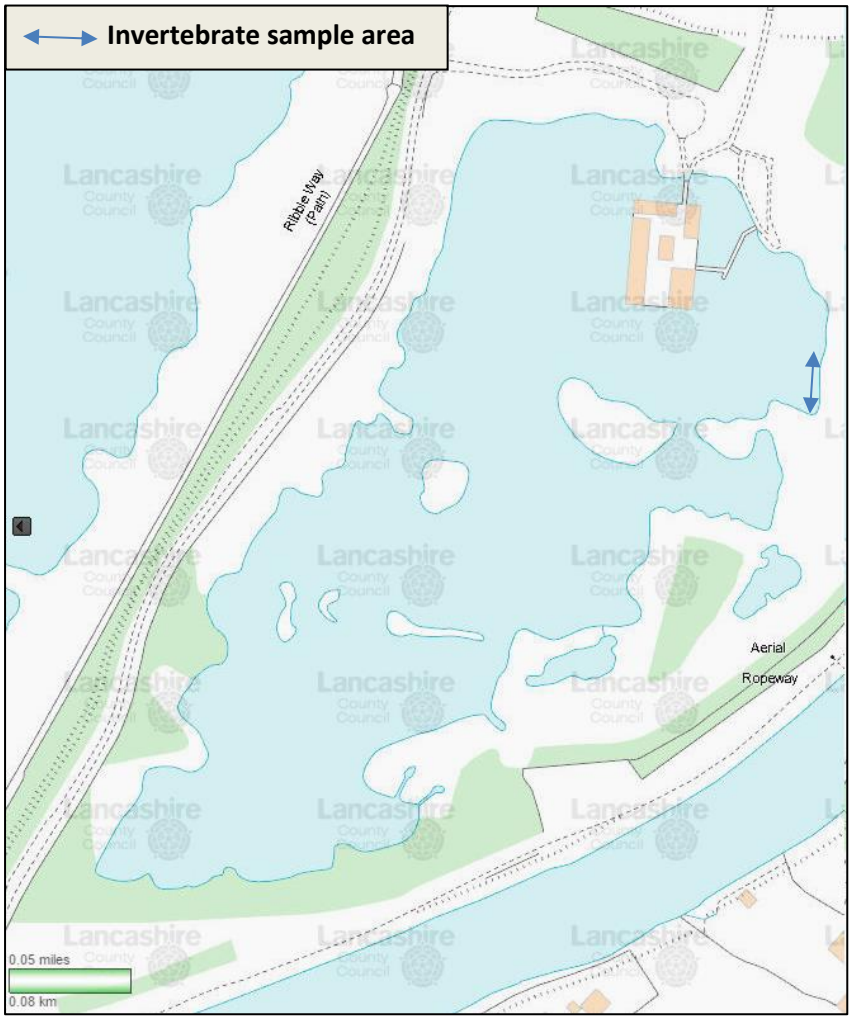


Figure 13 - Brockholes Nature Reserve Sampling Areas (adapted from MarioMap)

The Brockholes Nature Reserve (SD58849,30666) is located next to the River Ribble in Preston and was opened to the public in 2011. The site was previously used for commercial gravel extraction. The site contains a number of man-made ponds that were flooded in 2008 and has also seen the creation of extensive reed beds which was started in 2009 (LWT, n.d). This location represents an opportunity to sample at a site distinct from previous sample locations but also of fairly recent establishment. It has been suggested that *Prostoma* spp are more widely distributed than previously considered and it is difficulties associated with sampling that have resulted in their very limited recorded occurrence. It was thought that sampling at this new location

(connected to the River Ribble) might provide further information related to species distribution but also provide individuals for further comparative DNA analysis. The sample location at this site was dictated by the site management in an area away from public view and in order to cause the least amount of disturbance. Samples were taken within the beds of *Phragmites*, in areas easy to access (see *figure 13)*. In comparison with other sites the water was much shallower in the area sampled. Sampling was undertaken during a single visit.

4 .1.6 Hesketh Out Marsh

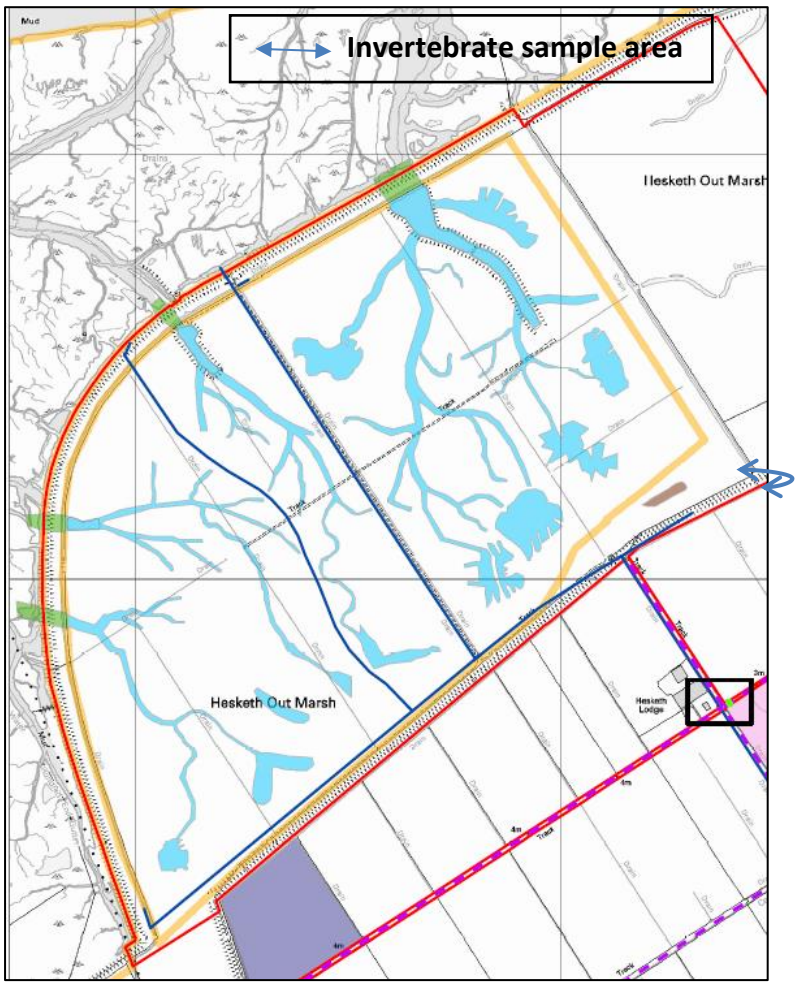


Figure 14 - Hesketh Out Marsh Sampling Areas (adapted from RSPB map)

Hesketh Out Marsh (RSPB reserve SD41981,25157), an area of tidal salt marsh on the River Ribble estuary, was also sampled – see *figure 14* by request of LWT. The site was chosen to link with the suggestion made by the preliminary research that *Prostoma* is able to exist within a brackish environment, given the brackish nature of the pond at Hesketh Bank, and the elevated salinity levels at the Clay 'Ole and Twin Lakes. The site was visited at low tide and exhibited very little vegetation with the exception of marsh grasses. Estuarine in nature, the site was extremely exposed. Samples were undertaken during one visit and the sample area chosen for ease of access and water bodies sampled were tidally influenced (see *figure 15* for Hesketh Out Marsh in relation to other sites).

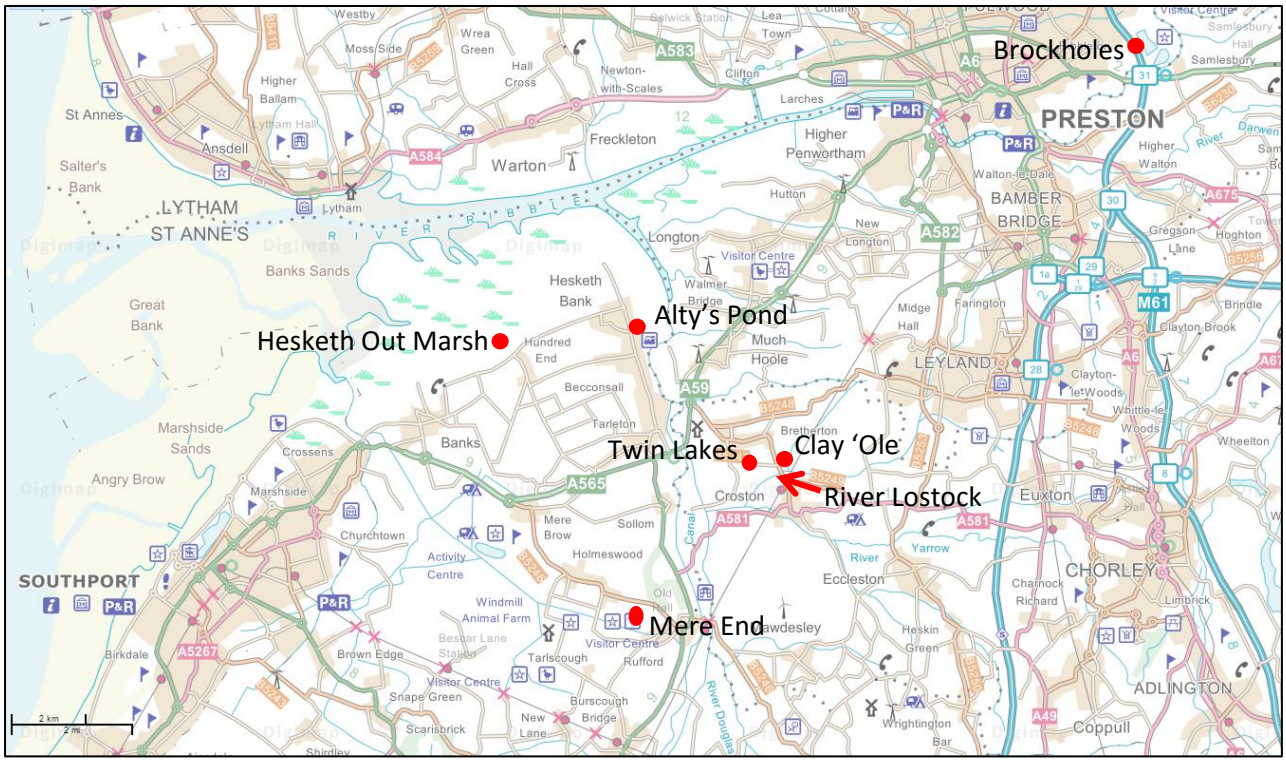


Figure 15 – Map of Study Locations (adapted from Edina Digimap)

4.2 Laboratory Processing

Laboratory analysis was undertaken as described in the preliminary research (see Chapter 3 section 3.5; Quigg & Lowe (2012b) appendix 1). Any *Prostoma* spp were visually identified, removed from the sample container and placed into a new container of original pond water with air holes in the lid. They were then stored in an incubator at 10°C. for further investigation under the binocular microscope as *Prostoma* spp are more easily identifiable when alive (Gibson 2012). Once identification was confirmed, specimens were preserved in 100% ethanol.

4.3 Results - Invertebrate Survey

Table 1 –Number of *Prostoma* spp. Recovered from Sampling Sites

Location	No. of *Prostoma* spp
Clay 'Ole (CO)	67
Twin Lakes (TL)	81
River Lostock (RL)	14
Mere End (ME)	66
Brockholes (B)	0
Hesketh Out Marsh (HOM)	0

Table 1 shows the total number of *Prostoma* spp collected at study locations as a result of all sampling events. The highest number collected was from Twin Lakes, with no specimens recovered from Brockholes or Hesketh Out Marsh.

Chapter 5 - Materials and methods – DNA

5.0 Materials and methods – DNA: Introduction

This Chapter describes the materials and methods used in the DNA analysis of *Prostoma* specimens. As discussed in the previous Chapter, to add robustness and validity of the study and to overcome potential genetic variation within populations a target of 50 individuals (with a minimum threshold of 30 individuals) per site was established. Due to the limitations of the study (time frame) it was decided that, where sample size allowed, DNA would be extracted from 30 individuals (14 for the River Lostock).

5.1 PCR Primers

Using accession numbers provided by Andrade *et al.* (2011), all *Prostoma* sequences were downloaded from GenBank and BOLD. Primers described by Andrade *et al.* (2011), for the 18S ribosomal RNA gene were used (18S1F- 5'tacctggttgatcctgccagtag3' and 18S5R- 5'cttggcaaatgctttcgc3' for a 978bp product. COI primers used by Andrade *et al*. (2011,) in their study (LCO1490 ⁄HCO2198 previously described by Folmer *et al.* (1994), as a universal set of primers for invertebrates amplifying 710-bp were unsuccessful in this study. Hence, new primers (PROSTOMACOIF- 5'ggagtttgatctgggttagttgg3' and PROSTOMACOIR- 5'agaaagtcgctcaaatgtatcc3') were designed using Primer3 software (http://primer3.wi.mit.edu/) using the downloaded *Prostoma* COI sequence (Accession number HQ848594) for a 480bp product.

5.2 DNA extraction

DNA was extracted using a QIAamp® DNA Mini kit with a few modifications to the manufacturer's spin column protocol as follows: During the final elution (step 7 of the

protocol – (see Appendix 2) 100 µL of Buffer AE was added directly onto the column membrane in order to increase the final DNA concentration. This was then left to incubate at room temperature for 10 minutes. Step 8 of the manufacturer protocol was not followed.

5.3 DNA Quantification

DNA extracts were then visualised on a 1% agarose gel (50ml in 1X TAE buffer) alongside known amounts of Lambda DNA (Promega, UK) with 2 µL of GelRed™ Nucleic Acid Gel Stain, (10,000X, Biotium). Lambda standards (54 µg/µL, 27 µg/µL and 14 µg/µL) and DNA were prepared as follows: 1 µL DNA; 1 µL 6X Tracking Dye and 4 µL PCR grade water. 2 µL 100bp Ladder (NBS Biologicals) was loaded into an appropriate well alongside 6 µL each of the prepared Lambda DNA standards and *Prostoma* DNA samples.

5.4 PCR Amplification

PCR amplification in a total volume of 25 µL was undertaken for both 18s (nuclear) and COI (mitochondrial) regions for all samples containing DNA. A master-mix was first prepared made up of 12.5 µL ThermoPrime 2x ReddyMix PCR Master Mix (ThermoFisher Scientific), 0.5 µL forward and reverse primer (10 µM) and 9.0 µL PCR grade water. Once thoroughly mixed, 22.5 µL of master-mix was added to labelled PCR tubes followed by 2.5 µL of DNA.

PCR tubes were then placed into a Thermo Cycler and the following cycling parameters were used: For COI - 3-minute initial denaturation at 94 °C followed by 30 cycles of 30 seconds denaturation at 94 °C; 30 seconds annealing at 51 °C; 90 seconds extension at 72 °C; followed by a final extension step of 10 minutes at 72 °C. For 18s - 3-minute initial denaturation at 94 °C followed by 30 cycles of 30 seconds

denaturation at 94 °C; 30 seconds annealing at 50 °C; 90 seconds extension at 72 °C; followed by a final extension step of 10 minutes at 72 °C.

DNA products were then visualised to check for successful amplification on a 1% agarose gel as described in section 5.3.

5.5 PCR product purification and cycle sequencing

PCR products were purified using a Micro Elute Cycle Pure kit (Omega bio-tek) following the manufacturer's protocols. Purified products were then cycle-sequenced with the forward primer in PCR, using the BigDye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific) as follows: 0.32 µL of sequencing primer (10 µM), 4.0 µL water, 0.6 µL Big Dye Reaction Mix, 1.76 µL 5X sequencing buffer and 6.0 µL (12 ng) of purified DNA template (200-500 bp, 3-10 ng; 500-1000 bp, 5-20 ng) were added and cycle sequenced using cycling of 1 minute at 96 °C followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes.

Cycle sequencing products were further purified as follows: a master-mix of 1.0 µL Sodium Acetate (3M), 1.0 µL Glycogen (20µg/µl), 1.0 µl EDTA (100 mM) and 30.0 µL ethanol (cold) per PCR was made. 33 µL of master-mix was added to each tube and left at room temperature overnight.

Tubes were centrifuged for 30 minutes at 4 °C. The supernatant was removed and then pellets washed using cold 70% alcohol. The tubes were then spun at full speed for 15 minutes and the supernatant removed leaving the pellet in place. The pellets were washed using cold 70% alcohol and spun at full speed for 15 minutes once again.

The supernatant was removed and the pellets placed in the PCR machine with tubes open to dry at 50 °C for 10 minutes.

After samples were dried, 13 µL HiDi formamide (ThermoFisher Scientific) was added just prior to loading on the ABI 3500.

5.6 Phylogenetic Analysis

All available *Prostoma* COI sequences were downloaded from GenBank and BOLD along with a sister taxon *Emplectonema gracile* (NC_016952.1) in order to carry out phylogenetic analyses.

The sequence alignment editor and sequence analysis programme BioEdit (Hall, 1999) was used to prepare a sequence alignment and phylogenetic analysis was carried out using the software package *MEGA* version 6 (Tamura *et al*., 2013). Pairwise uncorrected distance estimates were obtained for every sequence. Sequences were then grouped by geographical location and both within group and between group average distances were estimated.

A maximum likelihood (ML) tree based on the best nucleotide substitution model was also generated using MEGA with 100 bootstraps (Iyengar, unpublished).

Chapter 6 - DNA Results

6.0 DNA Results: Introduction

This Chapter provides the results for all successfully amplified DNA products. Firstly, it will provide details on the 18s (nuclear) region, moving on to results for the COI (mitochondrial) region.

6.1. Amplification Success - 18s (nuclear)

Of 104 samples, 71 were successfully amplified. Sequence alignment using BioEdit (Hall, 1999) indicated that there were no differences between all specimens over all sampled sites. Furthermore, *Prostoma* sequences (*P. eilhardi* JF293027.1; *P. eilhardi* U28494.1; *P. graecense* AY928355.1; *P. graecense* AY928356.1; *P. graecense* AY039666.1; *P. graecense* JX017297 -Lake Ohrid) added to the alignment from GenBank (Benson *et al*, 2016) were also identical – see Appendix 3 for image of sequence alignment.

6.2 Amplification Success COI (mitochondrial)

Of 104 samples, 50 were successfully amplified. Sequence alignment using Bioedit (Hall, 1999) indicated that differences were present between collected specimens and downloaded sequences. Furthermore, differences occurred between available samples taken from online databases BOLD (Ratnashingham & Hebert, 2007) and GenBank (Benson *et al*, 2016) - (*P. graecense* JX017298.1 - Lake Ohrid; *P. graecense* EF208981.1 & EU489490.1 – Sweden; *Prostoma spp* HQ848594.1 – Mass USA; *Prostoma spp* voucher HQ938796.1 & HQ939311.1 – CA USA; *Prostoma sp*; *Prostoma spp.* BOLDCFWIE357- CA USA) and a sister taxon (*Emplectonema gracile* NC_016952.1) See Appendix 4 for image of sequence alignment.

6.2.3 Final COI Average Uncorrected P differences between different geographical regions

Table – 2 Final COI Average Uncorrected P differences between different geographical regions

		Location	1	2	3	4	5	6
1	*Prostoma* spp	Lancashire, UK						
2	*P. graecense*	Lake Ohrid (Macedonia)	0.006					
3	*P. graecense*	Sweden	0.004	0.002				
4	*Prostoma* spp	Mass_USA	0.013	0.011	0.009			
5	*Prostoma* spp	CA_USA	0.041	0.040	0.038	0.038		
6	*Prostoma* spp *(?)*	CA_USA	0.248	0.247	0.247	0.247	0.265	
7	*Emplectonema_gracile*	Unknown	0.166	0.166	0.163	0.159	0.166	0.307

Table 2 shows the average distance between different regions: Lancashire UK (includes all specimens collected in this study), all available *Prostoma* sequences from Genbank and BOLD from Sweden, Macedonia, and USA (Massachusetts and California) and a sister taxon *E. gracile*. It can be seen that differences between UK samples and those from Europe (Lake Ohrid and from Sweden) are low. Differences between UK and European samples and those taken from Massachusetts (East coast of USA) are higher. Differences are even higher between samples taken from California (west coast of USA) and those from Europe. One Californian specimen was split from the group as it showed much higher variation and was considered unlikely to be *Prostoma* (BOLDCFWI.COI-5PE357-10). As expected, the sister taxon *E. gracile* is distinct, with high distance estimates with all *Prostoma* sequences.

6.2.1 Final COI within group P Differences

Table – 3 Final COI within group P Differences

Species	Location	No. of Specimens	P Differences
Prostoma spp	Lancashire, UK	50	0.003461429

Table 3 shows the within group COI differences - the distance estimate within the Lancashire group is low.

6.2.2 Final COI within group P Differences with split differences for Lancashire Locations

Table 4 Final COI within group P Differences showing split differences for Lancashire locations

Species	Location	No. of Specimens	Differences
Prostoma spp	Clay 'Ole (CL) UK	12	0.002327915
Prostoma spp	Twin Lakes(TL) UK	12	0.005050505
Prostoma spp	Mere End (ME) UK	18	0.001933371
Prostoma spp	Riover Lostock (RL) UK	8	0.005518764

Table 4 shows distance estimates with locations within Lancashire separated. Differences between UK locations are low, however slightly more variation can be seen from the River Lostock.

6.3.4 Maximum likelihood phylogenetic tree generated by MEGA using 100 bootstraps to show relationships

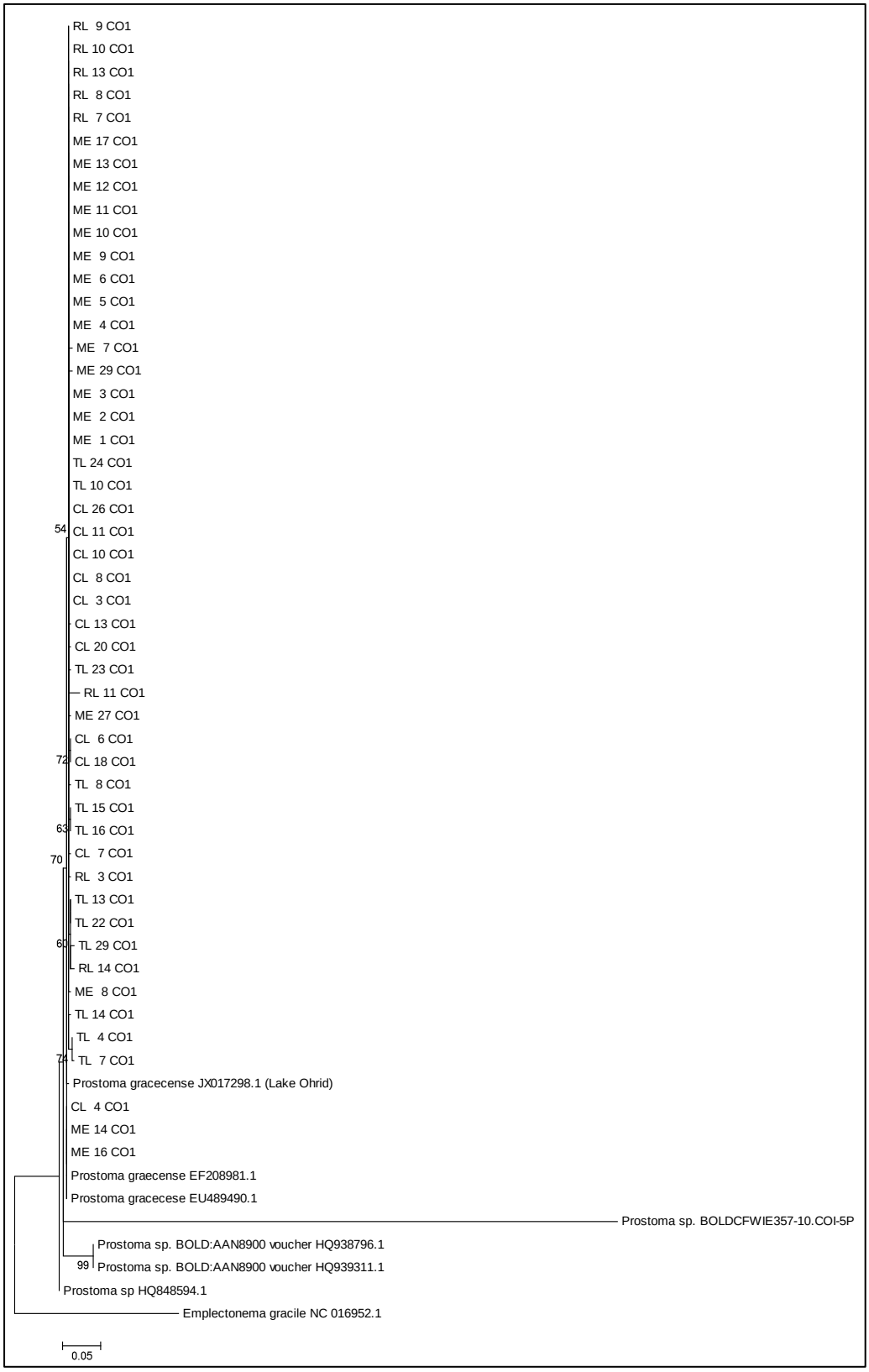


Figure 16 – Maximum likelihood phylogenetic tree generated by MEGA using 100 bootstraps to show relationships

As can be seen in *figure 16 a*ll *Prostoma* specimens collected within the UK group together within one clade (RL9 – ME16). Those from Lake Ohrid (*P. graecense* JX017298.1) and Sweden (*P. graecense* EF208981.1; *P. graecense* EU489490.1) are very similar to the UK sequences and cannot be resolved into separate clades. The sample collected from Massachusetts (*Prostoma*. spp HQ848594.1) did not fall within this clade and is separated from the European samples. Two Californian samples (*Prostoma* spp. BOLD: AAN8900 voucher HQ938796.1; *Prostoma* spp. BOLD: AAN8900 voucher HQ939311.1) are strongly grouped together separately with a very high bootstrap of 99 – high bootstrap values indicate high levels of statistical support for the grouping. The third (separated) Californian sample (*Prostoma* spp. BOLDCFWIE357-10.COI-5P) is markedly different as also indicated by the distance estimates (Table 2). The tree was rooted using *E. gracile*.

Chapter 7 – Discussion

7.0 Discussion: Introduction

The following chapter discusses aspects of the research undertaken and associated results. Firstly, it examines issues surrounding the ecology of *Prostoma* spp before evaluating the location and collection of specimens, leading to a discussion surrounding the status of *P. jenningsi and* concluding with recommendations and future work.

7.1 *Prostoma* Ecology

As stated in the review of the literature, very little is known surrounding the ecology of freshwater Nemertea. While the main focus of this study was to confirm the status of *P. jenningsi*, some ecological observations were recorded that can add to previous findings and observations surrounding *P. eilhardi*; *P. graecense* and *P. jenningsi.*

In general appearance, all specimens recovered displayed 4 to 8 eyespots, most commonly 6. They were mostly pink in colouration; however, variations did occur, with larger specimens being darker in appearance. Recovered specimens ranged in length from 4 to 6 mm, with a maximum recorded length of 20 mm.

Prostoma had previously been reported as widely distributed on a global basis, although locally sporadic in occurrence; this was, however, considered to be reflective of sampling efforts rather than true species distribution, a hypothesis that can be supported by this study, its occurrence having been confirmed at five locations within Lancashire. However, distributions at specific locations did appear to be restricted (see section 7.3). *P. graecense* had previously been reported to be tolerant to brackish water (Crozier, 1917) – an observation supported in this study through the recovery of

Prostoma spp from Hesketh Bank during preliminary research; however, its exact tolerance limits remain unknown.

As with previous studies, it was observed that specimens were able to attach themselves to plants within the water column – such a feature would lend itself more to conditions where water is fast-flowing (e.g. a river), rather than a pond location. Given that *Prostoma* may have evolved to become a freshwater species via estuarine routes (Moore & Gibson, 1973), it is thus considered likely that *Prostoma* may predominantly be located in rivers – see section 7.4.2.2 for further discussion.

Gibson & Moore (1985) suggested that *Prostoma* are able to secrete a sticky mucus that is considered to serve a number of purposes, as discussed in the literature review. During observation throughout this study, specimens were observed to exude such a sticky mucus when under stress, particularly during fixation for histological analysis. The mucus collected any small fragments of debris floating within the water, thus making specimens difficult to locate within sample pots.

7.2 Location and Collection of *Prostoma*

The net sampling technique employed during this study was considered an effective method of collecting *Prostoma* in the respect that, in locations where populations of *Prostoma* spp were found (the Clay 'Ole, Twin Lakes, Mere End), with the exception of the River Lostock, the target number of specimens per site was obtained (N = 50). It is important to note, however, sites where *Prostoma* spp were present were visited more than once to obtain the required number of individuals. As the actual population sizes remain unknown, the efficiency of the net sampling method cannot be assumed. It is possible that any specimens of *Prostoma* spp present during each sampling event may have simply been missed by the net. O'Connor *et al.* (2004) suggest that, when

sampling for macro-invertebrates, a pond net is not a highly effective method of collecting specimens that anchor themselves to the substrata; and nor is it when sampling within dense vegetation (conditions faced by the current study). They suggest that, whilst the pond net is effective at sampling within the water column, it is less effective in its ability to capture organisms hidden in more complex areas of the littoral layer and substrata, thus making it unlikely to reveal the full diversity of macro-invertebrates in a study area. However, due to the lack of knowledge surrounding the exact location of *Prostoma* within the water column, conclusions surrounding net sampling cannot be drawn. In locations where higher numbers of *Prostoma* were recovered, it may purely be due to a higher population density in that particular sampling area.

Box samplers, such as the Modified Gerking Box Sampler, as trialled in the preliminary research, would have provided quantitative data, allowing for population densities to be estimated. Where conditions were suitable, a box sampler may have yielded a higher number of specimens per sample, through collecting everything within the given area, and population 'hotspots' may have been revealed. As suggested by Cook & Herrmann (1997), freshwater Nemertea are often found to exist within one small habitat, when adjacent areas appear to be virtually identical. Due to the nature of the study sites, as outlined in section 3.3.1, a box sampling technique was not considered appropriate for use in this study.

7.3 Specimen Recovery

Sixty-seven *Prostoma* specimens were collected from the Clay 'Ole site - see table 1; however, individuals were not recovered from the Middle Bay of the pond, where they had previously been reported – see Quigg & Lowe (2011a, 2012a, 2012b –Appendix

1) - thus supporting the hypothesis that the population had migrated to another area of the pond. It must be considered, however, that a population may still remain within the Middle Bay of the pond and simply have been missed by the net sampling technique. All specimens were recovered from a limited area within the stands of *Phragmites* and found in, on and around the littoral layer, as suggested by Gibson & Young (1971; 1976). Such a result is unsurprising; as suggested above, Nemertea have been reported to exhibit limited local distribution, being found to exist in one small habitat, when nearby areas appear to be virtually identical (Williams, 1980; Thorp, 1991; Cook & Herrmann, 1997). The recovered Nemertea could not, however, be confirmed as *P. jenningsi,* as DNA analysis suggested the specimens recovered from the pond are the closely related *P. eilhardi or P. graecense* (see section 7.4 for further discussion).

Eighty-one specimens of *Prostoma* were recovered from the Twin Lakes site, 14 from the River Lostock and 66 from Mere End (table 1). Again, all specimens were recovered from within the stands of *Phragmites* and found in, on and around the littoral layer, as suggested by Gibson & Young (1971; 1976). Specimens recovered from Twin Lakes were found in the same location as during the preliminary research. Sampling at the River Lostock took place following a period of heavy rain and subsequent storm-flow and high levels of water; it is thus possible that populations of Nemertea may have been dispersed by strong currents and fast-flowing water and greater numbers may have been recovered had this event not taken place. It is also important to note that Mere End is not linked to any of the river networks that connect all other sampling sites, therefore representing an isolated population within Lancashire. *Prostoma* spp must have reached the site via means other than the river, such as by means of wading birds and the introduction of plant, as suggested by Gibson (1972), and discussed by

Quigg & Lowe (2012a; 2012b – Appendix 1). DNA analysis has confirmed the species to be *P. eilhardi* / *P. graecense* – see 7.4 for further discussion.

No *Prostoma* were recovered from Hesketh Out Marsh. This result was unsurprising, as *Prostoma* are considered to be freshwater species. The waterbodies sampled were tidally influenced, relatively shallow and temporary. No stands of *Phragmites,* which are thought to be the species' preferred habitat, were present.

Prostoma were not recovered from sampling at Brockholes, despite the site exhibiting large beds of *Phragmites*. This result may be associated with the relatively recent establishment of the ponds which have only been present for approximately 10 years. Given the suggested sedentary nature of Nemertea, a much longer time frame may be required for colonisation. However, as in the case for Mere End, Nemertea could be introduced by other means. Furthermore, the site is beside the River Ribble, so, if present in the river, *Prostoma* could be introduced via flooding events. As with all sites, it is possible *Prostoma* is present within the pond and its exact preferred location simply wasn't sampled; thus further, more widespread sampling at the site may reveal a population.

7.4 Status of *Prostoma jenningsi*

7.4.1 18S (Nuclear) DNA

As stated in the results, of the 71 successfully amplified genetic samples collected from Lancashire sites, all sequences were identical. This includes the specimens collected from the Clay 'Ole. Of particular note are no differences between the externally obtained sequences for *P. graecense,* (AY928355.1, AY928356.1, and AY039666.1 – all from Sweden, and *P. graecense* JX017297 from Lake Ohrid - see Sundberg *et al.*, 2010; Strand & Sundberg, 2005) and the samples collected in the

current study. The voucher specimen for *P. eilhardi* (JF293027.1 from Massachusetts used by Andrade *et al.,* 2012; and *P. eilhardi* U28494.1 from Spain) are also identical to all specimens collected throughout the study, and *P. graecense* samples (as listed above) in GenBank.

Such results may be accounted for by a number of hypotheses. Firstly, the externally obtained *P. graecense* and/or *P. eilhardi* sequences have been entered individually into the database (based on their original designation), and the specimens collected during the study are either *P. graecense* or *P. eilhardi*. – two individual species. Secondly, individuals previously recorded as either *P. graecense* or *P. eilhardi* are actually one species and that specimens collected during the study are from this single species. Further work, utilising a multi-loci approach would be required to confirm such a suggestion (Andrade *et. al*., 2011)

Strand and Sundberg (2005) suggest that difficulty in identifying Nemertea through their external characteristics and internal morphology alone, has led to inadequate species description, misidentification and classification, thus leading to conflict between the relationships identified by molecular methods and morphological based taxonomy.

7.4.2 COI (mitochondrial) DNA

As stated in the results, 50 samples were successfully amplified and sequence alignment indicated that differences were present between collected Lancashire specimens and those available from the databases. All substitutions (differences) were checked and confirmed, prior to the analysis being undertaken.

7.4.2.1 Between group p differences

It can be seen from the results (Table 2; *Figure 16*), that average genetic distances between the geographically closer regions of the UK and Europe (Lake Ohrid – JXD17298.1 and Sweden - EF208981.1; EU489490.1) are low, with the geographically more distant samples from Massachusetts (east coast of USA – HQ848594.1) displaying a higher difference and the highest difference being observed in samples from the furthest geographically distant region of California (west coast of USA). These differences can be attributed to geographical isolation.

Genetic isolation by distance (IBD) has been extensively described in many species (Weiss & Leese, 2016). Wang *et al.,* (2012) suggested that patterns of genetic variation often reflect spatial variation in gene flow. Spatially separated populations may experience a restricted gene flow due to landscape barriers and geographical distances. Differences in ecological environments may also contribute to variation, due to local adaptation (environmental isolation). Thorpe *et al.* (2008) suggested that divergence among populations can be associated with both ecological and geographical influences.

Leasi *et al*. (2016) suggest that most meiofaunal species (which include Nemertea) are known to have a cosmopolitan distribution, with no apparent barriers to their dispersal. When studying *Ototyphlotonemertes* - a group of marine Nemertea - they found a positive correlation between genetic and geographical distance; furthermore, they suggest that geological and ecological conditions are also barriers to the dispersion of, and gene flow in, such organisms. In the case of *Ototyphlotonemertes,* which have only been recorded in shallow habitats, they consider the deep ocean to represent a barrier.

As suggested by Ranasingham & Hebert (2007), some barcode records may not be fully verified due to misidentification or analytical error. One of the Californian specimen (BOLDCFWIE357-10COI-5P) showed the greatest distance to all other groups (even greater than that seen with sister taxon *E. gracile, as seen in the phylogenetic tree – figure 16*), and is thus considered unlikely to be *Prostoma*.

7.4.2.2 Number COI within group P Differences

Although no within group differences were observed in sequences from Lake Ohrid, Sweden or the USA, due to only 1 or 2 sequences being available (table 4), variation of within group P distances were observed between specimens collected within Lancashire (0.0035), with the River Lostock showing the highest level of variation (despite having the fewest number of specimens) (Table 4). Variation displayed by specimens taken from the River Lostock may support the proposition that the river acts as a reservoir to the Clay 'Ole and Twin Lakes populations. Furthermore, Mere End, the most isolated of all Lancashire sites, displays the least variation, despite the greatest number of successfully amplified samples. Specimens found within the river may have a less restricted range than those found within pond locations; thus, greater interaction between populations may occur and flowing water may aid dispersal. No other sampled locations within Lancashire exhibited flowing water; therefore, this ecological difference may have an influence on the gene flow, as suggested by Leasi *et al*. (2016).

The within-group distance observed in Lancashire is only marginally smaller than the distance seen between there and Sweden (0.004) and higher than the distance seen between Lake Ohrid and Sweden (0.002). Furthermore, when assessing the individual P distances (see spreadsheet in Appendix 5), it can be seen that sequence CL4 from the Clay 'Ole, and sequences ME16 and ME17 from Mere End are 100% identical to

both Swedish sequences for *P. graecense*. This is also demonstrated through their grouping on the phylogenetic tree – *figure 16.* This result is perhaps surprising, given the geographical distances between the populations.

Three possible hypotheses could be considered for such limited differences. Firstly, the *Prostoma* spp may exhibit slow evolutionary responses, with little variation having occurred across the species. In this scenario, the within group P distance for specimens collected in Lancashire could be attributed to genetic drift at local sites following initial colonisation (Weiss & Leese, 2016).

Secondly, movement and colonisation via transportation on migratory birds, or through import and export of aquatic plants, should be considered (Gibson, 1972; Sundberg & Gibson, 2008). Populations may have been dispersed from one original region or locality.

Thirdly, all *Prostoma* spp are considered to be hermaphroditic; however, their reported modes of reproduction should be questioned. No record of reproduction of *P. graecense* is available in the literature, but mention of egg deposits found within plant tissue has been reported (Gibson & Moore, 1971). Young & Gibson (1975) suggested reproduction within *P. eilhardi* is sexual, and Gibson & Young (1976) remained unsure as to whether cross or self-fertilisation was the normal means of reproduction for *P. jenningsi*. High rates of self-fertilisation have been recorded in the marine species *Prosorhochmus americanus* (Caplins & Turbeville, 2015). Self-fertilisation provides a means of reproductive assurance, particularly in species with limited distribution, thus securing genetic transmission and increasing an organism's colonisation ability. However, organisms displaying this mode of reproduction may suffer from limited adaptive ability, reduced fitness and genetic diversity (Charlesworth & Charlesworth,

1987). It is conceivable that *Prostoma* spp may be able to self-fertilise; however, additional experiments, and further DNA studies using nuclear markers, would need to be undertaken in order to confirm such an idea.

7.4.3 18s and COI regions

In research concerning the dispersion of *Ototyphylonemertes*, Leasi *et al*. (2016) found that the nuclear gene 18s was ineffective in revealing species diversity, suggesting that it disclosed fewer variables than traditional taxonomy considered this region to be unreliable for such studies. They found the mitochondrial gene COI more effective in detangling diversity.

Similarly, when researching genetic diversity of *Daphnia pulex* in reaches of the Yangtze River, Wang *et al.* (2016) found that only a 0 to 2% distance was revealed when using 18s and a larger distance of 0 to 11.3% was found using COI for specimens taken between middle and lower reaches. It is important to note, however, that these distance estimates are not directly comparable with data from this study, as an alternative distance estimate (Kimura-2) was used, but demonstrated that 18s distance estimates are lower than COI, in line with study findings.

Leasi & Norenburg (2014) suggested that great importance should be placed on utilising both morphological and genetic information, when untangling the diversity of meiofaunal organisms. They consider the COI barcoding region to be effective in resolving species identity in Nemertea and that it has demonstrated cases of likely morphological misidentification.

7.4.4 *Prostoma jenningsi*?

Results for this study strongly suggest that the original identification of *P. jenningsi* individuals from the Clay ‘Ole as a new species (*P. jenningsi)* was not appropriate and

that the *Prostoma* species originally recorded (and present today) is likely to be either *P. eilhardi* or *P. graecense*. The original misidentification may be directly attributed to difficulties associated with histological methodologies employed in the original classification process used by Gibson (1971). More extensive sampling across the UK and Europe will allow a clearer evaluation of the genetic diversity present within the populations of *Prostoma* within the UK.

7.5 Future conservation and issues

In a world where biodiversity is decreasing at an alarming rate, DNA barcoding is playing an increasingly important role in its conservation. It provides a fundamental understanding surrounding species boundaries, community ecology and trophic interactions and is being increasingly utilised for the identification of endangered species (Kress *et al.,* 2015). In the case of species such as *P. jenningsi*, where misidentification has occurred, undue focus and conservation status can be removed and placed on species with 'true' priorities. In the case of *P. jenningsi,* its current position in the British Red Data Book is no longer necessary, nor is its listing under the Natural Environment and Communities Act. With such confusion surrounding the identity of cryptic species through original designations, there may be many more cases where conservation focus is unnecessary. On the other hand, the use of DNA barcoding in species conservation may reveal new species, where they have been previously grouped together (Padial, *et al.,* 2010).

7.6 Recommendations and Future Work

Although this study has gained insights into *Prostoma* spp, given the limited knowledge available, there is a vast array of future work that could be undertaken.

It has been established that *Prostoma* spp are present within a number of locations in Lancashire and two previous records have found *Prostoma* to be present within the River Ouse and River Cam (Gibson & Moore, 1976). Expansion of study sites beyond the locality would confirm if *Prostoma* is found within many water bodies across the UK and beyond. More appropriate quantitative sampling methods could be developed, in order to ascertain population densities, both within previously sampled locations and potential new locations. Ecological research surrounding habitat preference (e.g. exact location within the water column) could be explored - such insights may also aid the location of populations within new water bodies. Further ecological studies could be undertaken to increase knowledge surrounding *Prostoma,* such as exploring predator-prey interactions, to confirm *Prostoma*'s role within freshwater communities; laboratory experiments, investigating mode of reproduction and life cycle parameters, could be undertaken and tolerance to varying water quality conditions tested.

As discussed, further studies using DNA analysis could be undertaken, to explore divergence among populations through both ecological and geographical influences, such as those found between samples from the UK and California, USA. Increased sampling across the UK would allow for clearer evaluation of genetic diversity present within populations of *Prostoma*, with particular interest in the River Lostock. Further sampling and subsequent DNA analysis using a wider set of genetic markers may be able to ascertain whether *P. eilhardi* and *P. graecense* are two separate species, or, as proposed, a single species that has been misidentified. Further DNA studies using nuclear markers could answer questions surrounding mode of reproduction and explore the idea that the species may be able to self-fertilise.

Chapter 8 – Conclusions

8.0 Conclusions

- A population of *Prostoma* spp was located at the Clay ‘Ole, the location in which *P. jenningsi* was originally discovered; however, no specimens were recovered from the Middle Bay (the stretch from which *P. jenningsi* had previously been found). Sampling was expanded to include additional locations within Lancashire and an additional four populations were found to exist.
- DNA analysis found *Prostoma* specimens to be identical to either *P. graecense or P. eilhardi,* when comparing the 18s gene, and *P. graecense or Prostoma* spp, when comparing the COI gene, with global databases.
- Limited genetic diversity was displayed between Lancashire populations and populations in both Europe and USA.
- This study confirmed the mis-identification of *P. jenningsi* as a separate species made through traditional (histological) methods.
- In the case of *P. jenningsi,* its current position in the British Red Data Book is no longer necessary, nor is its listing under the Natural Environment and Communities Act. As yet, Lancashire has not found its own endemic species.

References

Anderson, B.J., Armsworth, P.R., Eigenbrod, F., Thomas, C.D, Gilllings, S., Heinemeyer, A., Roy, D.B. & Gaston, K.J. (2009). Spatial covariance between biodiversity and other ecosystem service priorities.*Journal of Applied Ecology.* **46**, 4, pp 888-896

Andrade, S.C.S., Norenburg, J.L. & Solferini, V.N. (2011). Worms without borders: genetic diversity patterns in four Brazilian *Ototyphlonemertes* species (Nemertea, HoploNemertea). *Mar Biol***. 158,** pp 2109-2124

Andrade, S., Strand, M., Schwartz, M., Chen, H., Kajihara, H., Dohrenf, J V., Sung,S., Junoyh, J., Thieli, M., Norenburg, J N.,, James M.Turbeville J M., Giribet, G. and Sundberg, P. (2012). Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea. *Cladistics.* **28,** pp 141-159

Andrade, S.C.S., Montenegro, H., Strand, M. Schwartz, M., Kajihara, H., Norenburg, J. L., Turbeville, J. M., Sundberg, P., & Giribet, G. (2014). A transcription approach to ribbon worm systematics (Nemertea): reolving the Pilidiophora problem. *Mol Biol Evol*. **33**, 12, pp 3206 - 3215

Avise, J.C (2009) Phylogeography: retrospect and prospect. *Journal of Biogeography*. **36,** pp 3 -15

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. *Nucleic Acids Research*. 2016;45(Database Issue):37-42

Bilton, D.T., McBendroth, L., Bedford, A. & Ramsay, P.M. (2006). How wide to cast the net? Cross-taxon congruence of species richness, community similarity and indicator taxa in ponds. *Freshwater Biology.* **51**, pp 578-590.

Candeck, K. & Kuntner, M. (2015). DNA barcoding gap: reliable species identification. over morphological scales. *Mol. Ecol. Resour*. **15**, 2 pp 268 -77

Caplins, S.A., Norenburg, J.L. & Turbeville, J.M. (2012) Molecular and morphological variation in the barnacle predetor *Nemetopsis bivitatta* (Nemertea, HoploNemertea). *Integrative and Comparative Biology.* **52,** pp E24 - E24

Caplins, S. & Turberville, J.M. (2015). High Rates of Self- Fertilisation in a Marine Ribbon Worm (Nemertea). *Biol. Bull.* **229,** pp 255-264

Cook, J.L. & Herrmann, S.J. (1997) New Record of Prostoma graecense (Nemertea: HoploNemertea) from Southern Colorado, with Observations on its Feeding Behaviour and Habitat. *Southwestern Association of Naturalists*. **42,** 2, pp 162-169

Charlesworth, D & Charlesworth, B (1987). Inbreeding Depression and its Evolutionary Consequences. *Annual Review of Ecology and Systematics.* **18,** pp 237-268

Cox, R., Wright, J.F., Furse, T.M. & Moss D (1997). RIVPACS III© (River InVertebrate Prediction and Classification System).Environment Agency and Institute of Freshwater Ecology. Available at: http://nora.nerc.ac.uk/12936/1/N012936CR.pdf Accessed: August 2015

Edina Dgimap (2016). Available at: http:digimap.edina.ac.uk/digimap/home. Accessed: December 2016

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R.C. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, pp 294-299

Freshwater Biological Assosiation (2016). Available at: http://www.fba.org.uk/sites/default/files/CourseInvertSamplingProtocol.pdf . Accessed: October 2016

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. *Nucleic Acids Research*. 2005;33(Database Issue):D34-D38

Gerking, S.D. (1957). A Method of Sampling the Littoral Macrofauna and Its Application. *Ecology.* **38,** 2 pp 219-226

Gibson, R. (1972). *Nemerteans.* Hutchinson & Co, London.

Gibson, R. (1982). *British Nemerteans*. Cambridge University Press, Cambridge.

Gibson, R. (1995). Nemertean genera and species of the world: An annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats, and recored zoogeographical distribution. *Journal of Natural History,* **29,** pp.271-562

Gibson, R. (1998) Epilogue – one hundred years of Nemertean research:Burger(1895) to the present. *Hydrobiologia,* **365,** pp 301–310

Gibson, R. (2011). Pers comm. –Telephone conversation. 22/08/2011

Gibson, R. (2012). Pers comm. – email conversation. 14/11/2012

Gibson, R. & Moore, J. (1976). Freshwater Nemerteans. *Zoological Journal of the Linnean Society,* **58,** pp 177-218

Gibson, R & Moore, J. (1989). Functional requirements for the invasion of land and freshwater habitats by Nemertean worms. *J. Zool., Lond.* **219,** pp 517- 526

Gibson, R. & Young, J.O. (1971). *Prostoma jenningsi* sp. Nov., a new British freshwater hoploNemertean. *Freshwater Biuology.***1,** pp 121 -127

Gibson, R. & Young, J.O. (1976) Ecological observations on a population of the freshwater hoploNemertean *Prostoma jeninngsi. Archives of Hydrobiology.* **78,** 1, pp 42-50

Giribet, G., Carranza, S,Bagui, J.,Riutort, M. and Ribera, C. (1996). First Molecular Evidence for the Existence of a Tardigrada Arthropoda Clade*. Mol. Biol. Evol.* **13**,1, pp 76-84

Hall, T.A (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucl. Acids Symp Ser.* **41**, pp 95-95

Hebert, P.D., Cywinska A, Ball S.L. & deWaard J.R. (2003a). Biological Identifications through through DNA barcodes. *Proc Biol Sci.***7**; 270, pp 313-21

Hebert, P.D., Ratnasingham, S. & deWaard, J.R (2003b). Barcoding animal life: cytochrome *c* oxidise subunit 1 divergences among closely related species. *Proc. R. Soc Lond B.* **270,** pp 96-99

Hebert, P.D., Penton, E.H., Burns, J.M., Janzens, D.H. & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in th eneotropical skipper butterfly Astraptes fulgerator. *PNAS. **101**, 41.*

Hinchcliffe, S. (2007). *Geographies of Nature: Societies, environments, ecologies.* Sage, London.

IUCN (2004). *IUCN Red List of Threatened Species a Global Assessment.* Baillie, J.E.M., Hilton-Taylor, C. & Stuart, S.N (eds.) IUCN, Gland, Switzerland andCambridge, UK. Available at: http://portals.iucn.org/library/sites/files/documents/RL-2004-001.pdf. Accessed: November 2016.

IUCN. (2012). *IUCN Red List Categories and Criteria: Version 3.1.* Second edition. Gland, Switzerland and Cambridge, UK: IUCN.

IUCN (2016) Available at: http://www.iucn.org/ Accessed: October 2016

Iwata, F. (1954). The Fauna of Akkeshi Bay. Memertini in Hokkaido. *Zoology* **12**, 1-2, pp 1-39.

Iyengar, A. (2016) Unpublished - Phylogenetic Tree

JNCC (2015). The UK Biodiversity Action Plan. Available at: http://jncc.defra.gov.uk/page-5155. Accessed October 2016

JNCC on behalf of 4CBG. (2013). *Fifth National Report to the United Nations Convention on Biological Diversity: United Kingdom. Consultation Draft*. Peterborough: JNCC

Jones, A., Duck, R. & Wayers, J. (2000). *Practical Skills in Environmental Science*. Pearson Education Limited, London.

Kress, J.W., Garcíia- Robledo, C., Uriarte, M. & Erickson. D.L. (2015). DNA barcodes for ecology, evouloution and conservation.*Trends in Ecology & Evoulotion.* **30,** 1, pp 25-35

Kvist, S. (2013). Barcoding in the dark?: A critical view of the sufficiency of zoological DNA barcoding databases and a plea for broader interration of taxanomic knowledge. *Molecular Phylogenetics and Evoloution.* **69**, 1 pp 39-45

Kvist, S., Laumer, C.E., Junoy, J. & Giribet, G. (2014). New insights into the phylogeny, systematics and DNA barcoding of Nemertea. *Invertebrate Systematics*, **28**, pp 287–308

Laumer, C. (2012). Pers comm. Email received 20th June 2012

Leasi, F., Andrade, C.S.C. & Norenburg, J. (2016). At least some meiofaunal species are not everywhere. Indication of geographic, ecological and geological barriers affecting the dispersion of species of *Ototyphlonemertes* (Nemertea, HoploNemertea). *Molecular Ecology. **25**,* pp 1381-1397.

Leasi, F. & Norenburg, J.N. (2014). The Necessity of DNA Taxonomy to Reveal Cryptic Diversity and Spatial Distribution of Meiofauna, with a focus on Nemertea. *PLoS:ONE.* **9,** 8

LWT (2001). Prostoma jenningsi (A Ribbon Worm). Available at: hhttp://www.lancspartners.org/bap/plans/**Prostoma_jenningsi**_Final_2001.doc. Accessed: April 2014

LWT (n.d) Available at: http://www.lancswt.org.uk/places-to-see/mere-sands-wood-nature-reserve Accessed: November 2014

LWT (n.d) Available at: http://www.brockholes.org/our-journey Accessed: February 2015

Mace, G.M. (2004) The role of taxonomy in species conservation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **359**, pp 711-719

Mackley, E.A., Godfrey, A & Hardman, C.J. (2010) RSPB/NE Countdown 2010: Bringing Reedbeds to Life Project; Wildlife surveys. Available: https://www.rspb.org.uk/Images/Technical%20Report%20C7%20Aquatic%20Inverts_tcm9-276265.pdf: Accessed: September 2015

MarioMap (2017). Available at: http://mario.lancashire.gov.uk/agsmario/. Accessed March 2017

McDermott, J.J. (1993). Nemertea inhabiting the *Haploopls* (Amphipoda) community of the northern Øresund with special reference to the biology of *Nipponemertes pulcher* (HoploNemertea). *Hydrobiologia* **266**, pp 15–28

McDermott, J.J. & Roe, P (1985). Food, Feeding Behaviour and Feeding Ecology of Nemerteans. *Amer. Zool.,* **25**, pp.113-125

Meyer, C.P & Paulay, G. (2005). DNA Barcoding: Error Rates Based on Comprehensive Sampling. *PLoS Biology*, **3**, 12, pp 422

Moore, J. & Gibson, R. (1973). A new genus of freshwater hoploNemertean from New Zealand. *Zoological Journal of the Linnean Society*. **52,** pp 141–157

Moore, J. & Gibson, R. (1985). The evolution and comparative physiology of terrestrial and freshwater Nemertean. *Biol. Rev*. **60**, pp. 257-312

Natural England (2010). Lost life: England's lost and threatened species (NE233). Available at: http://publications.naturalengland.org.uk/publication/32023. Accessed: November 2016.

O'Connell, M & Yallop, M. (2002). Research needs in relation to the conservation of biodiversity in the UK. *Biol. Conserv*. **103**, pp 115-123

O'Connor, A., Bradish, S., Reed, T., Moran, J., Regan, E., Visser, M., Gormally, M. & Skeffington, S. (2004). A comparison of the efficacy of pond-net and box sampling methods in turloughs – Irish ephemeral aquatic systems. *Hydrobiologia.* **524,** pp 133-144.

Padial, J.M., Mirales, A., De la Riva, I. & Vences, M. (2010). The intergrative future of taxonomy. *Front Zool*. **7**, 1, pp16

Pauw, N. & Vanhooren, G. (1983). Method for biological quality assessment of watercourses in Belgium. *Hydrobiologia.* **100,** 1, pp 153-168

Poluhowich, J.J. (1968) Notes on the freshwater Nemertean *Prostoma rubrum. Turtox News.* **46,** 1, pp 2-7

Primer3 (n.d.) Avaialble at: http://primer3.wi.mit.edu/ Accesed: July 2014

Quigg , S., and Lowe, C.N. (2011a) *Confirming the existence of the Croston worm (Prostoma jenningsi).* September 2011. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2011b*) Confirming the existence of the Croston worm (Prostoma jenningsi) Stage 2. Winter 2011.* Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2012a) *Confirming the existence of the Croston worm (Prostoma jenningsi) Stage 3. Spring 2012*. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2012b) *Confirming the existence of the Croston worm (Prostoma jenningsi) Stage 4. Autumn 2012*. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Ratnashingham, S. & Hebert, P.D.N. (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Resources*. Wiley Online Library

Ratnashingham, S. & Hebert, P. D.N. (2013). A DNA=Based Registry for All Animal Species: The Barcode Index Number (BIN) Sytstem. *PlosONE.* **8**, 7, pp e66213

Rodrigues, A.S.L., Pilgrim, J.D., Lamoreux, J.F.mHoffmann, M. & Brooks, T.M. (2006). The value of the IUCN Red List for conservation. *Trends Ecol. Evol*. **21**, pp 71-76

Roe, P., Norenburg, J.L., & Maslakova, S.A.(2007). Nemertea. *The Light and Smith Manual: Intertidal Invertebrates from Central California to Oregon*, (ed. Carlton, J.). University of California Press, Berkeley.

Shen, Y., Guan, L., Wang, D. & Gan, X. (2013). DNA barcoding and evaluation of genetic diversity in Cyrinidae fish in the midstream of the Yangtze River. *Ecology and Evolution.* **6,** 9, pp 2702-2713

Smith, D.G. 2001. *Pennak's Freshwater Invertebrates of the United States: Porifera to Crusacea* (4th edition). Wiley & Sons, Cnanda.

Soumille, H. & Thiery, A. (1997). A new quantitative sediment corer for sampling invertebrates across the mud water interface and soil of shallow rice field. *Annals of Liminology.* **33,** 3, pp 197-203

Storey, R. (2007). *Aquatic invertebrate diversity and distribution at Aammiq, Marsh, Lebanon.* Available at: http://www.arocha.org/int-en/work/research/ecosystems/1969-DSY.html. Accessed: August 2014

Strand, M. and Sundberg, P. (2005) Genus *Tetrastemma* Ehrenberg, 1891 (Phylum Nemertea) – A natural group? Phylogenetic relationships inferred from partial 18S rRNA sequences. *Molecular Phylogenetics and Evolution*. **37,** pp 114-152.

Strand, M. & Sundberg, P. (2011). A DNA-based description of a new menertean (phylum Nemertea) species. *Marine Biology Research.* **7**, pp 63-70

Sundberg, P. (2015). Thirty-Five years of Nemertean (Nemertea) Research – Past, Present, and Future. *BioOne,* **32**, 6 pp 501-506

Sundberg, P. & Gibson, R. (2008) Global diversity of Nemerteans (Nemertea) in freshwater. *Hydrobiologia* **595,** pp 61-66

Sundberg, P. & Strand, M. (2010). Nemertean taxonomy – time to change lane? *J Zool Syst Evol Res.* **48**, 3, pp 383-284

Sundberg, P., Turbeville, J. M. & Lindh, S. (2010) Phylogenetic Relationships among Higher Nemertean (Nemertea) Taxa Inferred from 18S rDNA Sequences. *Molecular Phylogenetics and Evolution.* **20**, 3, pp 327–334

Sundberg, P., Andrade, S.C.S., Bartolomeaeus, T., Bekers, P., Dohren, J, V,. Kramer, D,. Gibson, R., Giribet, G., Herrera-Bachiller, A.,Junoy, J., Kajihara, H., Kvist, S.,Kanneby, T., Sun, S., Thiel, M., Turbeville, J.M. & Strand, M (2016a). The future of Nemertean taxonomy (phylum Nemertea) – a proposal. *Zoologica Scripta.* **45**, 6, pp 579-582.

Sundberg, P; Kvist, S & Strand, M (2016b). Evaluating the Utility of Single-Locus DNA Barcoding for the Identification of Ribbon Worms (Phylum Nemertea). *PLOS ONE.* **11,** 5, pp e0155541

Sychra, J. & Adamek, Z. (2010). Sampling Efficiency of Gerking Sampler and Sweep Net in Pond Emergent Littoral Macrophyte beds – a Pilot Study. *Turkish Journal of Fisheries and Aquatic Sciences.* **10,** pp 161-167.

Tamura, K.,Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). Mega6: Molecular Evoulutionary Genetics Analysis Verion 6.0. *Mol Biol Evol.* **30**, 12, pp 2725 -2729.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2003). A plea for DNA taxonomy. *Trens Ecol.Evol*. **18**, pp 70-74

Thorp, J.H. (1991) *Ecology and Classification of North American Freshwater Invertebrates.* Academic Press, California

Thorpe, R.S., Surget-Groba, Y. & Johansson, H. (2008). The relative importance of ecology and geographic isolation for speciation in anoles. *Phil. Trans. R. Soc B.* **363,** pp 3071 – 3081.

Turbevile, J.M.(2002). Progress in Nemertean Biology: Development and Phylogeny. *Integral and Comparative Biology.* **42,** 3, pp 692-703

Wang, W.,Zhang, K., Deng, D., Zhang, Y., Peng, S,. & Xu, X., (2016). Genetic Diversity of *Daphnia pulex* in the Reaches of the Yangtze River. *PLoS ONE.* **11**, 3, pp e0152436

Waterton, C., Ellis, R. & Wynne, B. (2013) *Barcoding nature: shifting cultures of taxonomy in an age of biodiversity loss.* Routledge, London

Weiss, M. & Leese, F. (2016). Widely distributed and regionally isolated! Drivers of genetic structure in *Gammarus fossarum* in a human-impacted landscape. *BMC Evoulutionary Biology.* **16,** 1, pp153

Williams, W.D. (1980) *Australian freshwater life*. Macmillian Publishers, Australia

Wilson, W.H. (1991) Competition and Predation in Marine Soft-Sediment Communities. *Annual Review Ecological Systems.* **21,** pp 221-241

Zhao, Y & Sun, SC. (2006). Effects of salinity, temperature and pH on the survival of the Nemertean *Procephalothrix stimulus* Iwata, 1952. *Journal of Experimental Marine Biology and Ecology.* **328**, 2, pp 168 -176

Young, J.O. (1975). Seasonal and Diurnal Changes in the Water Temperature of a temperate Pond (England) and a Tropical Pond (Kenya). *Hydrobiologia* **47,** 3-4, pp 513-526

Young, J.O. & Gibson, R. (1975). Some ecological studies on two populations of the freshwater hoploNemertean Prostoma eilhardi (Montgomery 1894) from Kenya. *Verh. Internat. Verein. Limnol.* **19,** pp 2803-2810

Appendix 1 – Unpublished Reports

Unpublished reports by Quigg & Lowe 2011a; 2011b; 2012a, prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside detailing stages 1 – 4 of the preliminary research.

Appendix 2 – DNA extraction protocol

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol).

1. Add *Prostoma* specimen a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Earmark the animal appropriately.

2. Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.

3. Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.

4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard flow-through and collection tube

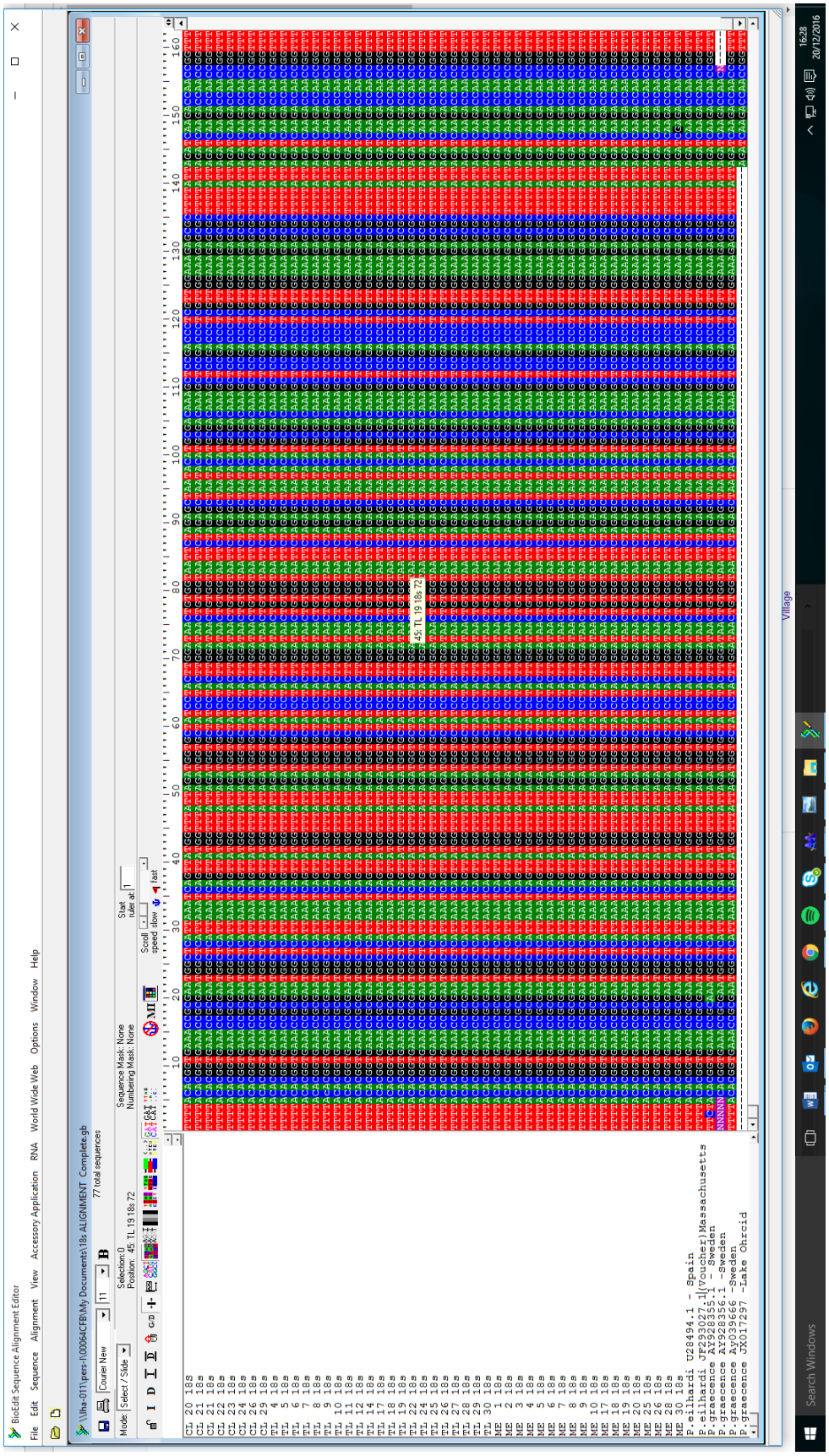
6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

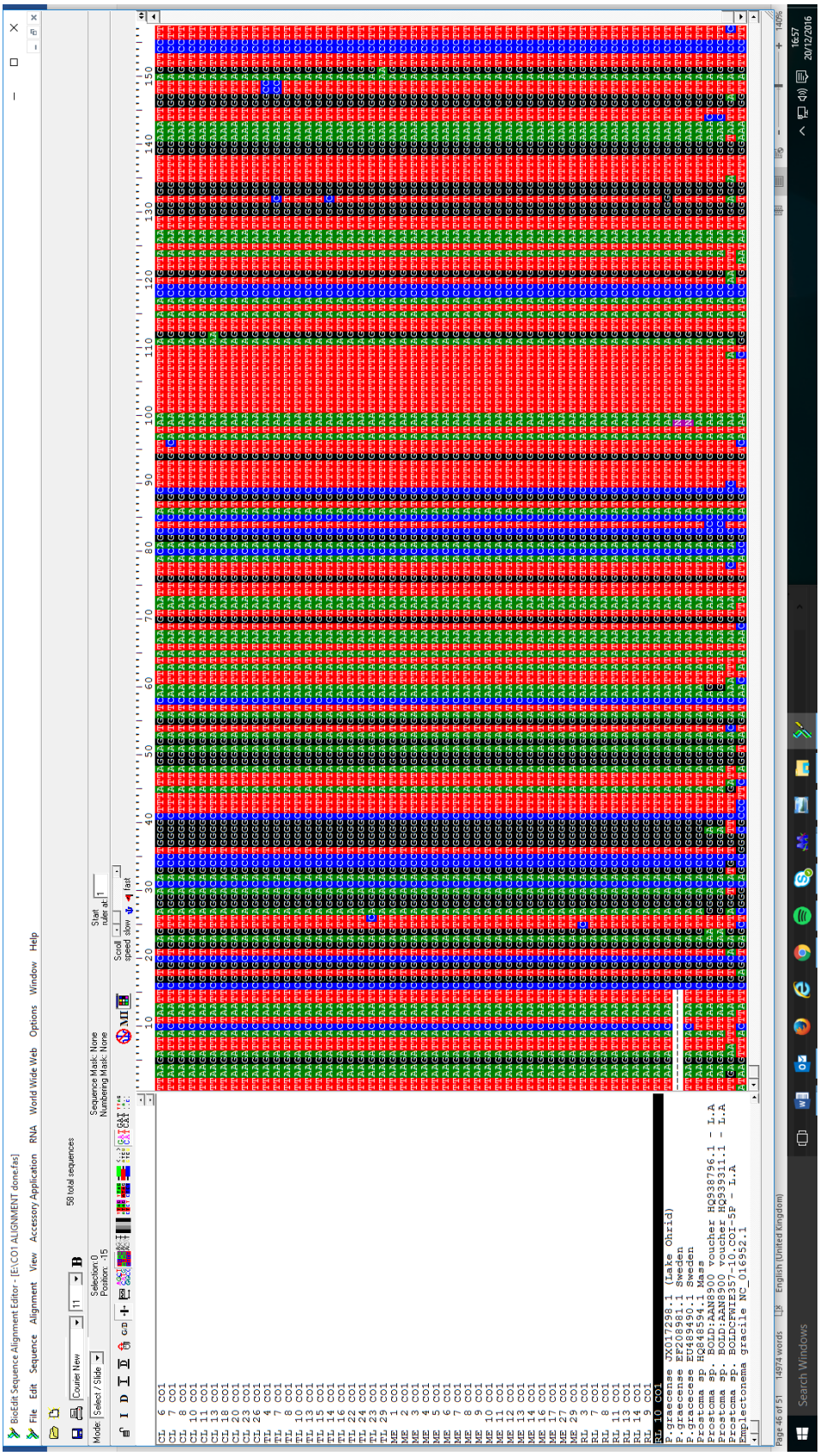
8. Recommended: For maximum DNA yield, repeat elution once as described in step 7.

DNeasy® Blood & Tissue Handbook – July 2006

Appendix 3 – 18s Sequence Alignment



Appendix 4 - COI Sequence Alignment



Appendix 5 - Individual Pairwise Differences

Please see attached Excel spreadsheet.

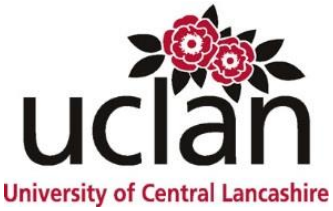
Confirming the existence of the

‘Croston Worm’ (*Prostoma jenningsi*)

The Clay ‘Ole, Bretherton

A report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside

The research detailed in this report was undertaken by Siobhan Quigg and Dr Chris Lowe from the School of Built and Natural Environment, University of Central Lancashire. The project was funded by Natural England.



Summary

The existence of the fresh water nemertean *Prostoma jenningsi,* more commonly known as the 'Croston Worm', has not been confirmed since 1999. The key actions of the project sought to confirm the existence of the species through systematically surveying the single pond location in which it is known to have existed. A full site history was compiled and Sampling techniques investigated. A net sampling technique was adopted, falling in line with previous research. The study was unable to confirm the continued existence of the species at the Clay 'Ole site however, knowledge surrounding *P.jenningsi* and the Clay 'Ole site was obtained, lessons were learnt and recommendations for future sampling attempts made - sampling during an alternative season and the use of a quantitative sampling method.

Table of Contents

Table of Figures

1.0 Introduction

The freshwater nemertean *Prostoma jenningsi* (the Croston Worm) is a UK BAP Species thought to be endemic to Lancashire. It is listed in the British Red Data book (1991) as Insufficiently Known. The species has been found solely at The Clay 'Ole, Bretherton (SD485198); however, its existence has not been confirmed at this location since 1999. The key actions of the project are to systematically survey the pond and establish the presence, and if possible estimate population density, of the species. Should the species be found, it would then be a priority to secure further populations where suitable conditions exist.

2.0 Nemertea

Phylum Nemertea is made up of approximately 1,150 nominal species, distributed between 250 genera (Gibson, 1995). Nemertean worms can be defined as unsegmented, bilaterally symmetrical, acoelomate animals, with a gut possessing separate mouth and anus, a blood vascular system, and a characteristic eversible proboscis situated dorsal to the gut in an enclosed tubular cavity, the rhyngchocoel (Gibson, 1972). The proboscis is a shared characteristic of the taxon and used primarily in prey capture. The species ranges in length from a few millimetres to about 30 metres, with a width that rarely exceeds a few millimetres. It occupies a broad range of habitats. The majority are found in marine or estuarine habitats, with a number of known terrestrial forms, and a small number have been recorded in freshwater environments (Turbevile, 2002).

To date the number of known freshwater nemerteans extends to only 22 reported species, representing less than 2% of the total number recorded. Like most nemerteans, the fresh water species are all free-living benthic, found under rocks and boulders, among algae and on mud bottoms on all depths from the littoral and down (Sundberg &Gibson, 2007). Most freshwater species belong to a monotypic genera and are known only from single localities; however, the genus *Prostoma* is an exception as it is widely distributed on a global basis, although locally, only sporadic in occurrence. (It is important to note, however, this known occurrence is largely reflective of sampling efforts, rather than the actual species distribution.) The genus *Prostoma* can be easily distinguished from its relatives; however, at a species level identification is more difficult, as its internal morphology is often key (Gibson, 1982; Gibson & Moore 1976).

3.0 Prostoma jenningsi

P. jenningsi was first recovered from samples collected at the site during July 1967 as part of a study by Johnstone O. Young, whilst he was thought to be researching freshwater Triclads (Gibson, pers comm). Young recognised *P.jenningsi* to be of the phylum nemertean; its identification was later officially confirmed with the help of Gibson (1971) and specimens held at the Natural History Museum.

P. jenningsi is a small slender hoplnemertean of elliptical body section. The species possess four to eight eyes (most commonly six) with the anterior two pairs being most well developed and generally larger than the others. Body colouration is dependent upon age and size, with no specimens below 6mm in length having displayed the full adult colouration. Young worms, with a length of approximately 0.5mm upon hatching, are an opaque-translucent white. From about 4 to 6 mm in length they gradually assume a yellowish hue, sometimes with a nervous system tinged pink or red, which generally deepens with age to the adult colour of darkish yellow to pale reddish brown. The maximum recorded length of the species is 18mm, as measured in normal loco-motory extension (Gibson & Young, 1971). Exact loco-motory movement of the species is unclear; however, amongst the terrestrial and freshwater genera, it is considered that adult nemerteans can only crawl, while small juveniles also swim and the use of the proboscis for rapid forward movement has been reported (Moore & Gibson, 1973).

P. Jenningsi is a true hermaphrodite and thought capable of breeding throughout the year; however, reproduction is considered to be more intensive between late autumn and early spring, due to previous studies having indicated that greater numbers of the species are found between late Autumn and Spring, with fewer numbers evident within the summer months. (It is also considered that a migratory event into deeper waters may have occurred at this period, thus accounting for fluctuations in numbers at this time.) As yet, neither the rate of reproduction nor the lifespan of the species is known (Gibson & Young, 1976).

On internal examination, it is the presence of eleven proboscidial nerves alone that distinguishes *P. Jenningsi* from all other *Prostoma* species within the genus; such is the closeness of the group's morphological similarity (Gibson & Young, 1971). *P. jenningsi* is thought to be predominately associated with the marginal vegetation, particularly the beds of *Phragmites* within the middle bay of the Clay 'Ole site, where it feeds on oligochatesetae, particularly Naididae.

As the Clay 'Ole is a relatively new pond, it is not possible for the nemertean to have evolved at this location. Questions are thus raised as to how the species came to be there. The species may have been introduced to the site as fish stocks have been replenished such as the nemertean populations

of *Apartronemertes albimiaculosa* found in freshwater aquarium tanks at the Dusseldorf City Aquarium, Germany and *Planolineus exsul* found in garden ponds at Buitenzorg, Java, both artificially introduced species that have never been found elsewhere. The possibility also exists that the species was introduced to the Clay 'Ole at the time when the pond was flooded, a point worth considering as it is thought that the freshwater genus *Prostoma* may be derived from estuarine/brackish water species. It could also be likely that the species has been introduced to the site after being carried from elsewhere on the feet of migratory birds (Sundberg & Gibson, 2008).

4.0 Site Description and History

The Clay 'Ole is a Biological Heritage site (BHS41 NEO6) made up of a flooded brick pit with a maximum length of 350m and breadth of 175m, surrounded by species-rich damp grassland and scrub amounting to approximately 8ha (Lancashire County Council, 1999). The water within the littoral zones of the site is generally shallow (approx. 0.5m); however, depths of 6m and beyond have been recorded where clay excavation has taken place (Gibson & Young, 1976). It is situated at grid reference SD485198, a lowland area lying on the border of the West Lancashire coastal plain and the River Douglas catchment area, at an elevation of approximately 5m above sea level (Mario, 2011). The underlying geology is made up of Permo-Triassic Keuper Marl, which is largely masked by glacial and post-glacial surface drifts of boulder clay, which has a strong influence over the area's landscape (Lancashire County Council, 2011). Alongside the Clay 'Ole sits the River Lostock, having been joined by its tributary, Wymott Brook, only a few hundred metres upstream.

The history of the surrounding area can be traced back as far as the early 16th century, when pressure on available land forced improvements and the reclamation of mosslands in the area (Lancashire County Council, 2011). In 1799, an Act of Parliament was passed and commissioners appointed to drain the lowlands of Croston, Mawdesly, Rufford, Tarleton and Bretherton to both improve the value of the land and enhance the health and comfort of its inhabitants (Baines, 1836).

Figure 1 – 1840' OS Map (source Edina)

The first edition (1840s) Ordinance Survey maps indicate that, prior to any clay excavation, the site was divided by a number of field boundaries, with small pits located in areas that are presently occupied by the Clay 'Ole.

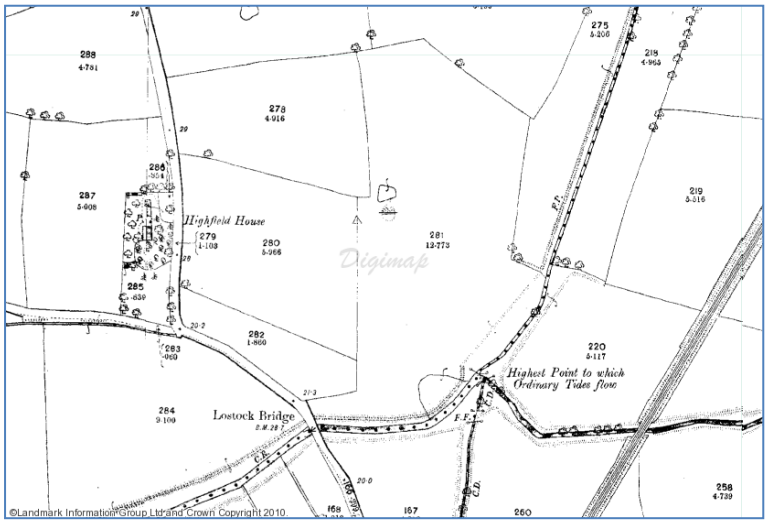


Figure 2 – 1890's OS Map (source Edina)

The 1890 edition Ordinance Survey map indicates a change to the field boundaries and relocation of small pits may have occurred; the accuracy of the maps during this period must, however, be considered. It is also clear that the point at which Wymott Brook and the River Lostock converge is the highest point to which ordinary tides flow, indicating that during this period, the area was within the tidal reach. Additional symbols suggest that the land was marshy and that saltings were present,

suggesting that any water within the ponds may have been brackish and that the area may possibly have been flooded by tidal water.

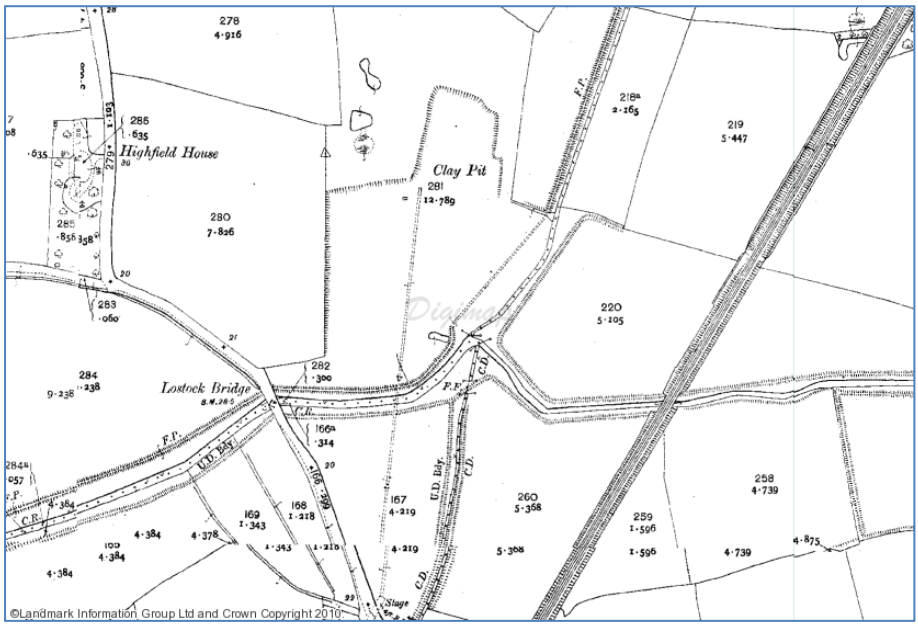


Figure 3 – 1910's OS Map (source Edina)

By the 1910s, clay extraction had begun at the site. An aerial ropeway had been put in place to transport clay from the site to Crompton & Co's brickworks. The 1910 Ordinance Survey map indicates that the area remained marshy with saltings present. Any ponds evident at this time do not sit in the place of the current pond; again however, it is important to consider the accuracy of the map at this time. The map also suggests that the wider area is prone to flooding by the River Lostock.

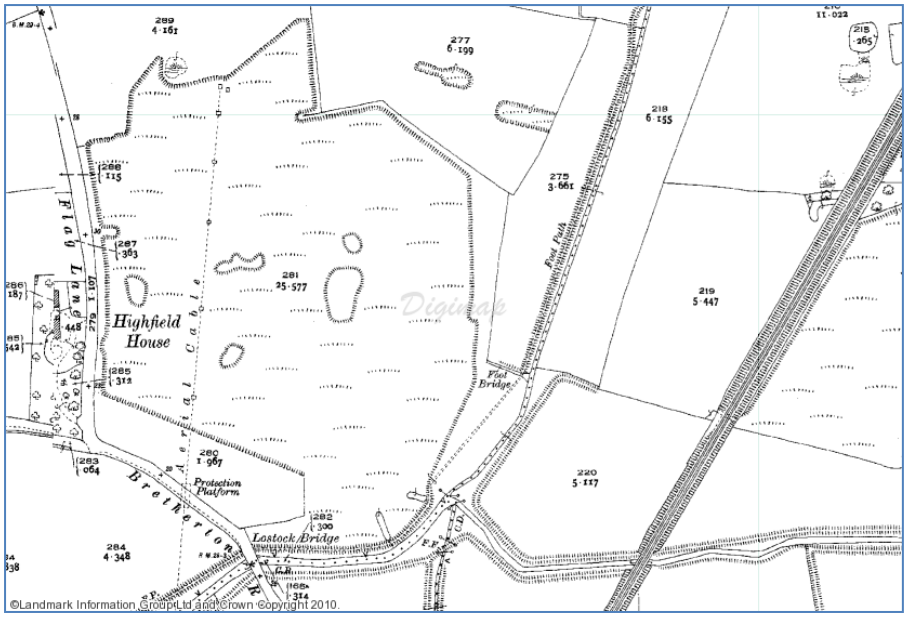


Figure 4 – 1920's OS Map (source Edina)

By the 1920s the majority of the site appears to be under excavation and the land predominately marshy with what appear to be five small pits. A new aerial cable is in place, again to transport clay from the site to the brickworks. Symbols indicate that saltings remain in the area.

A map produced by the River Douglas Catchment Board, dated 1935 (see Lancashire Records Office), depicts the site as being very much the same as the prior Ordinance Survey map (as seen in figure 4), with the general site remaining marshy and five small pits being present. It is thus considered that the present day Clay 'Ole was formed at some point between 1935 and 1955, where it becomes present on the Ordinance survey map - see figure 5. Whist writing about their ecological observations on *Prostoma jenningsi*, Gibson & Young (1971) suggest more specifically that the former clay pit was flooded approximately 25 years prior to their study, circa 1946. It is likely that the Clay 'Ole was flooded by water from the River Lostock, although suggestions of the site being spring fed have been made (however, there is no evidence available to support these claims).

Figure 5 1950's OS Map (Source Edina)

Throughout the period of 1932 – 1950, improvements were made to many of the rivers, brooks and streams in the River Douglas catchment area, undertaken by Lancashire County Council Land Drainage Department. Clay spoil was used from the Crossens excavations scheme for revetments across the area (Lancashire records office). During this time, revetment improvements were made to the River Lostock. In 1941 the Bretherton pumping station was constructed to deal with the flood waters within the catchment. These improvements did not however resolve the flooding in the area and flooding still occurred, possibly affecting the Clay 'Ole site. Evidence suggests that during 1961 further improvements were made downstream of Lostock Bridge and an accumulation of plastic silty caly removed. It is possible that these improvements may have extended upstream of the bridge adjacent to the Clay 'Ole site.

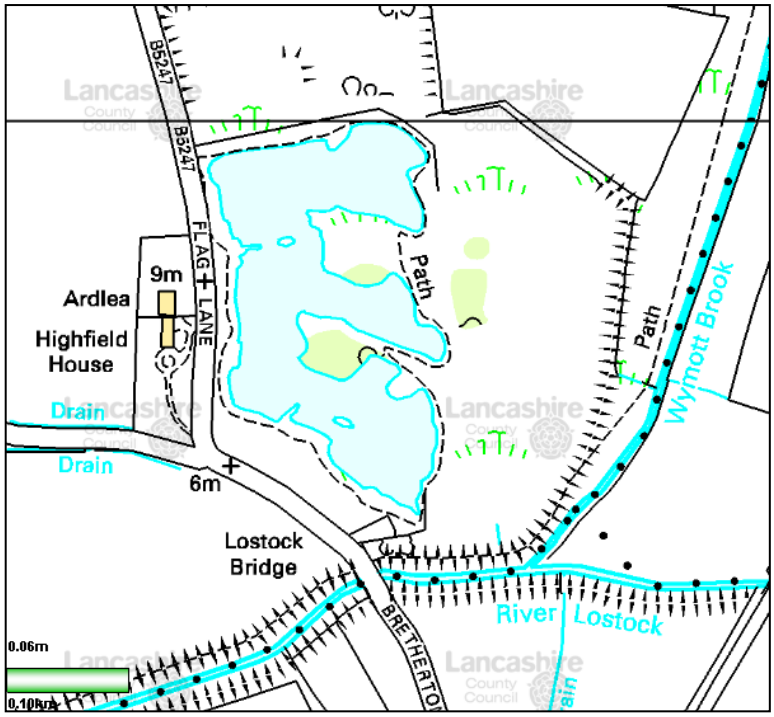


Figure 6 - Present Day Clay 'Ole (source Mario)

The Clay 'Ole has been used as a fishery since approximately 1955 and leased by Bretherton and Croston Angling Club from 1968 onwards, during which time the site has become a County Biological Heritage site. The site supports a wide variety of aquatic and terrestrial plants (including several very rare native plants), animals and bird species. The fishery has thirty-two permanent angling swims, with areas between swims being left as natural and undisturbed wildlife areas with the exception of clearance for pathways. Although the use of algaecide for the removal of weeds, particulary *Elodea canadiensis,* has been used in the past, it is thought that its use was limited and did not occur within the middle bay where P. *Jenningsi* has been previously found. A site survey carried out by Gibson (1998) confirmed that the use of chemicals on the site had not eradicated the species and the population remained. The only weed control within the middle bay has been limited to around the fishing pegs only and carried out by hand. Anglers currently describe the site as difficult to fish due to the large amounts of aquatic weed and abundance of natural bait within the water. The pond is thought to be well stocked with coarse fish such as carp, pike, bream, roach and eels.

5.0 Sampling Methods

During previous studies of *P. jenningsi*, specimens were collected by means of a standard F.B.A. zooplankton net mounted on a square frame. The net was used to sweep through the marginal vegetation (in which the nermerteans were predominately associated, particularly the beds of *Phragmites*), and to scoop up the substrates found in the locality of the plants (Gibson & Young, 1971). The procedure was carried out for a five-minute period in each of the weed beds sampled. Collections were placed in 3-litre narrow-necked glass containers. The containers were then returned to the laboratory and left to stand overnight at room temperature, allowing the oxygen in the water to become depleted. Animals present within the containers would move up to, on or near to, the surface of the film due to the depletion in oxygen, enabling specimens to be easily seen and removed. Samples were then subjected to a flotation technique (see Gibson & Young, 1971) for the separation of any worms previously missed. Gibson & Young acknowledged in their paper that due to difficulties of sampling among weed roots, the data they obtained could not be considered as quantitative; however, they stated that it was hoped that any changes in relative abundance might be discernible (Gibson & Young b, 1971).

For the purpose of this study, it was considered that a more quantitative approach should be considered, to better support the findings and give the data an element of robustness; however, through a review of the literature it became apparent that there is not a standardised procedure for the sampling of freshwater invertebrates in lentic conditions; nor is there a standard procedure for sampling within marginal vegetation, reedbeds and substrata. O'Conor *et al* (2004) suggest that within the literature there has been a disproportionate amount of focus placed on lotic macro-invertebrate sampling techniques in comparison with lentic methods, due to the use of aquatic invertebrates for biological monitoring of stream and river quality, such as RIVPACs adopted by Great Britain (Cox *et al*, 1997). Sampling methods considered included net sampling, grab sampling, sediment and core sampling and box sampling.

5.1 Net sampling

The pond net is possibly the most popular device employed in freshwater sampling; however, while procedures have been outlined for pond net sampling of lotic systems, the same is not true of lentic systems. A number of varying techniques are thus described in the literature. O'Conor *et al* (2004) suggest that pond nets may be swept or 'shuffled' (in a modification of the lotic kick-sampling method) and the size of the sample determined by time, distance, area or number of sweeps, with sweeping being best suited to macrophyte beds and soft substrata and 'shuffling' best when dealing with stony or gravelly substrata (see also Mackley *et al*, 2010; Bilton *et al,* 2006). Although a widely used method, the data obtained cannot be considered quantitative and questions can be raised about its

effectiveness when sampling for slower moving and bottom dwelling animals (O'Connor *et al*, 2004; Sychra & Adamek, 2010).

5.2 Sediment corers

Sediment corers have been used when sampling for macroinvertebrates in aquatic environments, particularly those associated with substrata and submerged roots. Corers can be used to both extract samples, including plant stems and submerged roots, and take stand-alone sediment samples. The advantage of coring devices is their ability to allow sampling to occur at different levels: in water, at water-mud interface and along a vertical profile within sediments which, theoretically, should remain undisturbed. Various sediment corers are available, such as gravity corers, piston corers and box corers (see Jones *et al,* 2000). Soumille & Thiery (1997) developed a sediment corer specifically for sampling invertebrates at different levels of rice plants within a shallow rice field, allowing stratified sampling to occur at varying vertical depths. Disadvantages of sediment coring techniques include the narrowness of the cores being extracted and the need for many replicates to be taken in order to gain a true representation of a given study area and provide comprehensive data for a thorough site survey; such methods are often time consuming.

5.3 Grab Samplers

Grab samplers, such as Ekman grab, van Veen grab and Surber samplers, are considered a good technique for sampling sediments that accumulate under water, working best in fine sediments such as muds and sands. Grabs collect bulk sediment using a scoop or bucket lowered to the bed with varying mechanisms depending on the design. Such samplers allow sampling to be quick and efficient, as large quantities are able to be collected per sampling attempt (size of sample is dependent on size of grab being used). The use of such samplers is, however, not best suited to areas of dense vegetation or where the samples are required to be taken between plants. Although they may result in the capture of organisms dwelling within the sediments, any animals present within the water column and on the submerged reeds are likely to be missed. (Jones *et al*, 2000). Pauw & Vanhooren (1983) found that the diversity of species in hand net samples was often greater than in samples taken with grab sampling devices. Although such devices can be effective, they are often more suited to areas of open water at greater depths and where techniques such as net sampling cannot be applied.

5.4 Box Samplers

Box sampling methods can be used to provide quantitative data when sampling for aquatic invertebrates, as they provide a known area and volume for each sample collected; they also allow for the inclusion of vegetation within the sample area. Like net sampling, various box sampling techniques exist, with no standard method apparent. Storey (2007) outlines a technique by which a round open-

bottomed barrel was placed around plants and into the water. Plant parts above the top of the barrel were removed and discarded, while the remaining plants were cut at ground level and retained for examination as part of the sample. A pail was then used to pour the water within the barrel through a sieve in order to separate any animals present. Samples were then returned to the laboratory for further investigation. O'Connor *et al* (2004) adopted a slightly different approach. A box frame was created by cutting out the bottom of a plastic storage box, which was lowered into the water and held firmly against the substrata; any organisms within the area were then removed using an aquarium-style fish net. Samples were then returned to the laboratory. Although both these methods allow for quantitative data to be obtained, they also present similar problems as net sampling, in that organisms may be missed. They can be tricky to use and require more than one person to carry out sampling. Gerking (1957) developed a box sampler specifically for surveying benthic macrofuana and phytomacrofauna within the littoral zone of lakes. The box, made up by a metal frame with mesh sides, is placed over the vegetation and into the water. Vegetation is then cut as near to the surface as possible. A sliding door is then closed across the bottom of the box and the box lifted from the water. The contents are then placed into a bucket and returned to the laboratory for further examination. This model has more recently been adapted to include poles for fixing the frame into the substratum and the sliding door replaced with a sliding cutting device. These additions allow for the plants to be cut below the surface and any creatures dwelling within the substratum to be collected (Sychra & Adamek, 2010). The Gerking box sampler and particularly its modified version appears to be a good solution for the sampling of freshwater invertebrates in lotic systems; they, however, are more expensive to produce than their simpler counterparts.

6.0 Study Method

Due to the nature of the study (with issues of time, availability of equipment and surveyor's limited experience), and with the priority of the project being to prove the existence of the nemertean, it was decided the study should adopt a net sampling technique in order to cover a greater area of the pond's marginal zone, whilst causing the least amount of disturbance. This was also in line with previous studies in which the species is known to have been found and would allow comparisons to be made with any data obtained.

As with Gibson & Young's study (1976) a five-minute sampling time per replicate was adopted. During this time a standard (1mm mesh) hand net was used in a jabbing/shuffling motion throughout the littoral and substrata and between the reeds. The contents of the net were emptied into 3.4-litre, rectangular, air-tight plastic containers. The sampling time included the time taken to empty the net's contents into the container – this occurred approximately 3 to 5 times per replicate. On occasions

when the container became full before the five-minute time period had been reached, sampling ceased, as it was considered a sufficient representative sample had been obtained. Once sampling was complete, the lids were placed on the air-tight containers and they were returned to the laboratory for processing.

6.1 Laboratory Analysis

Once back in the laboratory, larger pieces of debris and roots floating at the surface of the samples were removed from the containers, any organisms present on them having been carefully brushed off. The containers were then left to stand overnight at room temperature, allowing the oxygen in the water to become depleted, thus bringing creatures near to, on or up to, the surface film, as outlined by Gibson and Young (1976). Any 'worm-like' species were then removed from the container and preserved in 4% formalin for further investigation and identification under the microscope. This was felt to be necessary due to the inexperience of the investigator and the limited knowledge surrounding Prostoma species. Collection was limited to a one-hour period per container, due to the abundance of specimens per sample. It was considered that this would give a good representative sample, whilst adhering to the time constraints involved with processing samples of freshwater macro invertebrates. Care was taken, however, so as not to exclude any specimens of *P. jenningsi* present. Once preserved, specimens were identified under the microscope with the help of species key guides. Any specimens considered not to be *P. Jenningsi* were thus excluded from the results.

The study primarily focused on the stretch located within the middle bay of the Clay 'Ole site, in which previous studies have been concentrated and the species is known to have been found – Sample Site A (see figure 7). Ten replicates were taken, approximately 10 meters apart, between SD 4853419851 and SD 4863819821, with 5 being taken 18.07.11 and a further 5 on 20.07.11. (This was done in order to allow for the time taken to process the samples in the laboratory and to prevent samples from becoming stagnant). Garden shears were used to cut a path to the water's edge in order to sample. Where it was not possible to gain access at a ten-meter point, the nearest available access point was used.

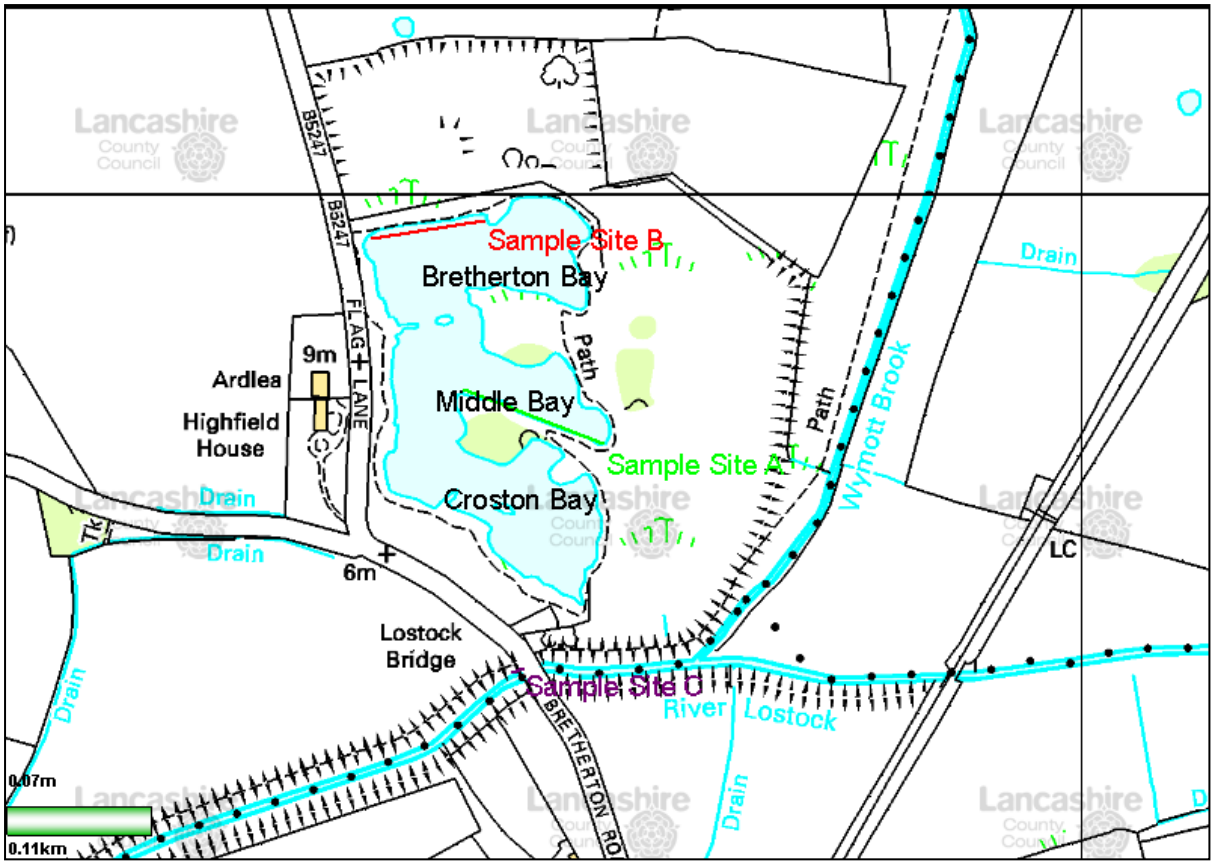


Figure 7 - Clay 'Ole Sampling Sites

Further samples were taken from study site B on 17.08.11 (see figure 7), a stretch of *Phragmite* stands within the Bretherton Bay of the Clay 'Ole, displaying similar conditions to those found at sample Site A. This was carried out as a follow-up to the initial study, enabling comparisons to be drawn between the sites and to establish whether populations of the species are present in an alternative area of the pond. Six replicates were taken at Sample Site B, between SD4847119976 and SD 4855019983, and the same procedures applied. Samples were also taken from an area of reedbed within the River Lostock, SD 4856419679, near to the Clay 'Ole – Sample Site C (see figure 7), to investigate the possibility of *P. jenningsi* population, and the river as a possible source for the population, within the Clay 'Ole. Two replicates were taken, again approximately 10 meters apart. It is important to note, in the days prior to sampling at the river, high water levels and storm flow were experienced due to heavy rainfall events in the area; thus the reed beds were very much flattened and not at their optimum condition.

7.0 Results

After careful observation of all samples taken, and further study under the microscope, no samples of *P. jenningsi* were recovered for the study period at any of the sample sites. Despite this, the Clay 'Ole appeared to be healthy, species-rich and abundant with life. Creatures such as hoglouse, water mites,

water beetles, waterboatmen, chrionomid, mollusca (including *Bithyria tentaculata, Ancylus lacustres, Planortses comlplanatus*), numerous leech species, bivalves, nymphs – possibly stonefly, caddis fly larvae, numerous flatworm species (these were present in abundance) and other oligochaetesetae including naididae were present in all samples collected. Fewer numbers and species were collected in samples taken from the River Lostock.

*Prostoma is often found when sampling flatworms (Gilbert, pers comm).

Can include tables of numbers of flatworms etc here.

8.0 Discussion

There are a number of possible explanations as to why no specimens of *P. jenningsi* were recovered during the study period, whilst other species appeared to be abundant. The time of year (July –August) at which the study was carried out coincides with the period in which the fewest specimens of *P. jenningsi* were recovered during Gibson & Young's research (1976). They suggest that fewest numbers of *Prostoma* occurred during the summer months, whilst higher numbers were recorded during the winter/spring; it is however noted that the results of their study were not quantitative due to the sampling method employed. Gibson and Young (1976) give various possible explanations for the decline in numbers during the summer months of their study, which should not be discounted from the present study. Firstly, they consider a possible migratory period into deeper waters during the summer months, although at the time of their study this was linked to trampling and disturbance by grazing cattle, an occurrence that no longer takes place at the site; other suggestions for possible migration, such as reproduction, could be considered. Further suggestions for the decline during this period include death after reproduction, senility and predation. As the present study was carried out during the period when the fewest specimens have been recorded in the past, it is possible that the findings are in line with previous data. It should also be considered that sampling over a limited period of time cannot produce a truly representative ecological sample, given the fluctuations that occur within natural environments.

The sampling method employed for the study was not quantitative and samples taken can only be considered representative of each point. It is possible that any specimens of *P. jenningsi* present at the site have simply been missed by the net. O'Connor *et al* (2004) suggest that when sampling for macroinvertebrates with a pond net, it is not a highly effective method when collecting specimens that anchor themselves to the substrata; and nor is it when sampling within dense vegetation. They suggest that, whilst the pond net is effective at sampling within the water column, it is less effective in its ability to capture organisms hidden in more complex areas of the littoral and substrata, thus

making it unlikely to reveal the full diversity of macroinvertibrates in a study area. Although Gibson and Young were able to recover a number of specimens throughout the summer months with the use of a net, greater numbers were recovered during periods when vegetation was less dense; it is possible that the fluctuations in recorded numbers are purely down to the success of the sampling method.

A further factor to take into account, when considering the failure to collect any samples, could be the inexperience of the surveyor; however, given the diversity and abundance of other species collected within the samples, it is unlikely that inexperience has had any effect on the data. Further to this, Gibson (2011) indicated that no specimens were recovered during his last sampling attempt (Gibson, 2011 pers comm). Weather conditions could also be considered, as, at the time of the study, the weather conditions were changeable and a particularly cold summer was experienced. Again however, the abundance and diversity of other species within the samples suggests that this should not have impacted on the numbers of *P. jenningsi.* The possibility that the population of *P.jenningsi* has become extinct at the site could be considered; however, this cannot be concluded, due to the limited time over which the study was executed.

9.0 Conclusion and Recommendations

Although the study has not proven the existence of *P.jenningsi* at the Clay 'Ole site, neither has it been disproved. It has been seen that the Clay 'Ole is a healthy, abundant and species-rich environment, thus suggesting no reason as to why the species should no longer be present. The time period over which the study has been carried out, and number of samples taken, cannot be considered conclusive, particularly given the ecological nature of the project and the fluctuations observed in previous studies of the species. The sampling method employed may have brought limitations to the quality and diversity of the samples obtained, with the possibility that any specimens of *P. jenningsi* present within the sampling area have simply been missed.

For any true conclusions to be drawn surrounding the existence of *P. jenningsi*, the study would benefit from, at a minimum, a further attempt at sampling during a period at which higher numbers of the species are thought to exist, such as during the winter months, and ideally samples being taken monthly over a one-year period, in order to account for seasonal changes, ecological life-cycles and any possible monthly sampling anomalies. The study would benefit from a more quantitative sampling method, so as to eliminate the possibility of specimens being missed within a given sampling area. Due to the difficulties presented by the site, such as sampling amongst reedbeds and attempting to collect specimens thought to exist within the substratum and littoral zones, as well as on the plants themselves, future studies should consider the use of a box sampler. Box samplers have the advantage of being able to capture organisms hidden within the littoral and substratum zones, allowing for a

greater reflection of the sampling area, whilst providing highly quantitative data, due to the known area and volume of the sample taken (O'Connor *et al*, 2004). For this particular study, and given the nature of the sampling area, a modified Gerking box sampler should be seriously considered, as it is designed specifically for sampling macroinvertibrates within littoral macrophyte beds. The modified Gerking box sampler is an adaptation of the original Gerking box sampler design – see Gerking 1957. The design has been modified to include a metallic frame box and a removable cutter, thus eliminating difficulties surrounding the cutting of stems within the substratum. When tested against the efficiency of a pond netting technique, the modified Gerking box sampler, although more labour consuming, was significantly more effective when capturing slow-moving sedentary animals such as gastropods, oligochaetes, leeches, water mites and chironomid larvae; therefore, if present, it should be able to successfully capture *P. Jenningsi.* It was considered to be a more suitable method for quantitative monitoring of macroinvertibrates in littoral zones of standing water bodies (Sychra & Adamek, 2010).

With an extended study period, and the use of a more effective and quantitative sampling method, the likelihood of success in capturing specimens of *P. jenningsi* would be greatly improved. Any data obtained would be more robust, thus enabling more substantial conclusions to be drawn as to the existence of the species within the Clay 'Ole site

References

Baines, E. (1836) History of The County Palatine And the Duchy of Lancaster,. Vol. III Leyland Hundred Parish of Croston. Available at: http://www.heskethbank.com/history/baines/baines_croston.html . Viewed 17/06/2011.

Bilton, D.T., McBendroth, L., Bedford, A. & Ramsay, P.M. (2006). How wide to cast the net? Cross-taxon congruence of species richness, community similarity and indicator taxa in ponds. *Freshwater Biology.* **51**, 578-590.

Cox, R, Wright, J.F, Furse, M.T & Moss, D. (1997) RIVPACS III (River InVertebtate Prediction and Classification System) User Manual. Environment Agency and Institute of Freshwater Ecology

Gerking, S.D. (1957). A Method of Sampling the Littoral Macrofauna and Its Application. *Ecology.* **38,** 2 pp219-226.

Gibson, R. (1972). *Nemerteans.* Hutchinson & Co. LTD, London.

Gibson, R. (1982). *British Nemerteans* . Cambridge University Press, Cambridge

Gibson, R. (1995). Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. *Journal of Natural History*. **29**. pp 271-562.

Gibson, R. & Moore, J. (1976). Freshwater Nemerteans. *Zoological Journal of the Linnean Society.* **58.** Pp 177-218.

Gibson, R. & Young, J.O. (1971). *Prostoma jenningsi* sp.nov., a new British freshwater hoplonemertean. *Freshwater Biology.* **1.** Pp 121-127.

Gibson, R. & Young, J.O. (1976). Ecological observations on a population of the freshwater hoplonemertean *Prostoma jeninngsi. Archives of Hydrobiology.* **78.** 1. Pp 42-50.

Jones, A., Duck, R. & Wayers, J. (2000). *Practical Skills in Environmental Science*. Pearson Education Limited, London.

Lancashire County Council (2011). A Landscape Strategy for Lancashire – Landscape Character Assesment: Coastal Plain. Available at: http://www.lancashire.gov.uk/environment/landscape/landscapecharacass/charcarea15.asp Viewed 17/06/2011.

Mackleyi, E.A., Godfrey, A & Hardman, C.J. (2010) RSPB/NE Countdown 2010: Bringing Reedbeds to Life Project; Wildlife surveys.

Moore, J. & Gibson, R. (1973) A new genus of freshwater hoplonemertean from New Zealand. *Zoological Journal of the Linnean Society*. **52** pp141–157

O'Connor, A., Bradish, S., Reed, T., Moran, J., Regan, E., Visser, M., Gormally, M. & Skeffington, S. (2004). A comparison of the efficacy of pond-net and box sampling methods in turloughs – Irish ephemeral aquatic systems. *Hydrobiologia.* **524,** pp 133-144.

Pauw, N. & Vanhooren, G. Method for biological quality assessment of watercourses in Belgium. *Hydrobiologia.* **100,** 1 pp 153-168.

Soumille, H. & Thiery, A. (1997). A new quantitative sediment corer for sampling invertebrates across the mud water interface and soil of shallow rice field. *Annals of Liminology.* **33:** 3 pp 197 -203.

Storey, R. (2007). *Aquatic invertebrate diversity and distribution at Aammiq, Marsh, Lebanon.* Available at: http://www.arocha.org/int-en/work/research/ecosystems/1969-DSY.html. Accessed 08.08.2011.

Sundberg, P. & Gibson, R (2008). Global diversity of Nemerteans (Nemertea) in freshwater. Hydrobiologia **595,** pp 61-66.

Sychra, J. & Adamek, Z. (2010). Sampling Efficiency of Gerking Sampler and Sweep Net in Pond Emergent Littoral Macrophyte beds – a Pilot Study. *Turkish Journal of Fisheries and Aquatic Sciences.* **10,** pp161-167.

Turbevile, J.M.(2002). Progress in Nemertean Biology: Development and Phylogeny. *Integral and Comparative Biology.* **42:** 692-703.

Confirming the existence of the

‘Croston Worm’ (*Prostoma jenningsi*)

Stage 2: Winter 2011

The Clay ‘Ole, Bretherton

A report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside

The research detailed in this report was undertaken by Siobhan Quigg and Dr Chris Lowe from the School of Built and Natural Environment, University of Central Lancashire. The project was funded by Natural England.

Summary

The existence of the fresh water nemertean *Prostoma jenningsi,* more commonly known as the 'Croston Worm', has not been confirmed since 1999. The key actions of the project sought to confirm the existence of the species through systematically surveying the single pond location in which it is known to have existed. As a follow up to a study that took place during summer 2011, stage 2 of the project sought to conduct further surveys of the pond using previous knowledge and recommendations made in stage 1. A modified Gerking Box sampler was used alongside the net sampling technique used during stage one of the project. Sampling was carried out during a different season (November-December 2011) as recommended by the previous report and in line with findings from previous research. The study was unable to confirm the continued existence of the species at the Clay 'Ole site and the modified Gerking box sampler was not fully utilised due to a number of logistical factors; however, knowledge of seasonal variations that occur at the site was obtained, lessons were learnt and recommendations made regarding future sampling attempts.

Recommendations

This report makes several recommendations related to future studies:

- Further sampling events are required to account for seasonal changes, ecological life-cycles and any possible monthly sampling anomalies.
- The collection of quantitative data using the modified Gerking box sampler (when conditions are more favourable) would allow for more robust data collection. This would also enable a more representative comparison to be drawn between sampling methods, which would be beneficial to the study and to the research of aquatic phytophilous macro-invertebrates in hard emergent littoral macrophyte beds in general.
- The survey should be widened to include similar pond locations in the surrounding area.

Contents

1.0 Introduction

The freshwater nemertean *Prostoma jenningsi* (the Croston Worm) is a UK BAP Species thought to be endemic to Lancashire. It is listed in the British Red Data book as Insufficiently Known. The species has been found solely at The Clay 'Ole, Bretherton (SD485198); however, its existence has not been confirmed at this location since 1999. The key actions of the project set out to systematically survey the pond and establish the presence, and if possible estimate population density, of the species. Should the species be found, it would then be a priority to secure further populations where suitable conditions exist.

1.1 *Prostoma jenningsi*

P. jenningsi was first recovered from samples collected at the study site during July 1969 as part of a study by Johnstone O. Young of Liverpool University, whilst he was thought to be researching freshwater Triclads (Gibson, pers comm., 2011). Young recognised *P.jenningsi* to be of the phylum nemertean; its identification was later officially confirmed with the help of Gibson in 1971 and specimens are held at the Natural History Museum. *P. jenningsi* is thought to be predominately associated with the marginal vegetation, particularly the beds of *Phragmites* within the middle bay of the Clay 'Ole site, where it feeds on oligochatesetae, particularly Naididae (for more in depth background of the species, study site and current project, please see phase 1 report – Quigg & Lowe, 2011).

1.2 Phase two study

Phase two of the project sought to fulfil the recommendations made by phase one; to re-survey the pond at a more appropriate time of year, and to implement a more quantitative sampling method. It was considered that, for any true conclusions to be drawn surrounding the existence of *P. jenningsi*, the study would benefit from, at a minimum, a further attempt at sampling during a period at which higher numbers of the species are thought to exist, such as during the winter months (see Gibson & Young, 1976), and ideally for samples to be taken monthly over a one-year period, in order to account for seasonal changes, ecological life-cycles and any possible monthly sampling anomalies. It was also considered that the study would benefit from a more quantitative sampling method, so as to eliminate the possibility of specimens being missed within a given sampling area. Due to the difficulties presented by the site, such as sampling amongst reed beds and attempting to collect specimens thought to exist within the substratum and littoral zones, as well as on the plants themselves, the use of a box sampling device was recommended, due to its advantage of being able to capture organisms hidden within the littoral and substratum zones, thus allowing for a greater reflection of the sampling area, whilst providing highly quantitative data, due to the known area and volume of the sample taken, as suggested by O'Connor *et al* (2004).

With an extended study period, and the use of a more effective and quantitative sampling method, it was considered that the likelihood of success in collecting specimens of *P. jenningsi* would be greatly improved. It was thought any data obtained would be more robust, thus enabling more substantial conclusions to be drawn as to the existence of the species within the Clay 'Ole site.

2.0 Sampling methods

Drawing on the recommendations made by phase one of the Croston worm project (Quigg & Lowe, 2011), the use of a modified Gerking box sampler, in addition to the previously employed net sampling technique, was deemed appropriate for the survey in order to obtain more conclusive results. It was considered that a continuation of the net sampling technique (as used during stage one of the project) would be advisable, as the effectiveness of the box sampling method was yet unknown. In addition, the continuation of the net sampling method would allow for results to be compared with previous data obtained during phase one, and comparisons to be drawn surrounding the effectiveness of the sampling methods.

2.1 Net sampling

The pond net is possibly the most popular device employed in freshwater sampling; however, while procedures have been outlined for pond net sampling of lotic systems, the same is not true of lentic systems. A number of varying techniques are thus described in the literature. O'Connor et al (2004) suggest that pond nets may be swept or 'shuffled' (in a modification of the lotic kick-sampling method) and the size of the sample determined by time, distance, area or number of sweeps, with sweeping being best suited to macrophyte beds and soft substrata and 'shuffling' best when dealing with stony or gravelly substrata (see also Mackley et al, 2010; Bilton et al, 2006). Although a widely used method, the data obtained cannot be considered quantitative and questions can be raised about its effectiveness when sampling for slower moving and bottom dwelling animals (O'Connor et al, 2004; Sychra & Adamek, 2010).

2.2 Box sampling

Box sampling methods can be used to provide quantitative data when sampling for aquatic invertebrates, as they provide a known area and volume for each sample collected; they also allow for the inclusion of vegetation within the sample area. As with net sampling, various box sampling techniques exist, with no standard method apparent. Although various box sampling methods allow for quantitative data to be obtained, they also present similar problems as net sampling, in that organisms may be missed, as often the contents from within the box frame are required to be collected or scooped out by hand. They can be tricky to use and require more than one person to carry out sampling (see O'Connor et al, 2004; Storey, 2007).

2.2.1 Gerking box sampler

Gerking (1957) developed a box sampler specifically for surveying benthic macrofauna and phytomacrofauna within the littoral zone of lakes. The box, made up of a metal frame with mesh sides, was designed to be placed over the vegetation and into the water. Vegetation was then cut by hand as near to the surface as possible. A sliding door was then closed across the bottom of the box and the box lifted from the water, the contents placed into a bucket and returned to the laboratory for further examination (see Gerking, 1957).

2.2.2 Modified Gerking box sampler

Figure 1 - Modified Gerking box sampler with cutting blade retracted.

Figure 2 - View from above modified Gerking box sampler with cutting blade retracted to show size of sample obtainable.

Figure 3- Modified Gerking box sampler within reed bed.

The modified Gerking box sampler (see *figure 1)* is an adaptation of the original Gerking box sampler design as outlined above (see also; Gerking, 1957), its purpose to provide a quantitative sampling method as an alternative to the qualitative or semi-quantitative sweep net sampling technique more commonly used for research in aquatic environments. It is designed specifically for the sampling and collection of aquatic phytophilious macroinvertebrates in hard emergent littoral macrophyte beds. The modified device comprises of an open metal frame (height 75cm, base 25 x 45 cm inside dimension – see *figures 1-3*) and a movable cutter. Three sides are fitted with 500 µm mesh; the fourth is sheet metal. The corners of the base frame are fitted with sharpened poles for fixing the sampler into the substratum. The removable cutter slides through slots fitted along the long edges of the base (see fig 1). The addition of fixing poles and a sliding cutting device allow for the plants to be cut below the surface, (thus eliminating difficulties surrounding the cutting of stems within the substratum) and any creatures dwelling within the substratum to be collected. During a trial of the sampling device, Sychra & Adamek (2009) found the modified Gerking box sampler to be significantly more effective in capturing slow-moving or sedentary animals in comparison with sweep net samples, with the ability to capture all higher taxa (for full details of the trial method see - Sychra & Adamek, 2010).

3.0 Study methods

The study focused on the stretch located within the middle bay of the Clay 'Ole site, in which previous studies have been concentrated and the species is known to have been found (see figure 4; see also phase 1 report). A number of replicates were taken using the net sampling technique, approximately 10 metres apart, between SD 4853419851 and SD 4863819821. In addition, replicates were taken using the modified Gerking box sampler during the period 22.02.11 to 08.12.11. (This was done in order to allow for the time taken to process the samples in the laboratory and to prevent samples from becoming stagnant). Garden shears were used to cut a path through the vegetation to the water's edge in order to sample. Where it was not possible to gain access at a ten-metre point, the nearest available access point was used.

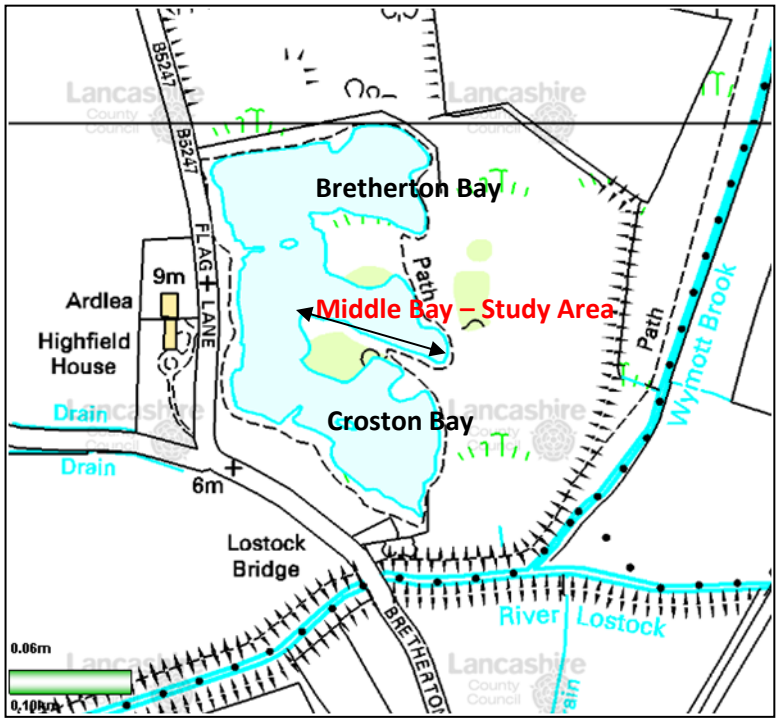


Figure 4 - The Clay 'Ole

3.1 Net sampling technique

As with Gibson & Young's study (1976), and during stage one of the Croston Worm project, a five minute sampling time per replicate was adopted. During this time a standard (1mm mesh) hand net was used in a jabbing/shuffling motion throughout the littoral and substrata and between the reeds (see figure 5). The contents of the net were emptied into 3.4-litre, rectangular, air-tight plastic containers. The sampling time included the time taken to empty the net's contents into the container – this occurred approximately 3 to 5 times per replicate. On occasions, when the container became full before the five-minute time period had been reached, sampling ceased, as it was considered a sufficient representative sample had been obtained. Once sampling was complete, the lids were placed on the air-tight containers and they were returned to the laboratory for processing.

Figure 5 - Net sampling

3.2 Modified Gerking box sampler

The box sampler was placed in the marginal macrophyte bed areas of the littoral zones in the middle bay of the Clay 'Ole (*see figure 4*). At each sampling site, where necessary, the upper, emerged part of the reeds were first cut off to allow positioning of the box. The device was then submerged into the substratum with the cutter blade retracted and the poles fixing it into place. Where necessary, reeds were cut away around the bottom of the box frame to allow it to sit flat to the bottom of the pond. Once in place, the sliding cutter was closed. The sampler was then removed from the water and the contents

poured into 3.4-litre, air-tight plastic containers to be returned to the laboratory for processing. The sampling device was cleaned between taking each replicate using a watering can to pour water over the mesh.

3.3 Laboratory analysis

Once back in the laboratory, larger pieces of debris and roots floating at the surface of the samples were removed from the containers, any organisms present on them having been carefully brushed off. The containers were then left to stand overnight at room temperature, allowing the oxygen in the water to become depleted, thus bringing creatures near to, on or up to, the surface film, as outlined by Gibson and Young (1976). Any worm-like organisms were then removed from the container and preserved in 4% formalin for further investigation and identification under the microscope. This was felt to be necessary due to the inexperience of the investigator and the limited knowledge surrounding *Prostoma* species. Collection was limited to a one-hour period per container, due to the abundance of specimens per sample. It was considered that this would give a good representative sample, whilst adhering to the time constraints involved with processing samples of freshwater macro invertebrates. Care was taken, however, so as not to exclude any specimens of *P. jenningsi* present. Once preserved, specimens were identified under the microscope with the help of species key guides. Any specimens considered not to be *P. Jenningsi* were thus excluded from the results.

N.B. Samples taken by the net were processed in the same way as those taken by the box sampler.

4.0 Results

After careful observation of all samples, and further study under the microscope, no samples of *P. jenningsi* were recovered for the study period using either sampling technique. Despite this, the Clay 'Ole appeared to remain healthy, species-rich and abundant with life. Creatures such as hoglouse, water mites, water beetles, waterboatmen, chrionomid, mollusca, numerous leech species, bivalves, numerous flatworm species (these were present in abundance) and other oligochaetes ncluding naididae were present in all samples collected.

The graphs below indicate the abundance of worm-like creatures (as outlined above) that were extracted from the samples and preserved for investigation, thus giving an indication of conditions found at the sampling sites. It is important to point out *Prostoma* is often found when sampling for flatworms (Gilbert, pers comm. 2011).

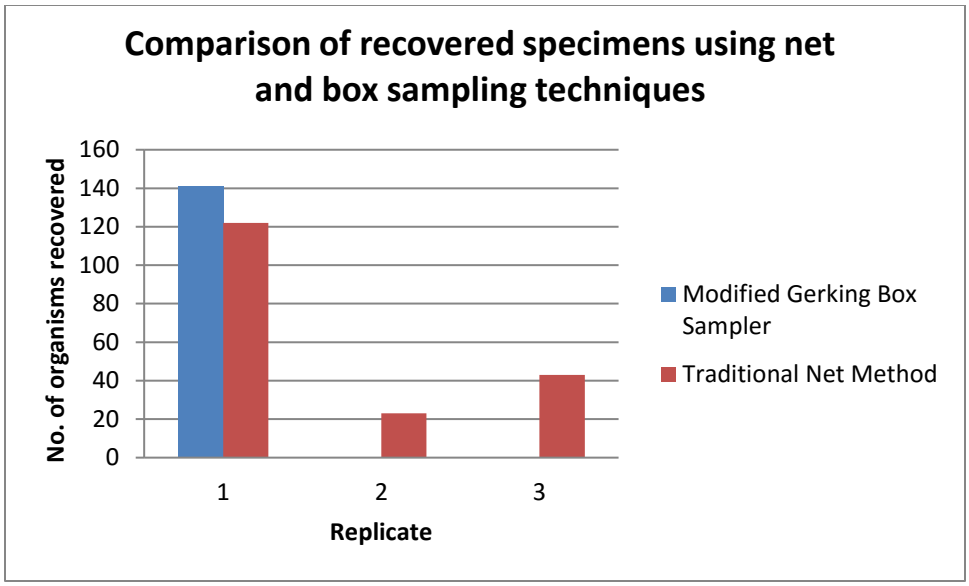


Figure 6 - Graph to show comparison of sampling methods

Figure 6 shows the number of worm-like organisms recovered by replicates 1, 2 and 3, using both the net and the box sampling devices. It can be seen that the box sampling device recovered a greater number of specimens at replicate one than the net method. At replicate sites 2 and 3, the box sampler was unsuccessful in capturing any specimens; however, the numbers captured using the net sampling method were very low.

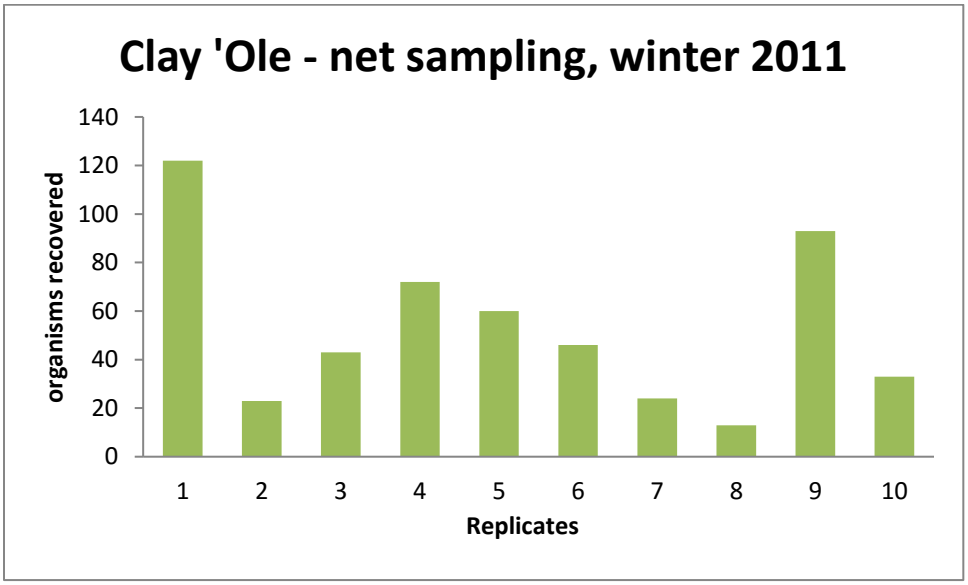


Figure 7 - Graph to show relative abundance of worm-like organisms collected using a sweep net technique during winter 2011.

Figure 7 shows the relative abundance of worm-like species collected using a sweep net technique over ten replicates within the Middle Bay (the area in which *P. jenningsi* has been previously found) of the Clay 'Ole site during phase two of the project, winter 2011. It can be seen that the fewest specimens were recovered from replicate 8, with the most abundant replicate being replicate 1.

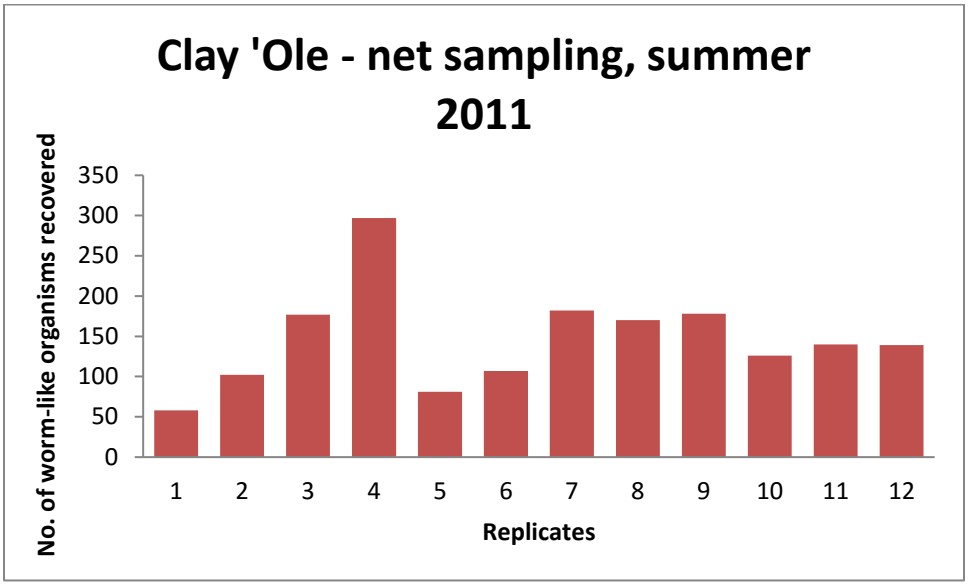


Figure 8 - Graph to show the relative abundance of worm-like organisms collected using a sweep net technique during summer 2011

Figure 8 shows the relative abundance of worm-like species collected using a sweep net technique over ten replicates within the Middle Bay (the area in which *P. jenningsi* has been previously found) of the Clay 'Ole site during phase one of the project, summer 2011. It can be seen that the greatest numbers occurred at replicate 4 and the fewest at replicate 1. Greater numbers were recorded over the summer study period in comparison with those recorded during the winter months.

5.0 Discussion

There are a number of possible explanations as to why no specimens of *P. jenningsi* were recovered during the study period, whilst other species appeared to be relatively abundant. The majority of the samples taken did not use a quantitative method, so can only be considered representative of each point. It is possible that any specimens of *P. jenningsi* present at the site have simply been missed by the net.

5.1 Modified Gerking box sampler

The modified Gerking box sampler was only used in three out of the ten replicates over the study period in which no specimens of *P. Jenningsi* were recovered. Unfortunately, adverse weather conditions (low

temperatures, rain, sleet, hail and strong winds) and consequently high water levels rendered the use of the box sampling device dangerous. As the pond is situated on the site of an old clay excavation pit (for full details and site history see phase one report), there is a steep drop off from the shelf/ledge at the pond's margins on which reed beds exist and the study is being conducted, as illustrated in *figure 9*. The box sampler is fairly labour-intensive and requires a certain amount of force and manipulation to be used when placing it into the substratum, cutting away reeds and sliding the cutter shut. The device was less effective in cutting dense reeds than was hoped (with the difficult conditions and high water levels contributing to the problems), leading to manual cutting with shears of the submerged reeds within and around the box frame. Surveyors were thus increasingly exposed to the cold water and put at risk of falling into deep water.

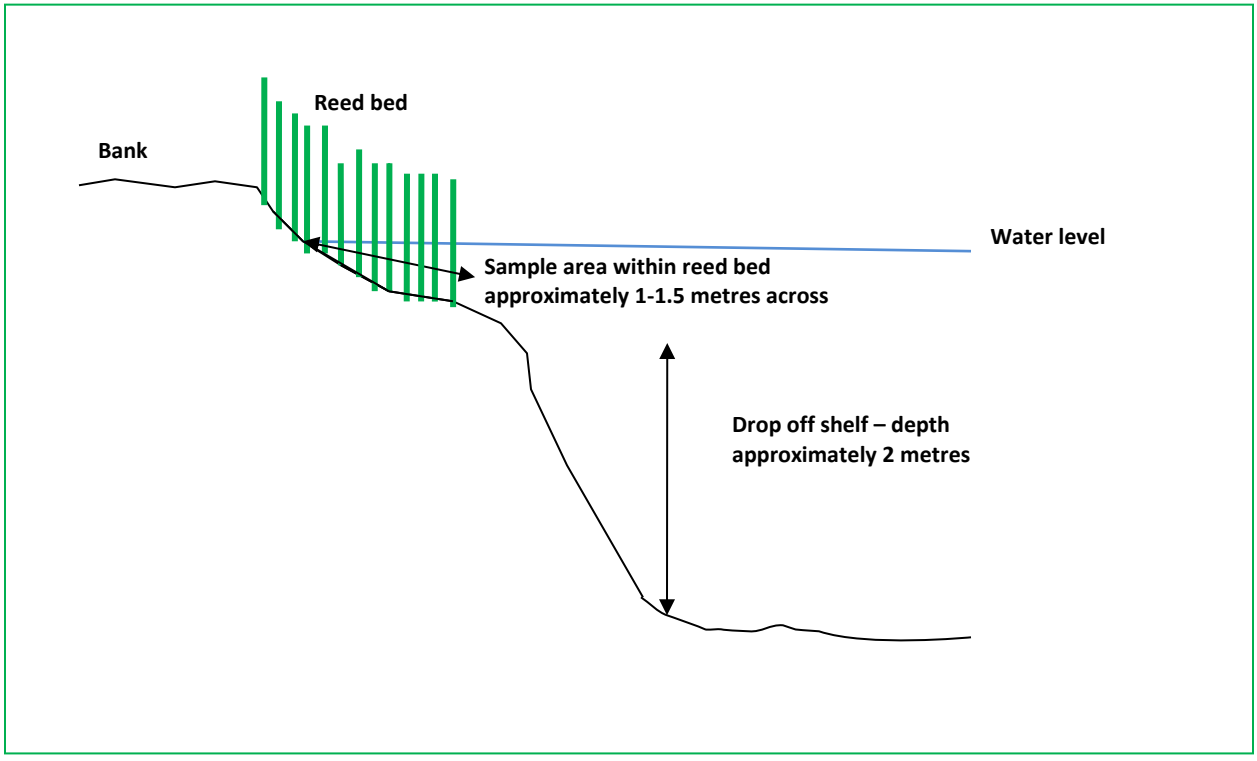


Figure 9 - Profile of the Clay 'Ole (not drawn to scale).

It can be seen in *figure 6* that, out of the three replicates taken by the Gerking box sampler, only the first replicate produced relevant specimens, with replicates 2 and 3 failing to collect any relevant specimens. The first replicate was taken in an area next to a marked fishing peg, where the reeds had been previously cleared; thus there were no hard stems to be cut through and sampling with the device was relatively

straightforward. In this instance the Gerking box sampler produced a good representative sample. When looking at the result of the net sampling technique in the same replicate, the box sampler collected a higher number of organisms. It is important to note that the net sampling technique covered a much greater area than that of the box. It could be considered that, if using a net in the same given area as the box, the net sampling result would have collected far fewer organisms. It can therefore be suggested that, during sampling at this particular replicate, the modified Gerking box sampler was more successful than the net sampling method in collecting specimens relevant to the study, thus falling in line with the research. During a comparative study conducted by Syrchra & Adamek (2010), comparisons were drawn with a net sampling device covering the same area as that of the modified Gerking box sampler. The study concluded that the box sampler collected significantly more gastropods, oligchaetes, leeches, chrionomid larve ($P>0.05$ in all groups) than that of the sweep net when conducted in the same given area. In both the net and box samples taken from replicate 1, an annelid species occurred (awaiting identification) measuring a maximum of 5mm, thus suggesting both methods are effective in the collection of sedentary macro-invertebrates.

The results of the Gerking box sampler in replicates 2 and 3 were disappointing, with no relevant organism being collected. This was perhaps due to the difficulties encountered with the sampling conditions, as outlined above. Much difficulty was encountered when placing the sampler on the substrata, due to the density of the surrounding reeds which prevented the frame from being placed directly onto the substrate. It is important to note, however, that the comparative net samples within replicates 2 and 3 produced relatively low results and, as explained above, the net samples were taken over a greater area; therefore, had they been restricted to the same area as the box sampler, it could be suggested that they, too, may have yielded no specimens.

On consultation with the manufacturer, it was suggested that the shutter be removed completely whilst the box is placed in position; once in position, it would then be possible to clear space for the shutter to slide in. Also it was suggested that it may be more effective on the reed bed's marginal zones; however, in the case of the Clay 'Ole, difficulties in accessing the reed bed's marginal zones occur due to the drop next to the ledge (see *figure 9*) (Syrchra 2011 pers comm). On reflection, it may be advisable to prepare the box sampling sites in advance of the sampling event, thus enabling the box sampler to be placed quickly and easily onto the substrata, whilst allowing for the area to settle after the initial disturbance.

5.2 Net sampling

The net sampling method employed for the study was not quantitative and samples taken can only be considered representative of each point. It is possible that any specimens of *P. jenningsi* present at the site have simply been missed by the net. O'Connor *et al* (2004) suggest that when sampling for macro-invertebrates with a pond net, it is not a highly effective method when collecting specimens that anchor themselves to the substrata; and nor is it when sampling within dense vegetation. It is suggested that, whilst the pond net is effective at sampling within the water column, it is less effective in its ability to capture organisms hidden in more complex areas of the littoral and substrata, thus making it unlikely to reveal the full diversity of macro-invertebrates in a study area.

5.3 *Prostoma jenningsi*?

In his last sampling attempt (date unknown), Gibson indicated that no specimens of *P. jenningsi* were recovered (Gibson, 2011 pers comm); this does not however allow any conclusions to be drawn surrounding the continued existence of the species. Further factors influencing the negative result could include weather conditions. Prior to the sampling, temperatures reached seasonal highs, whilst during sampling the weather was changeable with the occurrence of heavy rainfall and hail storm events. When drawing comparisons between summer and winter net sampling results (see figures 7 and 8), it can be seen that the relevant abundance of worm-like species collected in the winter months is significantly lower than those collected during the summer months. Although freshwater invertebrates occur throughout the year, seasonal variation is apparent, with the greatest richness and abundance being recorded in early spring. Fluctuations are thought to occur in response to a variety of factors which include food supply, intra-specific competition and temperature (Thorpe & Covich, 2009). Such factors should, however, not affect the population of *P. jenningsi* as, according to Gibson & Young (1971), greater numbers of the species are found between late autumn and spring, with fewer numbers evident within the summer months. An additional factor that may account for both lower abundance of relative species (when comparing winter and summer months) and the failure to collect specimens of *P. jenningsi* could be the increase in litter layer due to recently shed and decaying plant leaves. Increased litter provides additional shelter for benthic macro-invertebrates, thus making their collection more difficult during sampling (Thorpe & Covich, 2009). The increase in litter was evident when processing samples in the laboratory, thus making the procedure more lengthy and the samples more difficult to separate.

Although there are a number of explanations available to support the possible continued existence of *P. jenningsi* at the Clay 'Ole site, the likelihood that the species may no longer be present should also be

considered. Causes for the species to no longer exist in the pond could include changes in land and water management practices, inter-specific competition and predation.

At present the Clay 'Ole site is registered as a County Biological Heritage Site, a status granted in September 1993. This status imposes strict guidelines as to how the area is managed. Further to this, from 1999 onwards, the site has also been managed in accordance with the countryside stewardship agreement. At the time in which the species was first reported to exist at the site and during Gibson and Young's preliminary ecological investigation (see Gibson & Young, 1971; 1976), the site was under no form of protection. Cattle were grazed throughout the location and allowed access to the pond from which they drank. Concerns were raised, suggesting that the population of *P.jenningsi* may have been adversely affected by cattle trampling the pond's margins, following Gibson & Young's paper (1976). The paper proposed that interference by cattle during the summer months may have been a cause of fewer recorded numbers during this period. It could however be considered that the impact of the cattle within the pond may perhaps have given *P. jenningsi* an advantage, as Gibson & Young go on to suggest that the intrusion by the cattle could have led to the species migrating into deeper waters during this time, and later emerging when the disturbance ceased. The continued presence of the species during following sampling events suggested that the species was able to survive this pressure. The pond has since been fenced off from grazing animals, as suggested by the Lancashire Biodiversity Action Plan for the species, despite some suggestions that some trampling may be beneficial. It could be considered that these precautionary measures to protect the species have in fact had an adverse effect, as the balance in the pond's margins has been altered. There is however no evidence to support the suggestion that this proposed migration gave *P. jenningsi* an advantage and comparisons cannot be drawn with the numbers of other species, as no data is available.

It is also reported that algaecides were used to eradicate *Elodea canadiensis* after the discovery of *P. jenningsi* and concerns were raised as to whether this had had a detrimental effect on the population. A survey carried out by Ray Gibson during November 1998 confirmed that the species remained extant at this time (with an approximate 20 year gap between the previous studies), despite the chemical treatment of the pond. An emphasis was made at this point that the pond should be maintained in the best condition possible to ensure the species survival. The Lancashire Biodiversity Action Plan (1995) recommended that chemical usage on the pond should be avoided in the future. It is thus thought that all chemical weed control was ceased from this point onwards. The current management practice of aquatic weeds is through manual removal, which, in the Middle Bay of the Clay 'Ole is carried out around the marked fishing

pegs alone. Problems with aquatic weed growth have thus arisen. An aquatic macrophyte management report, following a site visit conducted in September 2005, suggested a number of possible weed control methods, including the use of herbicides, to deal with the problem. They concluded that, due to the lack of understanding surrounding the life cycle of *P. jenningsi* , any change in the regime of the lake, even just to leave the weed untreated, would have unknown consequences for the population. It was suggested that the only sensible option would be to continue the management practice that had been adopted since the time of the species discovery. Due to the fact the species was reported to still be present in 1999, any management strategies applied thus far had not had an adverse effect (A.G.A Group, 2005). It could be considered that an accumulation in aquatic weed growth may have had a negative effect on the population of *P. jenningsi.*

Anglers have reported that the accumulation of aquatic weeds has made the Clay 'Ole difficult to fish and the site has become unpopular. Rules are also in place as to the baiting techniques anglers are permitted to use at the pond, adding to their difficulties. In addition, anglers have suggested that due to the abundance of natural food sources available in the pond the fish are not easy to catch, although the Clay 'Ole is well stocked. It is unknown whether excessive predation by fish or other animals inhabiting the pond poses any threat to *P.jenningsi,* but it is possible that the decline in angling has led to possible predatory species becoming more reliant on natural food sources.

6.0 Conclusion and recommendations

Although the study has not shown the existence of *P.jenningsi* at the Clay 'Ole site, neither has it been disproved. The possibility that the population of *P.jenningsi* has become locally extinct at the site could be considered; however, this cannot be concluded, due to the limited time over which the study was executed and inadequate quantitative data. The Clay 'Ole continues to be a healthy and species-rich environment, following the normal fluctuations expected to be found in freshwater aquatic environments, thus suggesting no reason as to why the species should no longer be present. The time period over which the study has been carried out, and number of samples taken, cannot be considered conclusive, particularly given the ecological nature of the project and the fluctuations observed in previous studies of the species. The sampling method employed may have brought limitations to the quality and diversity of the samples obtained, with the possibility that any specimens of *P. jenningsi* present within the sampling area have simply been missed.

To add more depth to the data obtained thus far within the study, it would be beneficial to carry out a further sampling event to account for seasonal changes, ecological life-cycles and any possible monthly sampling anomalies. The collection of quantitative data using the modified Gerking box sampler would also be advantageous when conditions are more favourable, with the possibility of carrying out preparatory work prior to the sampling event. A further attempt at sampling with the box sampling device would also enable a more representative comparison to be drawn between sampling methods, which would be beneficial both to the study and to the research of aquatic phytophilous macro-invertebrates in hard emergent littoral macrophyte beds in general. In addition, given that the Clay 'Ole is a man-made site, dating back to as recently as circa 1955, the existence of *P.jenningsi* within the pond can only be through introduction (for more details and a comprehensive site history please see Quigg & Lowe, 2011). Due to the ecological nature of the project, it would be advisable to widen the survey to include similar sites in the surrounding area. As discussed by Quigg & Lowe (2011), it is possible that the species may have been introduced to the Clay 'Ole site through a number of different means, such as being transported from elsewhere on the feet of migratory birds (see also Sundberg & Gibson, 2008). It is also possible that P. jenningsi was introduced during periods of flooding in the area. If *P. jenningsi* were to have been introduced to the site through either of these means, it is probable that the species may occur elsewhere in the local vicinity.

Drawing on the knowledge gained during stages 1 and 2 of the project, any additional data obtained in future sampling events would allow for a more robust data set, thus enabling more substantial conclusions to be drawn as to the existence of the species within the Clay 'Ole site and, if applicable, the surrounding area.

References

A.G.A Group (2005) Unpublished Aquatic macrophite management report on behalf of Bretherton & Croston Angling Club Clay Ole Fishery.

Bilton, D.T., McBendroth, L., Bedford, A. & Ramsay, P.M. (2006) How wide to cast the net? Cross-taxon congruence of species richness, community similarity and indicator taxa in ponds. *Freshwater Biology.* **51**, 578-590.

Gerking, S.D. (1957) A Method of Sampling the Littoral Macrofauna and Its Application. *Ecology.* **38,** 2 pp219-226.

Gibson, R. (2011) Pers comm. – Telephone Conversation 22.08. 2011

Gibson, R. & Young, J.O. (1976) Ecological observations on a population of the freshwater hoplonemertean *Prostoma jeninngsi. Archives of Hydrobiology.* **78.** 1. Pp 42-50.

Mackley, E.A., Godfrey, A & Hardman, C.J. (2010) RSPB/NE Countdown 2010: Bringing Reedbeds to Life Project; Wildlife surveys

O'Connor, A., Bradish, S., Reed, T., Moran, J., Regan, E., Visser, M., Gormally, M. & Skeffington, S. (2004) A comparison of the efficacy of pond-net and box sampling methods in turloughs – Irish ephemeral aquatic systems. *Hydrobiologia.* **524,** pp 133-144.

Quigg, S., Lowe, C.N. (2011) Confirming the existence of the Croston Worm (*Prostoma jenningsi*), September 2011. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Storey, R. (2007) *Aquatic invertebrate diversity and distribution at Aammiq, Marsh, Lebanon.* Available at: http://www.arocha.org/int-en/work/research/ecosystems/1969-DSY.html. Accessed 08.08.2011.

Syrchra, J. (2011) pers comm. Email received 24.11.2011

Sychra, J. & Adamek, Z. (2010) Sampling Efficiency of Gerking Sampler and Sweep Net in Pond Emergent Littoral Macrophyte beds – a Pilot Study. *Turkish Journal of Fisheries and Aquatic Sciences.* **10,** pp161-167.

Sundberg, P. & Gibson, R (2008) Global diversity of Nemerteans (Nemertea) in freshwater. Hydrobiologia **595,** pp 61-66.

Thorp, J.H. & Covich, A.P. (2009) *Ecology and Classification of North American Freshwater Invertebrates*. Academic Press, London.

Confirming the existence of the

‘Croston Worm’ (*Prostoma jenningsi*)

Stage 4: Autumn/Winter 2012

The Clay ‘Ole, Bretherton

A report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside

uclan
University of Central Lancashire

The research detailed in this report was undertaken by Siobhan Quigg and Dr Chris Lowe from the School of Built and Natural Environment, University of Central Lancashire. The project was funded by Natural England.

Summary

The existence of the fresh water nemertean *Prostoma jenningsi,* more commonly known as the 'Croston Worm', has not been confirmed since 1999. The key actions of the project sought to confirm the existence of the species through systematically surveying the single pond location in which it is known to have existed. As a follow up to the initial study that took place during summer 2011, stage 2 of the project carried out during winter 2011 and stage 3 in spring 2012, stage 4 of the project sought to conduct further surveys of the pond using previous knowledge and recommendations made in stages 1, 2 and 3. The search was widened to include additional areas of the Clay 'Ole and also included 2 further ponds in the area. The study was unable to confirm the continued existence of the species at the Clay 'Ole site; however populations of *Prostoma* were recovered from both the additional sites.

Contents

1.0 Introduction

The freshwater nemertean, *Prostoma jenningsi* (the Croston Worm), is a UK BAP species, thought to be endemic to Lancashire. It is listed in the British Red Data Book as Insufficiently Known. The species has been found solely at the Clay 'Ole, Bretherton (SD485198); however, its existence has not been confirmed at this location since 1999.

1.1 *Prostoma jenningsi*

Prostoma Jenningsi

Class: Enlopa

Order: Hoplonemertea

Family: Terastemmatidae

Genus: *Prostoma*

Species: *Prostoma jenningsi*

P. jenningsi was first recovered from samples collected at the study site during July 1969 as part of a study by Johnstone O. Young of Liverpool University, whilst he was thought to be researching freshwater Triclads (Gibson, pers comm., 2011). Young recognised *P. jenningsi* to be of the phylum nemertean; its identification was later officially confirmed with the help of Gibson in 1971 and specimens are held at the Natural History Museum. *P. jenningsi* is thought to be predominately associated with the marginal vegetation, particularly the beds of *Phragmites* within the middle bay of the Clay 'Ole site, where it feeds on oligochatesetae, particularly Naididae. (For more in-depth background of the species, study site and current project, please see phase 1 and 3 reports - Quigg & Lowe 2011a; Quigg & Lowe 2012).

1.2 Phase Four Study

Phase four of the project sought to continue sampling for *P. jenningsi* and to fulfil recommendations made by phase three; to re-survey the pond to account for seasonal changes, ecological life-cycles and possible sampling anomalies; to widen the survey area within the Clay 'Ole in order to investigate the possibility that the species may have migrated to another area of the pond. In addition it was recommended that the survey should be widened further to include similar sites in the surrounding area due to the ecological nature of the project and, as discussed by Quigg & Lowe (2011), it is likely that the species may have been introduced to the Clay 'Ole site through a number of different means (see also Sundberg & Gibson, 2008); it is thus considered probable that the species may occur elsewhere in the local vicinity.

2.0 Study Methods

Drawing on the recommendations made in phases one, two and three of the project (see Quigg & Lowe 2011a; 2011b, 2012), it was considered that a continuation of the previously employed net

sampling technique would be most appropriate for the survey in order to maintain consistency and allow comparisons to be drawn with previous results.

The study was widened to include a further two similar ponds in the area, drawing on recommendations made by Christopher Laumer, who suggested the search be widened to include other ponds that may have similar conditions today to how the Clay 'Ole may have looked when *P. jenningsi* was first discovered and before management practices may have changed (Laumer, 2012; pers comm; see also Quigg & Lowe 2012). The ponds were thus chosen due to their similarities with the Clay 'Ole; both were situated on the site of former clay excavation works and both were situated close to rivers (the Lostock and the Douglas).

Chemical analysis of water samples was also carried out for ponds sampled, in order to ascertain the conditions in which *P. jenningsi* may be found and also to compare the water chemistry at the Clay 'Ole with historical data collected in both 2003 and earlier in 2012 – see Quigg & Lowe 2012. In addition, comparisons could be drawn between the Clay 'Ole and similar ponds in the area.

2.1 Clay 'Ole

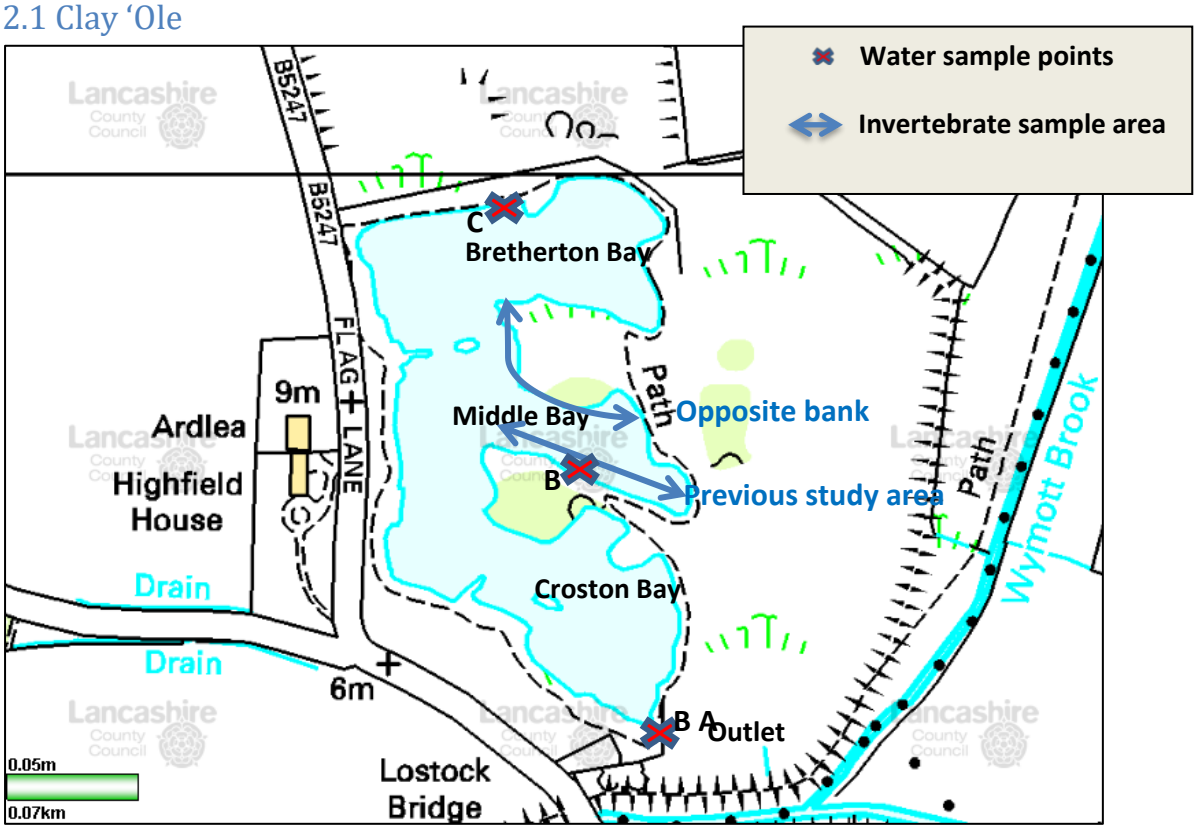


Figure 1 - the Clay 'Ole (source Mario)

The study primarily focused on the stretch of pond located within the Middle Bay of the Clay 'Ole site, in which previous studies have concentrated and the species is known to have been located (see *figure* 2; see also Quigg and Lowe ,2011a for a full outline of the site). Ten replicates were taken at 10 metre intervals using the net sampling technique, between SD 4853419851 and SD 4863819821. Furthermore, net sampling was also carried out along a stretch of *Phragmite* stands on the opposite side of the Middle Bay of the Clay 'Ole. Garden shears were used to cut a path through the vegetation to the water's edge in order to sample. Where it was not possible to gain access at a ten-metre point, the nearest available access point was used. Water samples were also taken from points A, B and C – see *figure 1*. Point A is within the Bretherton Bay of the pond, where water drains into the pond through drainage pipes from the surrounding land. Point B is within the Middle Bay of the pond and is the area in which *P. jenningsi* was previously located and point C is within the Croston Bay of the pond, where the water overflows and drains out into the River Lostock. The points were chosen to account for any variations that may occur within the site. At each point three replicates were taken.

2.2 Twin Lakes

Figure 2 - Twin Lakes

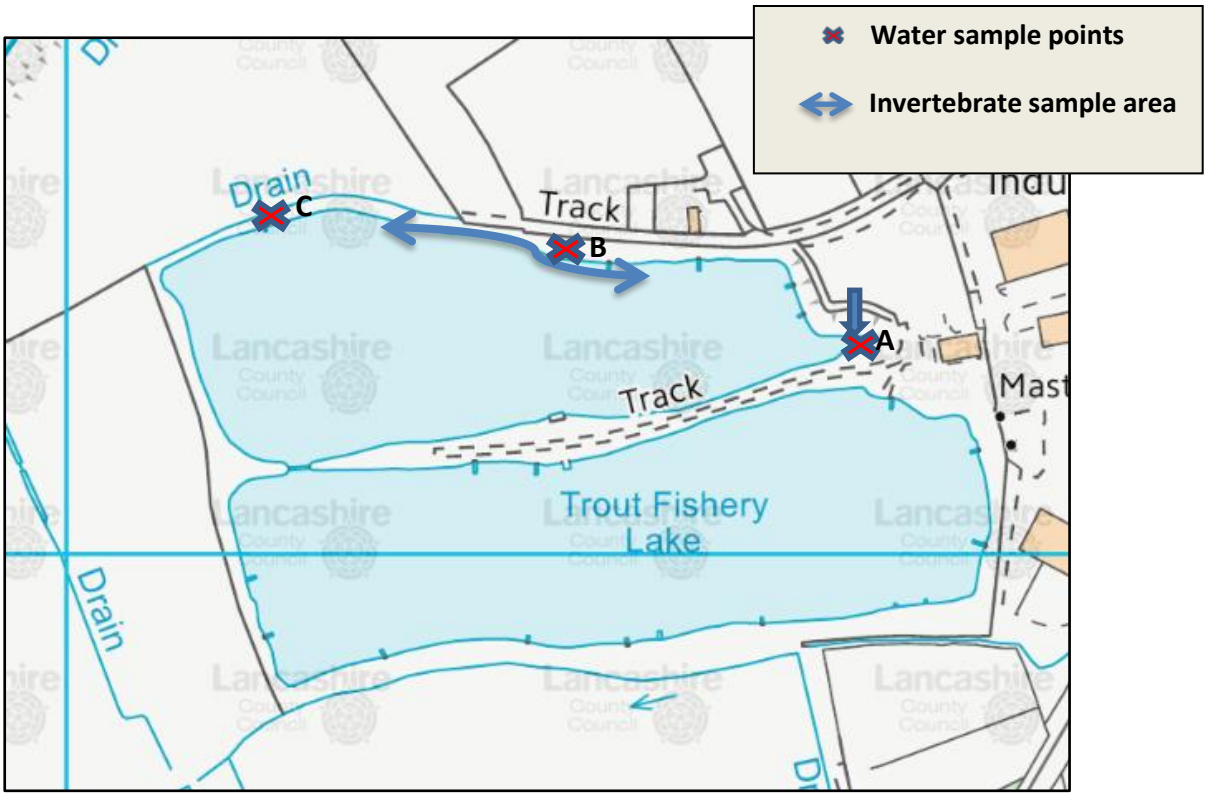


Figure 3- Twin Lakes sampling points

Samples were also taken from Twin Lakes, a similar pond in Croston, close to the Clay 'Ole (see *figure 7*). The Twin Lakes site is situated downstream of the Clay 'Ole, near to the River Lostock, on the site of a former clay extraction works**.** The site has been used as both a trout and course fishery for a number of years, having recently been taken on by Southport and District Angling Association. Access to the pond is gained by a path running around its perimeter and fishing pegs/small pontoons are in place. The general upkeep of the site is good. The surrounding area is relatively sparse, with a number of mature trees and some scrub present. The pond shares similar characteristics to those of the Clay 'Ole, exhibiting stands of *phragmites*, *Junctus* and other macrophytic vegetation around its margins (see *figure 2;* these are, however, limited to small areas and much of the upper zones of the pond's margins are artificial. The pond is very deep, dropping off almost immediately from the water's edge. Water levels within the pond are prone to fluctuation and the pond is linked by an outlet/inlet pipe to a drainage network which, in turn, is connected to the river Lostock - see *figure 3* A single sample was taken from a shallow area of the pond, with the remaining five being taken from the single stand of *phragmites* present - see *figure 3*. At these points aquatic invertebrate samples were taken, using the net sampling technique as outlined below. Water samples were also taken from points A, B and C – see *figure3.* Point A is at what is a gently sloping shallow part of the pond. B is within the *phragmite*

stand. C sits next to the inlet/outlet pipe. The points were chosen to account for any variations that may occur within the site. At each point three replicates were taken.

2.3 Hesketh Bank

Figure 4 - Hesketh Bank

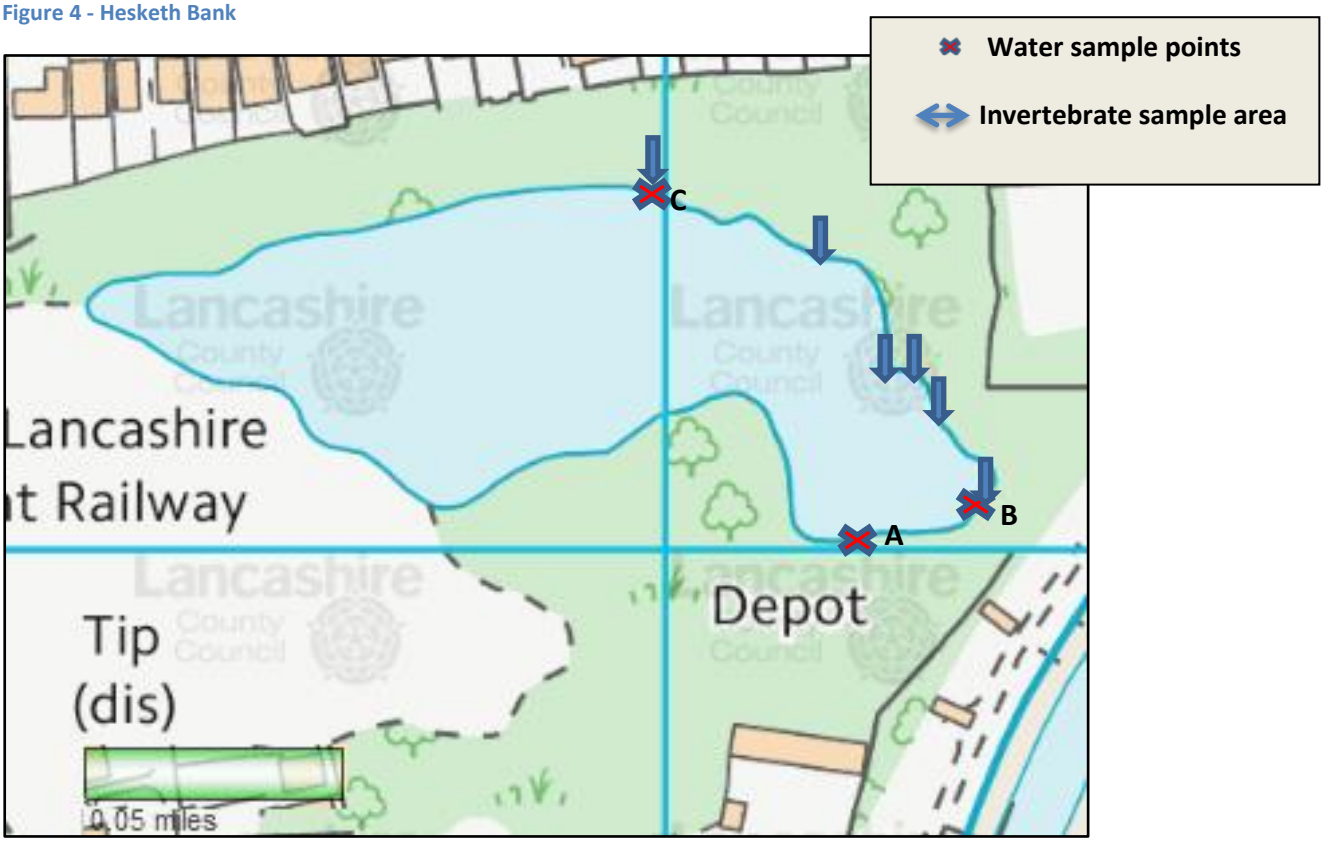


Figure 5 - Hesketh Bank sampling points (source Mario)

In addition to sampling at the Clay 'Ole site, samples were also taken from a pond in Hesketh Bank (see *figure 7* for location in relation to the Clay 'Ole). The Hesketh Bank pond is situated downstream of the Clay 'Ole and sits adjacent to the River Douglas on the site of a former clay extraction works**.** At this point the River Douglas (of which the River Lostock is a tributary) is well within the tidal reach and it was thus considered that salinity levels would be higher within this pond, allowing for comparisons to be made between this site and that of the Clay 'Ole, where questions have been previously raised concerning water quality – see Quigg & Lowe, 2012. The site is privately owned and managed by Alty's horticultural and building supplies, with fishing rights leased to Southport Fly Fishers. Although access to the pond can be gained by a path running around its perimeter, and fishing pegs/small pontoons are in place, the general upkeep is poor, with litter and industrial type waste both present around the pond's perimeter and within the water. A locally run website (see www.southportgb.co.uk) suggests that the pond has in the past been licensed to the builders yard as a landfill site and subsequently had waste bulldozed into it, reducing its size by up to a third of its original. The surrounding area is made up largely of mature trees and scrub, with the pond backed on to by residential gardens, Alty's horticultural and building supplies yard, the former Lancashire light railway and the River Douglas. The pond shares similar characteristics to those of the Clay 'Ole, exhibiting stands of *phragmites*, *Junctus* and other macrophytic vegetation within its margins (see *figure 4).* Water could be seen to be flowing into the pond through an inflow pipe from the river Douglas during high tide – see figure 6. Six locations were identified for sampling, as seen in *figure 5*. These points were chosen due to the areas of phragmites stands they supported and due to their ease of access. At these points aquatic invertebrate samples were taken, using the net sampling technique as outlined below. Water samples were also taken from points A, B and C – see *figure 5.* Point A is at what is considered to be the deepest part of the pond. B is the point at which the tidal water flows into the pond during high tide and point C sits close to the residential area. The points were chosen to account for any variations that may occur within the site. At each point three replicates were taken.

Figure 6- Tidal inflow at Hesketh Bank

2.4 Map of sample sites

Figure 2 - Map to show relation of Clay 'Ole to other sampling ponds

2.5 Sampling techniques

2.5.1 Net sampling technique

As with Gibson & Young's study (1976), and during stages one and two of the current project, a five-minute sampling time per replicate was adopted. During this time a standard (1mm mesh) hand net was used in a jabbing/shuffling motion throughout the littoral zone and substrata between the reeds. The contents of the net were emptied into 3.4-litre, rectangular, air-tight plastic container. The sampling time included the time taken to empty the net's contents into the container – this occurred approximately three to five times per replicate. On occasions, when the container became full before the five-minute time period had been reached, sampling ceased. Once sampling was complete, samples were returned to the laboratory for processing.

2.5.2 Laboratory analysis

In the laboratory, larger pieces of debris and roots floating at the surface of the samples were removed from the containers, any organisms present on them having carefully been removed. The sealed containers were then left to stand overnight at room temperature, allowing the oxygen in the water to become depleted, thus bringing organisms near to, on or up to, the surface film, as outlined by Gibson and Young (1976). Any 'worm-like' organisms were then removed from the container and preserved in 4% formalin for further investigation and identification under the microscope. Initial collection was limited to a one-hour period per container, due to the abundance of specimens per sample. It was considered that this would give a good representative sample, whilst adhering to the time constraints involved with processing samples of freshwater macro-invertebrates. However, care was taken not to exclude any potential specimens of *P. jenningsi*. Any specimens considered not to be *P. Jenningsi* were excluded from the results.

In the case where specimens of possible *P.jenningsi* were recovered, individuals were removed from the container and placed into a separate tub with air holes in the lid within some of their original pond water; samples would then be stored in an incubator at 10^{o}C (similar to that of the pond water). This would enable live specimens to be viewed under the microscope because, as suggested by Gibson, 2012, *Prostoma* are more easily identifiable when live. Once identification could be confirmed, specimens were either preserved in 100% ethanol or kept alive in the incubator for further investigation.

A number of specimens were prepared for histological investigation, following guidelines for fixation, sectioning and staining, as outlined by Gibson (1982). Further specimens were sent for DNA analysis.

2.5.3Water Testing

Water samples were taken in clean airtight plastic bottles. Each bottle was rinsed out with pond water, then filled with a water sample. Three replicates were taken per sampling point. Samples were taken from shallow areas around the pond's margins. Water was tested for pH (p Hep+ meter by HANNA calibrated to pH 4 and 7), Total Dissolved Solids (H1914d Dissolved Oxygen Meter by HANNA) Dissolved Oxygen and temperature (Hi 98311 Di STEC/TDS meter by HANNA) on site; the samples were then returned to the laboratory for analysis.

2.5.4Water sample laboratory analysis

On return to the laboratory, water samples were stored in the refrigerator until processing. A Chromeleon Dionex was used to test for nitrates, phosphates and chloride. In addition, potassium and sodium content were determined by Atomic Absorption Spectrometry (A.A.S.) & Flame Photometer.

3.0 Results

3.1 Invertebrate Survey

3.1.1 Clay 'Ole

After careful observation of all samples, and further study under the microscope, no samples of *P. jenningsi* were recovered for the study period. Despite this, a wide variety of invertebrates were recovered from the Clay 'Ole site. Samples were found to contain hoglouse, water mites, water beetles, waterboatmen, hydra, chrionomidae, mollusca, numerous leech species, bivalves, numerous flatworm species and other oligochaetes including naididae.

The graphs below indicate the abundance of 'worm-like' invertebrates (as outlined above) that were extracted from the samples and preserved for investigation, thus giving an indication of conditions found at the sampling sites. It is important to point out *Prostoma* is often found when sampling for flatworms (Gilbert, pers comm. 2011).

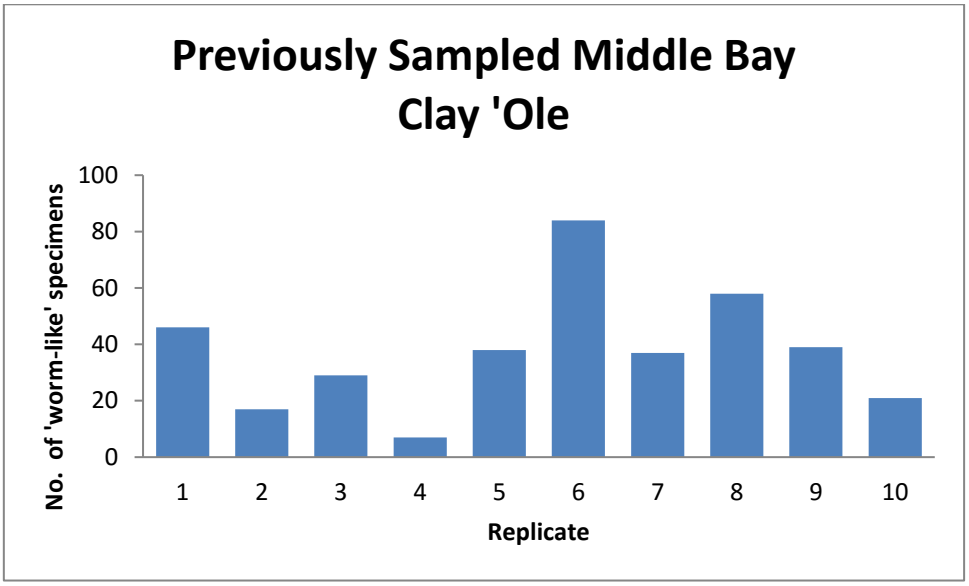


Figure 3 - Graph to show relative abundance of worm-like organisms collected from the Middle Bay of the Clay 'Ole during Autumn 2012.

Figure 8 shows the number of 'worm-like' organisms recovered using a sweep net technique over ten replicates within the Middle Bay (the area in which *P. jenningsi* has been previously found) of the Clay 'Ole site during phase three of the project, autumn 2012. It can be seen that the fewest specimens were recovered from replicate four, with the most abundant being replicate six.

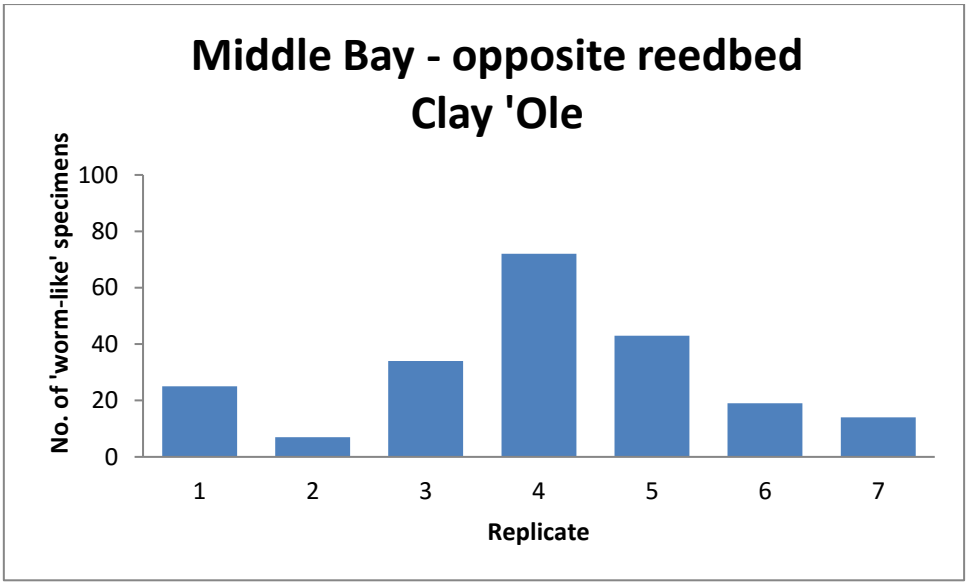


Figure 4 - Graph to show relative abundance of worm-like organisms collected from the Middle Bay – opposite reedbed of the Clay 'Ole during autumn 2012.

Figure 9 displays the relative abundance of 'worm-like' organisms collected using the sweep net technique over seven replicates on the opposite reedbed of the Middle Bay of the Clay' Ole site.

3.1.2 Twin Lakes

A wide variety of invertebrates were recovered from the Twin Lakes site. Samples were found to contain hoglouse, water mites, water beetles, water boatmen, backswimmers, water scorpion, hydra, chrionomidae, mollusca caddis fly larvae, damsel fly nymph, culicidae, biting and non-biting midge larvae, numerous leech species, bivalves, numerous flatworm species and other oligochaetes, including naididae. After careful observation of all samples, and further study under the microscope, 54 specimens of possible *P. jenningsi* were recovered from the sampling attempt.

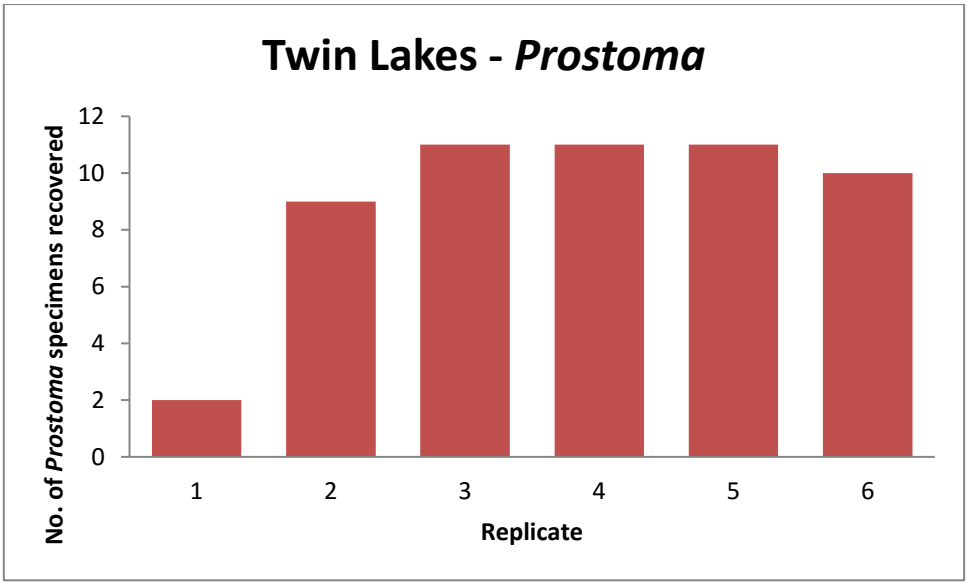


Figure 5 - Graph to show No. of *Prostoma* recovered from Twin Lakes during autumn 2012

*Figure 11 s*hows the relative abundance of *Prostoma* collected using the sweep net over six replicates at the Twin Lakes pond during autumn 2012. It can be seen specimens were recovered at all replicates.

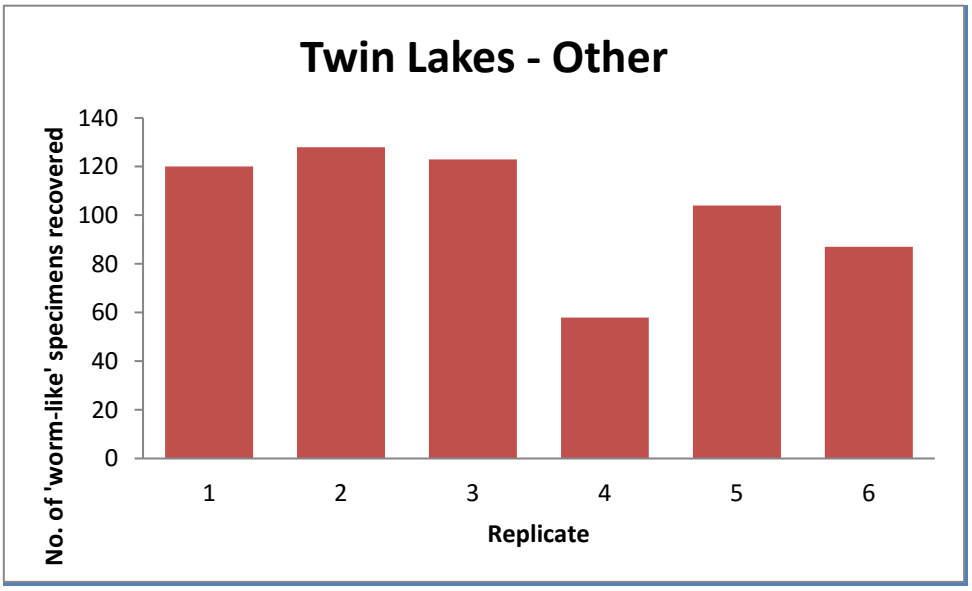


Figure 6 - Graph to show relative abundance of worm-like organisms collected from Twin Lakes during autumn 2012.

Figure 12 displays the relative abundance of 'worm-like' organisms collected using the sweep net technique over six replicates at the Twin Lakes site.

3.1.2 Hesketh Bank

The pond at Hesketh Bank showed very little species diversity. In comparison to other ponds studied throughout the project, a total of only three flatworms were recovered; however, crustaceans such as Gammarus (freshwater shrimp) were present in abundance, as were Palaemonidae – Palaemonetes varians (prawns). After careful observation of all samples, and further study under the microscope, three specimens of possible *P. jenningsi* were recovered from the sampling attempt. Following this discovery, a second site visit was made and a further four specimens were recovered.

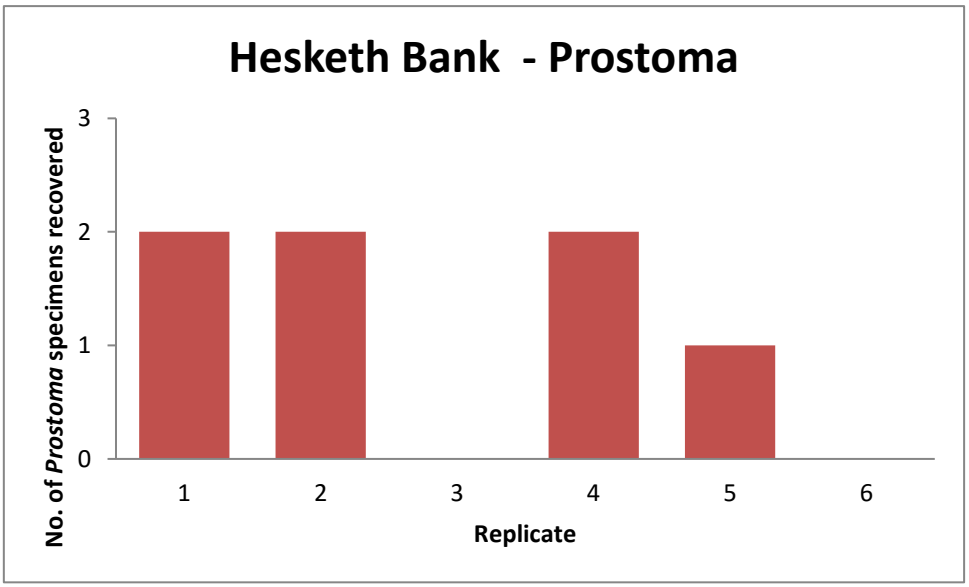


Figure 7 - Graph to show no. of *Prostoma* recovered from Hesketh Bank during autumn 2012

Figure 10 shows the relative abundance of *Prostoma* collected using the sweep net over six replicates at the Hesketh Bank pond during autumn 2012. It can be seen that, at replicates one and four, two specimens were recovered. One specimen was recovered at replicates two and five, with no specimens collected at replicates three and six.

3.2 Water Chemistry

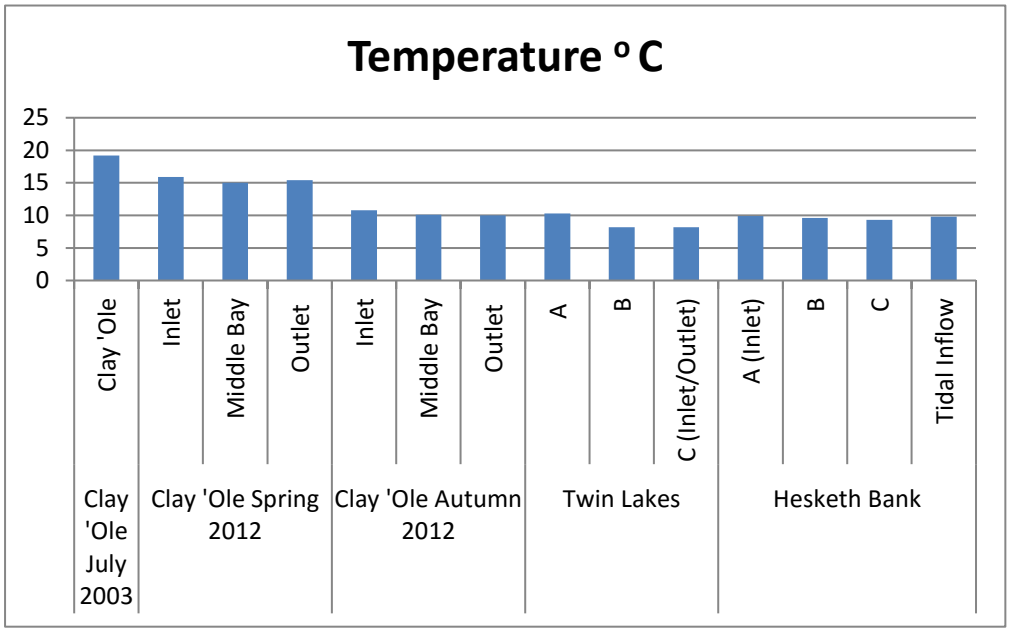


Figure 8 - graph to show water temperature recorded over the sample sites.

Figure 13 shows water temperature recorded over the sample sites. Temperatures range between 8.2ºC and 19.2ºC.

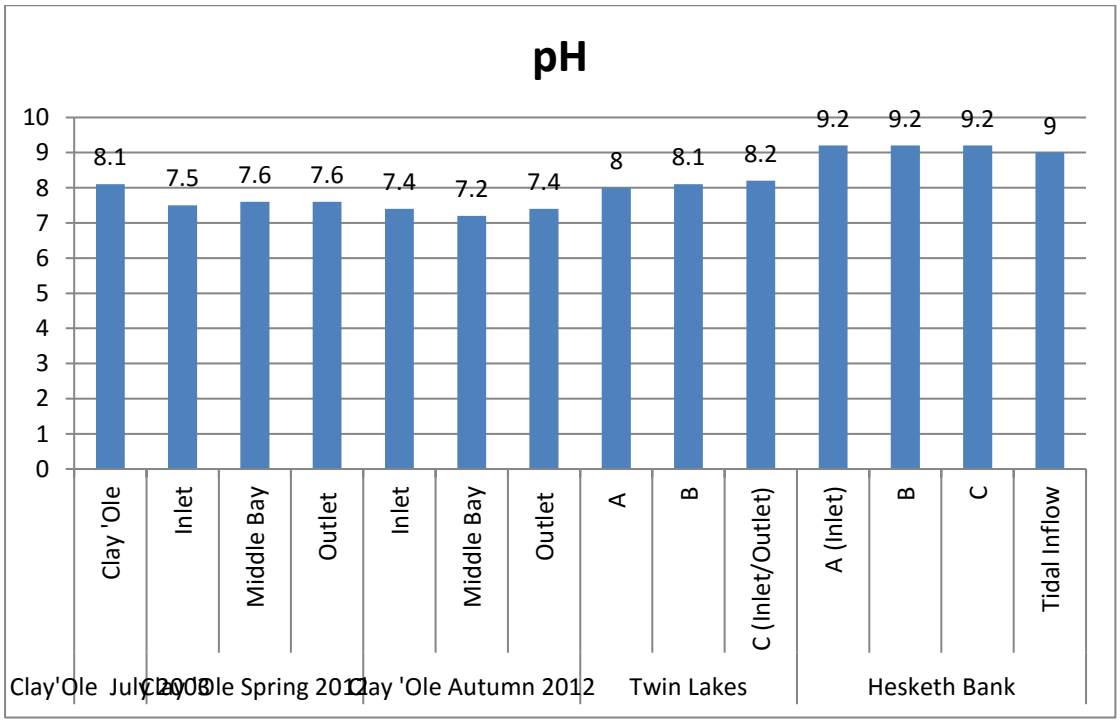


Figure 9 - Graph to show pH readings taken over sample sites.

Figure 14 shows pH levels recorded at the survey sites, in addition to data recorded at the Clay 'Ole in July 2003 and spring 2012. It can be seen that pH levels recorded at Hesketh Bank are higher than other sites.

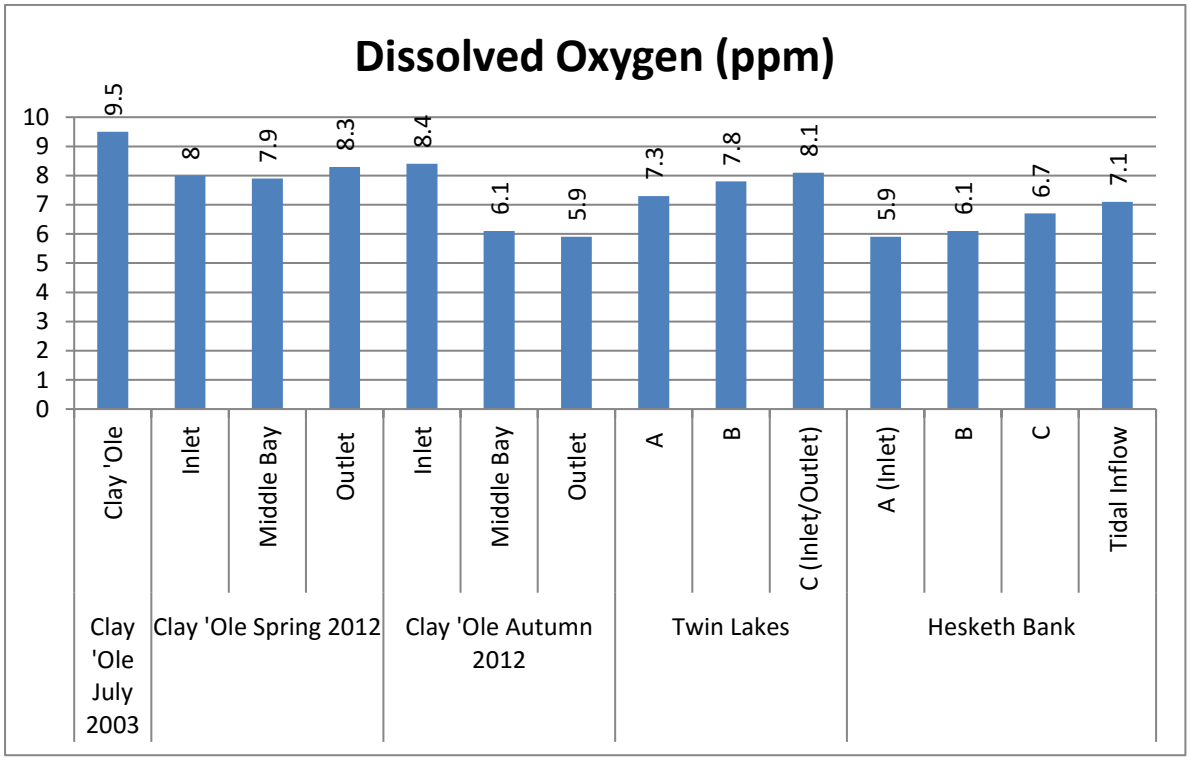


Figure 10 - Graph to show Dissolved Oxygen Content taken over sample sites.

Table 1 - Table to show mean values and standard deviation for Dissolved Oxygen Content across sample sites.

Dissolved Oxygen (ppm)	Mean Value	Standard Deviation
Clay Ole Spring 2012	8.07	0.2
Clay 'Ole Autumn 2012	6.8	1.4
Twin Lakes	6.23	0.42
Hesketh Bank	6.5	0.4

Figure 15 shows levels of dissolved oxygen (ppm) taken at survey sites during phase three project and July 2003. It can be seen that levels were slightly higher during July 2003. Table 1 displays mean values and standard deviation.

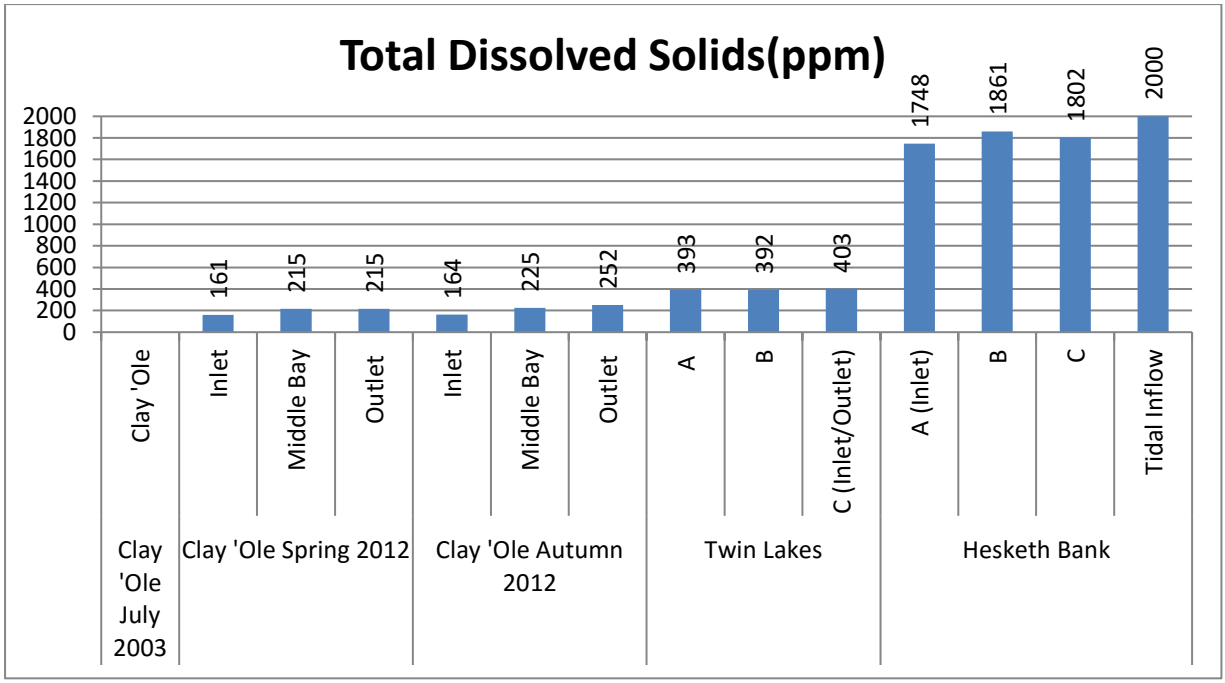


Figure 11 - Graph to show levels of Total Dissolved Solids (ppm) recorded at sample sites.

Table 2 -Table to show mean values and standard deviation for TDS across sample sites.

TDS (ppm)	Mean Value	Standard Deviation
Clay 'Ole Spring 2012	197.0	31.2
Clay 'Ole Autumn 2012	213.67	45.08
Twin Lakes	396	6.1
Hesketh Bank	1803.67	56.5

Figure 16 shows the levels of total dissolved solids recorded at the sample sites. It can be seen that levels recorded at Hesketh Bank are significantly higher than those recorded elsewhere. No

information was available for the Clay ‘Ole July 2003. Table 2 displays mean values and standard deviation. Readings from the tidal inflow at Hesketh Bank were not included.

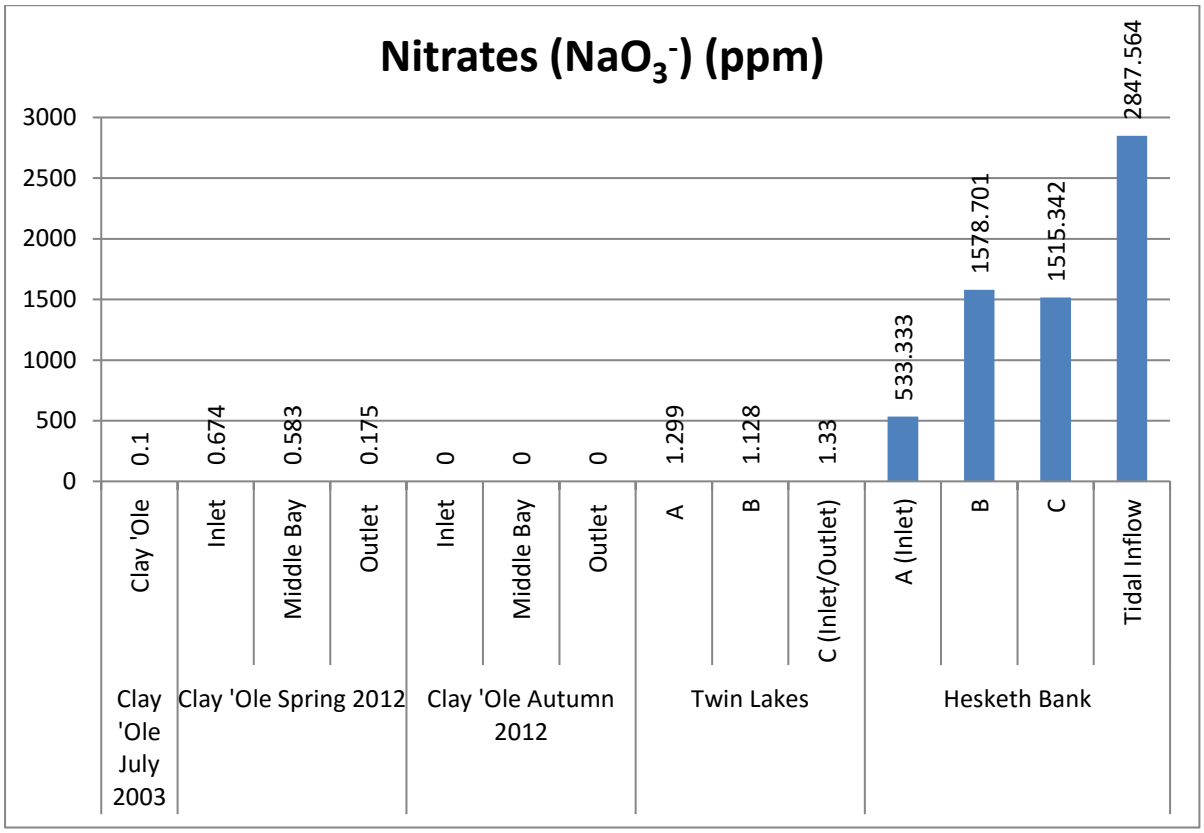


Figure 12 - Graph to show nitrate levels (ppm) recorded at sample sites.

Table 3 - Table to show mean values and standard deviation of nitrate levels at sample sites.

Nitrate nitrogen NaO_3^- (ppm)	Mean Value	Standard Deviation
Clay ‘Ole Spring 2012	0.48	0.27
Clay 'Ole Autumn 2012	0	0
Twin Lakes	1.23	0.1
Hesketh Bank	1209.12	586.11

Figure 17 shows trace nitrate levels recorded at the Clay ‘Ole site during July 2003 and Spring 2012. No trace was detected during autumn 2012. Trace levels were recorded at Twin Lakes. It can be seen that much greater levels were recorded at Hesketh Bank, with the highest recording having been taken from the tidal inflow. Table 3 displays mean values and standard deviation. Readings from the tidal inflow at Hesketh Bank were not included.

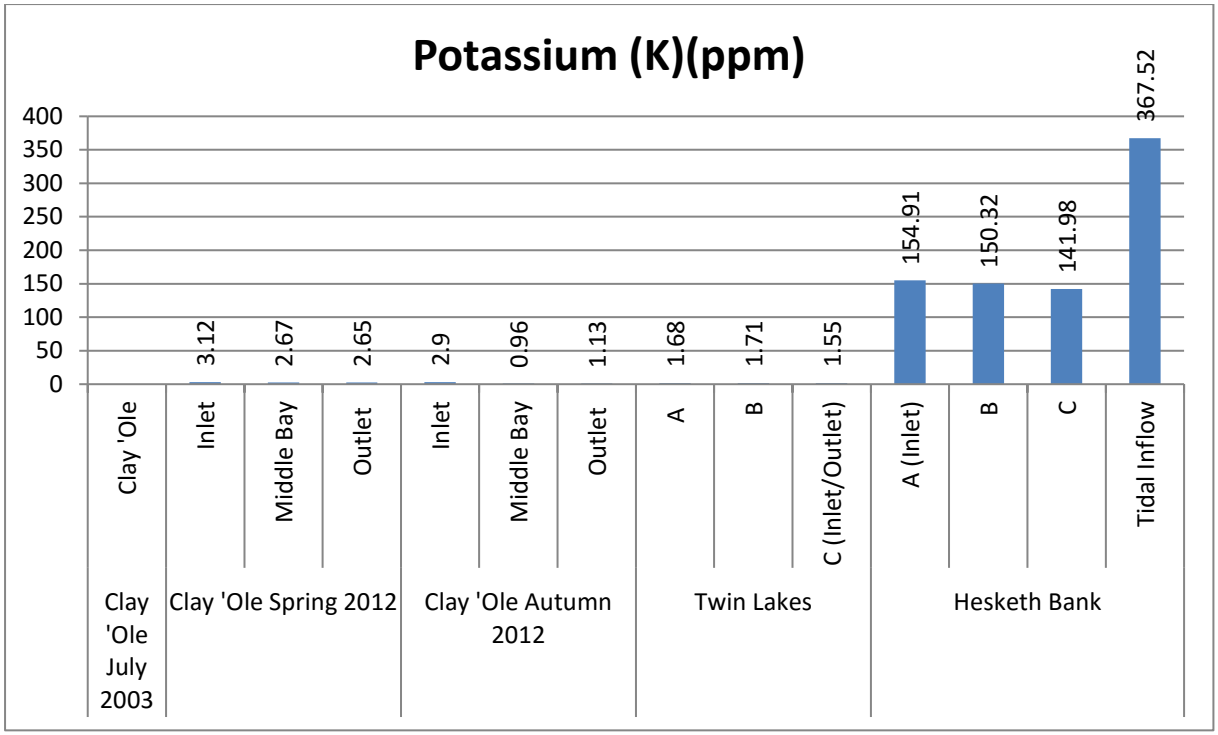


Figure 13 -Graph to show potassium levels (ppm) recorded over sample sites.

Table 4 - Table to show mean values and standard deviation of Potasium levels at sample sites.

Potassium (K) (ppm)	Mean Value	Standard Deviation
Clay 'Ole Spring 2012	2.81	0.27
Clay 'Ole Autumn 2012	1.66	1.1
Twin Lakes	1.65	0.09
Hesketh Bank	149.07	6.56

Figure 18 shows levels of potassium recorded across all sites. It can be seen that trace levels were recorded at the Clay 'Ole and Twin Lakes; however, much higher levels were recorded at Hesketh Bank. Table 4 displays mean values and standard deviation. Readings from the tidal inflow at Hesketh Bank were not included.

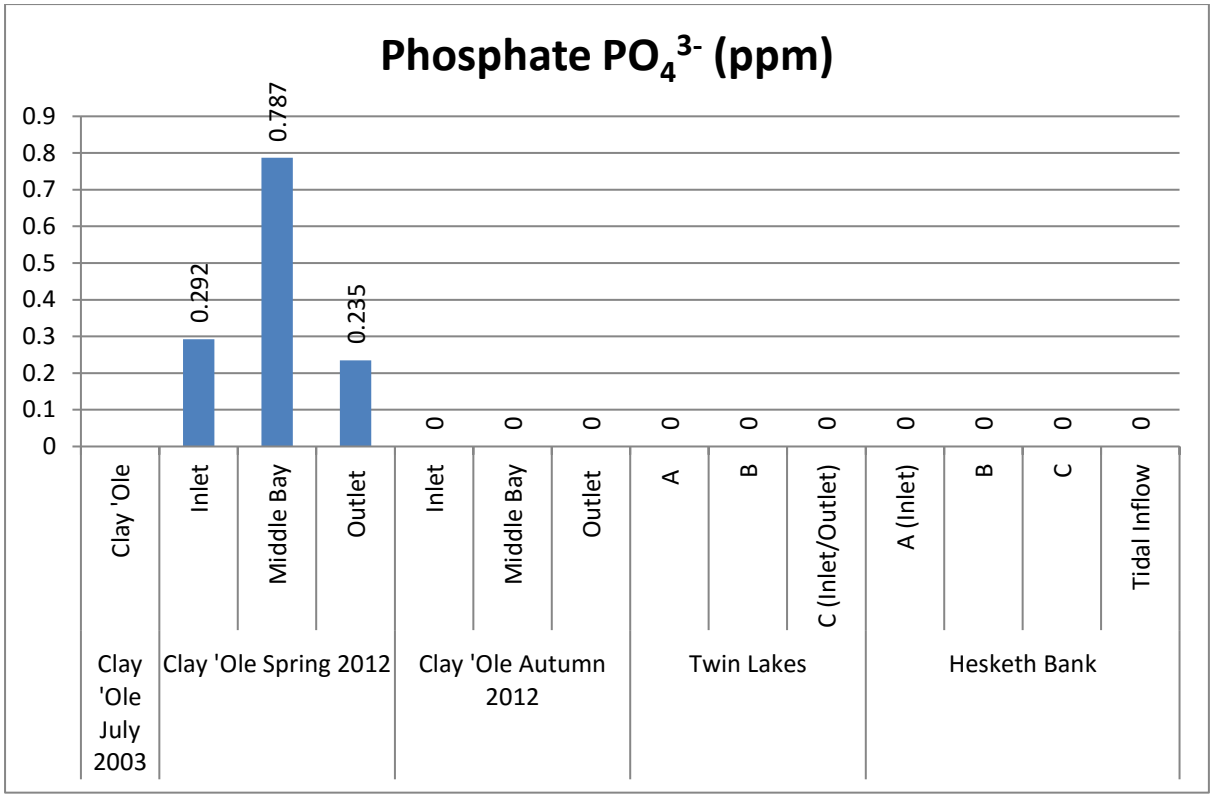


Figure 14 - Graph to show phosphate levels (ppm) recorded over sample sites.

Table 5 - Table to show mean values and standard deviation of phosphate levels at sample sites.

Phosphate (PO_4^{3-}) (ppm)	Mean Value	Standard Deviation
Clay 'Ole Spring 2012	0.44	0.30
Clay 'Ole Autumn 2012	0	0
Twin Lakes	0	0
Hesketh Bank	0	0

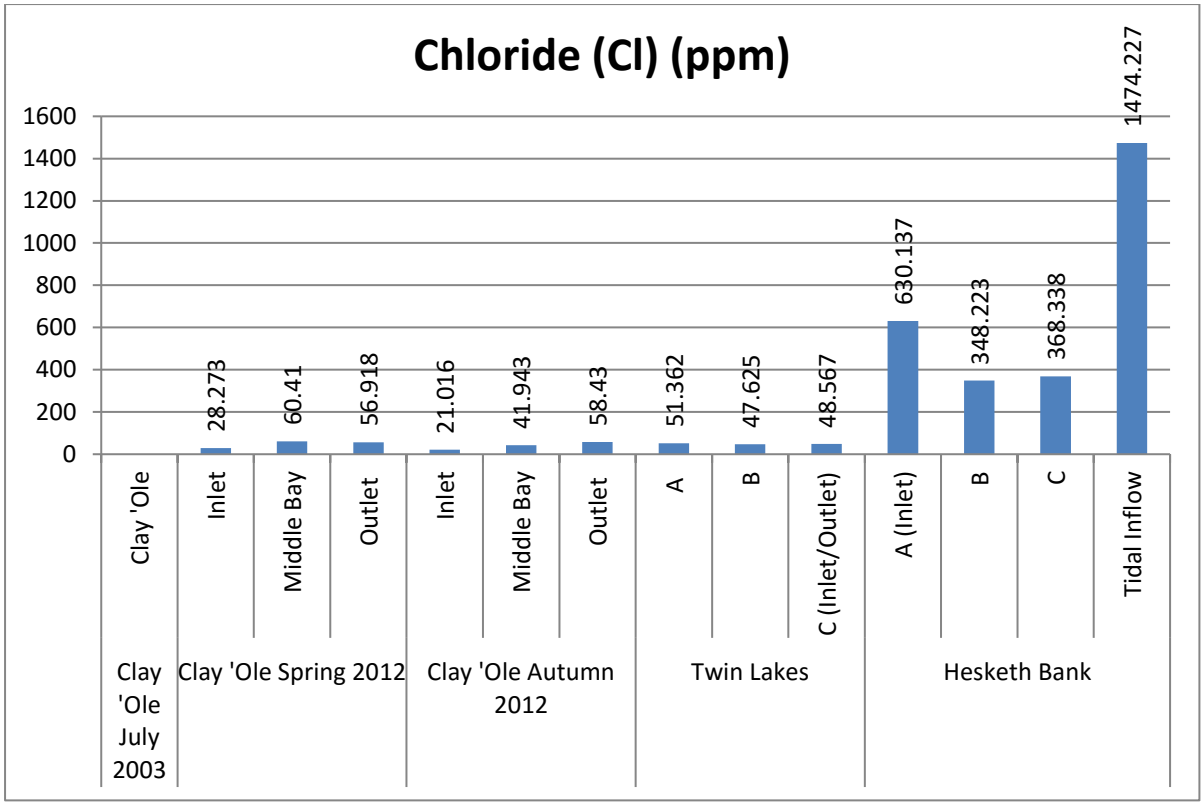


Figure 15 - Graph to show chloride levels (ppm)recorded over sample sites.

Table 6 - Table to show mean values and standard deviation of Chloride at sample sites.

Chloride (Cl) (ppm)	Mean Value	Standard Deviation
Clay 'Ole Spring 2012	48.53	17.63
Clay 'Ole Autumn 2012	40.43	18.7
Twin Lakes	49.12	1.94
Hesketh Bank	448.9	157.28

Figure 20 shows chloride levels recorded across all sites. It can be seen that levels are much higher at Hesketh Bank, with those at the tidal inflow being the greatest. Table 6 displays mean values and standard deviation. Readings from the tidal inflow at Hesketh Bank were not included. No data was available for the Clay 'Ole July 2003.

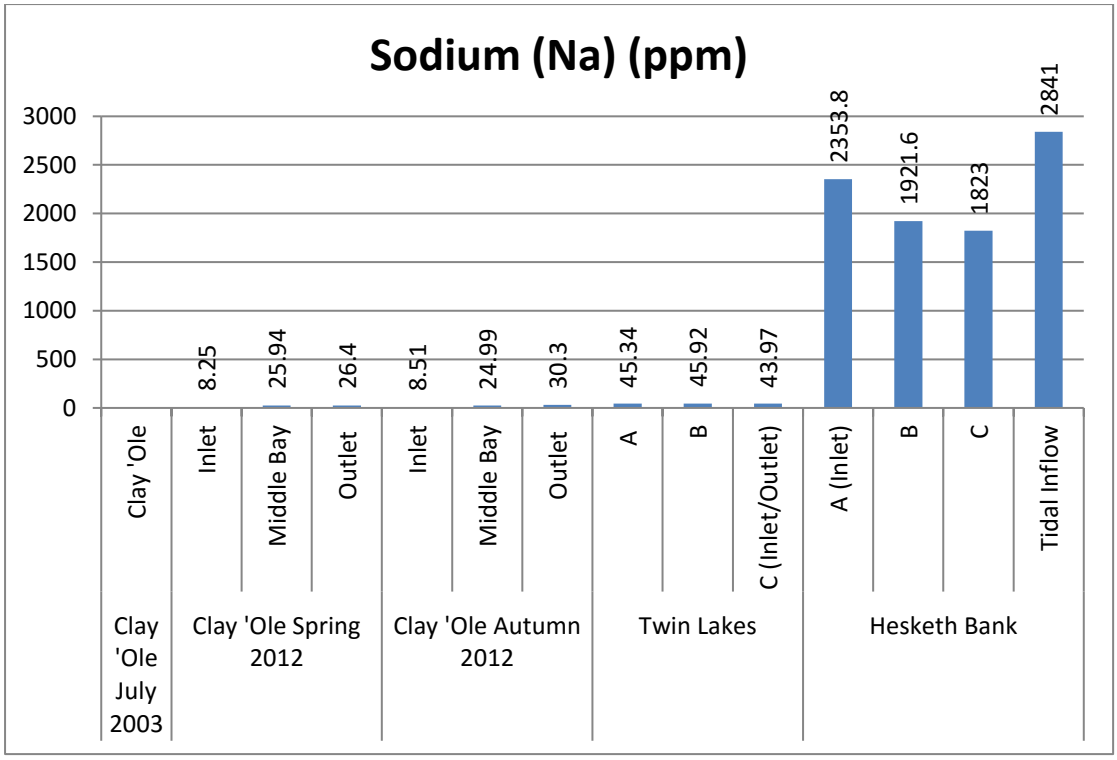


Figure 16 - Graph to show sodium levels (ppm) recorded over sample sites.

Table 7 - Table to show mean values and standard deviation of Sodium at sample sites.

Sodium (Na) (ppm)	Mean Value	Standard Deviation
Clay 'Ole Spring 2012	20.19	10.35
Clay 'Ole Autumn 2012	21.27	11.4
Twin Lakes	45.08	1
Hesketh Bank	2032.8	282.33
Twin Lakes	45.08	1

Figure 21 shows levels of sodium recorded across all sites. It can be seen that levels are greater at Hesketh Bank, particularly at the tidal inflow. Table 7 displays mean values and standard deviation. Readings from the tidal inflow at Hesketh Bank were not included. No data was available for the Clay 'Ole July 2003.

4.0 Discussion

4.1 Water Chemistry

4.1.1 pH

The 'normal' biological range for most freshwater habitats is approximately pH 4.4 to 8.6, with variations of as much as two full pH points not being uncommon over the course of a year. Variations

in pH are dependent upon photosynthesis, light, current, respiratory processes, biota circulation etc. to which common species adjust with no difficulty (Smith, 2001). It can be seen in *figure 14* that pH readings for both the Clay 'Ole and Twin lakes fall within the 'normal' range for freshwater. Hesketh Bank, however, displays a reading of 9.2, falling outside of 'normal' freshwater parameters. This is unsurprising given the inflow of brackish water into the pond during high tide events. Seawater is more alkaline than freshwater, due to the occurrence of natural buffering from carbonate and bicarbonate dissolved in water. Estuarine pH levels, however, fall typically from between 7.0 and 7.5 within the fresher sections, to between 8.0 and 8.6 in the more saline reaches; the Hesketh Bank pond thus falls outside of these 'expected' levels. This is a possible explanation for the limited diversity of aquatic macro invertebrates evident at the pond, as many species have difficulty surviving if pH levels rise above 9.0. Possible causes for such pH readings could be attributed to factors such as bacterial activity; chemical constituents in runoff flowing into the pond; sewage overflows and impacts from other human activities (Ohrel & Register, 2006). It is possible that waste that has previously been disposed of into the pond still has an impact on the water quality.

4.1.2 Total Dissolved Solids

Total dissolved solids account for the amount of dissolved chemical species in water and are a good indicator of the concentration of ionic substances. Commonly, freshwater has less than 1,500 mg/L of TDS, brackish water between 1500 and 5000 mg/L TDS and marine water has a TDS content of 30,000-40,000mg/L (Kegley & Andrews, 1998). Freshwater fauna are clearly distinguished by occurrence in waters that, while dilute, have a wide range of dissolved salts. Commonly, the lower limit for TDS in which freshwater fauna exists is in the region of 10 mg/L, with the upper limit being as high as 1000 mg/L (see *figure 22* below); although the species that exist within certain parameters vary (Smith, 2001).

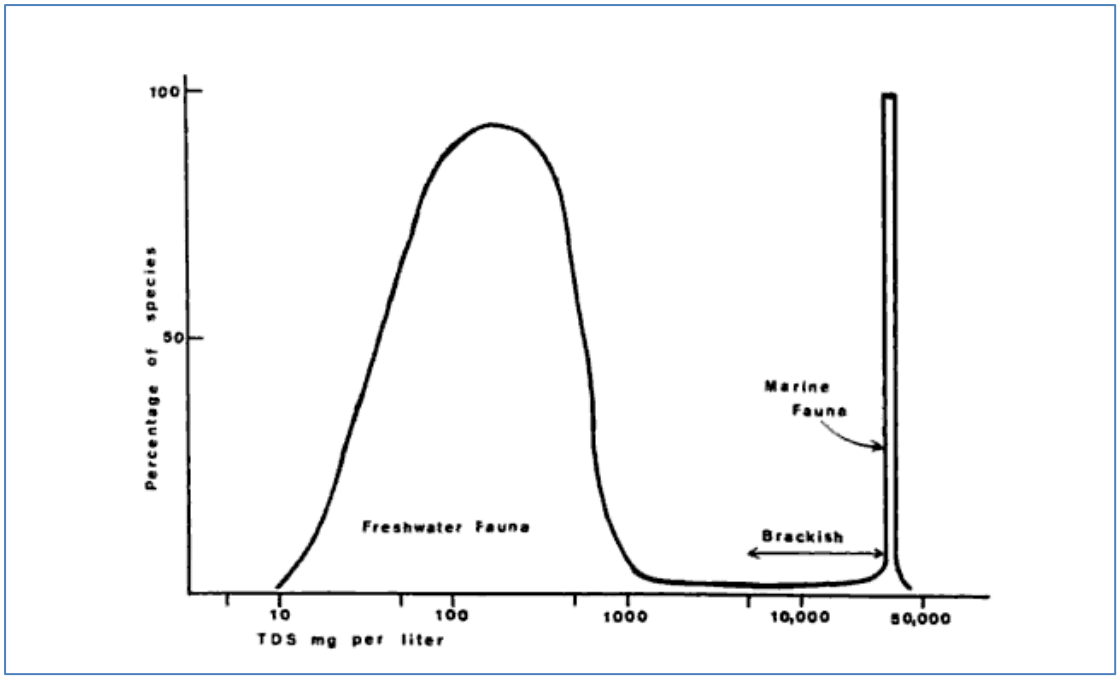


Figure 17 - Salt content and environmental preferences of the aquatic faunas. NB Figure does not show limits of tolerance of faunas.

Values recorded for total dissolved solids (TDS) at both the Clay 'Ole and Twin lakes fall comfortably within the range expected for freshwater fauna. It can be seen in *figure 16* that levels of TDS are slightly elevated within the Middle Bay and Croston Bay of the Clay 'Ole site, falling in line with data recorded during phase 3 of the project (see Quigg and Lowe 2012). TDS levels are slightly higher within the Twin Lakes site than at the Clay 'Ole. TDS levels recorded at Hesketh Bank reflect those expected to be found within brackish water; this ties in with the limited fauna recovered at this site. These results are reflected in results recorded for sodium and chloride - see *figures 20 and 21* and further discussion below.

4.1.3. Nitrate

Nitrates are present in natural freshwater ecosystems as normal biological degradation products of proteins and nucleic acids. High concentrations of NO_3^- can be found in surface waters, as a consequence of freshwater pollution. Nitrogen compounds can enter aquatic ecosystems through various means; point sources such as aquaculture operations, livestock and agricultural operations, industrial waste and sewage effluence; with non-point sources also related to agriculture, through means such as fertilization, manure and urbanisation, through runoff from septic systems etc. (Camargo & Ward, 1995; Soucek & Dickinson, 2012). It can be seen in *figure 17* that low levels of trace nitrates have been found at the Clay 'Ole during July 2003 and spring 2012, with no trace levels being detected in autumn 2012. Slightly higher trace levels can be seen within Twin Lakes during autumn

2012 compared with those previously recorded at the Clay 'Ole; however, they still fall within normal limits for freshwater (safe limit guidelines 50mg/L). Levels of nitrates detected at Hesketh Bank are extremely high. Higher levels of nitrate would be expected at Hesketh Bank when drawing comparisons with the freshwater ponds, due to the brackish nature of the pond and the ionic composition of marine water. Long term exposure guidelines for marine aquatic ecosystems suggest that concentrations should not exceed 200mg $NO_3^- N \cdot L^{-1}$ in terms of protection of all aquatic life; however, caution must be taken when applying marine nitrate guideline values in transitional environments, such as estuaries and brackish waters (Canadian Council of Ministers of the Environment, 2012). Such high levels of nitrates could be attributed to the industrial waste that is thought to have been previously dumped into the pond and which may have included fertilisers. The highest levels of nitrate concentrations can be seen at the tidal inflow. Again, levels would be expected to be higher at this point due to the concentration of salinity in the water; however, other factors or point sources of pollution must also be at play. Such high nitrate levels could account for the limited numbers and species of aquatic life recorded at the site. The pond is, however, still used for fishing.

4.1.4 Potassium

Freshwaters usually contain less than 10 mg/L of potassium, and this is often only in traces. Large quantities of potassium salts in freshwaters are known to be toxic to many freshwater invertebrates (Smith, 2001). It can be seen in *figure 18* that no trace levels of potassium were recorded for the Clay 'Ole or Twin Lakes. Potassium levels at Hesketh Bank are higher, falling in line with the nature of the brackish water. The concentration levels of potassium in seawater are thought to be approximately 380 mg/L (Kegley & Andrews, 1998). Levels at points A, B and C fall well below this, as would be expected with the tidal inflow, displaying levels near to those expected to be found in seawater.

4.1.5 Phosphates

Phosphorous occurs in natural waters almost exclusively as phosphates, playing a significant role in the eutrophication of surface waters. The main impact of raised phosphorus concentrations in rivers or lakes is to promote plant growth, which may then have detrimental effects on ecosystem quality and functioning. Phosphates can be introduced to freshwaters from sources such as fertilizers and other agricultural operations, wastewaters, farm waste products and the degradation of organic matter (Pichette *et al*, 2009). It can be seen in *figure 19* that no levels of phosphates were recorded during the most recent sampling event. Although possible, this is probably not reflective of the true levels within the water bodies; it is more likely to be due to a fault during the analysis process.

4.1.6 Sodium and Chloride

Sodium ion (Na+) is the metal ion with the lowest toxicity for aquatic organisms; however, chronic concentrations of chloride as low as 250 ppm have been recognised as harmful to freshwater life (Kaushal *et al,* 2005). It can be seen in *figures 20 and 21* that sodium and chloride levels remain low across the Clay 'Ole and Twin Lakes, with both sites experiencing similar levels. It can be seen that levels at the inlet of the Clay 'Ole are lower, suggesting that water entering the pond at this point is less saline than that of the water body itself. A salinity level of 5-8% is assumed to be the upper limit for freshwater invertebrates, with information concerning the lower limits being scarce. Oligochaetes and crustaceans are thought to be most tolerant to changes in water salinity, both through substantial increase and decrease (Berezina, 2003). It could be suggested that the salinity of the Clay 'Ole and Twin Lakes is slightly raised due to the possible influx of brackish water from the nearby river Lostock. It has been established that the river Lostock at this point remains within the limits of the high mean water, and that the area is prone to flooding (see phase one report, Quigg and Lowe, 2011a). During the previous study period (spring 2012), the nearby river Lostock is known to have flooded on two occasions, with one storm event being the most severe in the area for the last 25 years. Due to the lack of research surrounding *P.jenningsi*, it is unclear as to how sensitive the species would be to such changes to its environment. Although previous reports have discussed the possibility that over the years the surrounding area in which the Clay 'Ole is located may have been prone to flooding, no further data has been gathered surrounding the frequency and severity of such events.

4.2 Prostoma jenningsi?

4.2.1 Clay 'Ole

The current phase of the study has been unable to prove the existence of *P. jenningsi* at the Clay 'Ole site, despite investigating other areas of the pond. As with Gibson's last sampling attempt (date unknown), no specimens have been recovered (Gibson, 2011 pers com). Although the species has not been recovered, conclusions cannot be drawn regarding the species' continued existence at this site. It can be seen in *figures 8* and *9* that the pond continues to support an abundance of 'worm-like' organisms. For possible explanations as to why *P.jenningsi* may not have been recovered at the Clay 'Ole, please see Quigg and Lowe, 2012.

4.2.2 Twin Lakes

A number of possible specimens of *P.jenningsi* have been recovered from the Twin lakes site. The species, however, can not be confirmed until histological and DNA analysis have been completed, as its identification relies on aspects of its internal morphology - see Quigg and Lowe, 2012 for a full species description. The recovered specimens (see *figure 23*) have been confirmed as belonging to the

genus *Prostoma* through visual identification by Professor Gonzalo Gribert, Professor Jon Norenburg and Professor Ray Gibson, experts in the field.

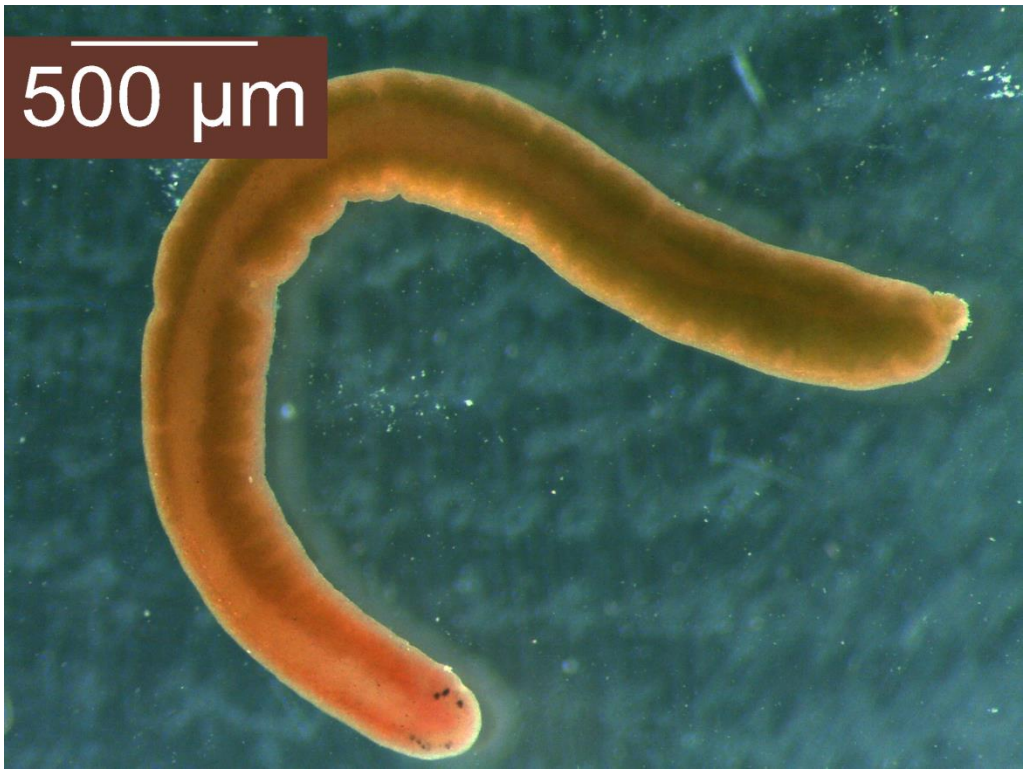


Figure 18 - *P. jenningsi?*

It is possible that *Prostoma* were present at Twin Lakes when *P.jenninsi* was first discovered at the Clay 'Ole during 1969, although it is not known whether the Twin Lakes site was included in the wider study of freshwater triclads that originally uncovered *P. jenningsi* (see Quigg and Lowe, 2012). The management of the Twin Lakes site appears to be far less intense than that of the Clay 'Ole with water chemistry slightly more saline; it is thus conceivable that Twin Lake is more representative of the conditions in which *P. jenningsi* was first recovered. All specimens were recovered from within the stands of *Phragmites* and found in, on and around the littoral layer, as suggested by Gibson & Young (1971; 1976).

4.2.3 Hesketh Bank

Possible specimens of *P. jenningsi* were also recovered at Hesketh Bank, but in much fewer numbers. Again the species cannot be confirmed without the results of histological and DNA analysis. As at Twin Lakes, specimens were recovered from within the stands of *Phragmites,* in, on and around the littoral layer. Again it could be suggested that conditions here may be more in line with those found at the Clay 'Ole when *P. jenningsi* was first recovered; however, the water at Hesketh Bank is of a brackish nature. As *Prostoma* species are considered to exist only within freshwater, this result is surprising. *P. eilhardi* from the USA is thought to have been found in coastal freshwater pools which were

occasionally subject to seawater invasion such as tidal, storm wave surges (Gibson, pers comm, 2012). Such a finding could be of significance to the study of freshwater nemerteans and could support Moore and Gibson (1973), who suggest that limnetic hoplonemerteans, such as *Prostoma,* may have evolved from their marine ancestors. It is possible that the *Prostoma* specimens found at the two different locations may be different species.

The Clay 'Ole, Twin Lakes and Hesketh Bank are all linked through their proximity to the rivers by which they sit, the river Lostock being a tributary to the River Douglas in Hesketh Bank. Given this fact, it is likely that further populations of *Prostoma* may exist in similar ponds, or possibly in the rivers themselves.

5.0 Conclusions and recommendations

The study has been unable to prove the existence of *P.jenningsi* at the Clay 'Ole site; however, two further populations of possible *P.jenningsi* have been found to exist at nearby locations. Although the species can not be confirmed as *P. jenningsi* until the completion of histological and DNA analysis, confirmation of the genus *Prostoma* has been made; this in itself is significant, as so few freshwater populations have been recorded within the UK.

Substantial differences in the water chemistry between Twin Lakes and Hesketh Bank can be seen. Little research has focused on ecological observations of freshwater nemerteans, as discussed in Quigg &Lowe, 2012. It is thus difficult to ascertain whether this is of great significance, particularly if the two populations are indeed the same species. Further study would allow a better understanding to be gained.

References

Berezina, 2003 Berizina, N.A. (2003). Tolerance of freshwater invertebrates to changes in water salinity. *Russiun Journal of Ecology.* **34,** 4, pp 261 -266.

Camargo, J.A. & Ward, J.V. (1995). Nitrate (NO_3-N) Toxicity to Aquatic Life: A Proposal Of Safe Concentrations For Two Species Of Neartic Freshwater Invertebrates. *Chemosphere* **13,** 5 pp 3211-3216.

Canadian Council of Ministers of the Environment (2012*). Canadian water quality guidelines for the protection of aquatic life: Nitrate.* In/; Canadian environmental quality guidelines, Canadian Council of Ministers of the Environment, Winnipeg.

Gibson, R. (1982). *British Nemerteans*. Cambridge University Press, Cambridge.

Gibson, R. (2011). Pers comm. –Telephone conversation. 22/08/2011.

Gibson, R. (2011). Pers comm. – email conversation. 14/11/2012.

Gibson, R. & Young, J.O. (1971). *Prostoma jenningsi* sp. Nov., a new British freshwater hoplonemertean. *Freshwater Biuology.***1** pp 121 -127.

Gibson, R. & Young, J.O. (1976) Ecological observations on a population of the freshwater hoplonemertean *Prostoma jeninngsi. Archives of Hydrobiology.* **78.** 1. Pp 42-50.

Kaushal, S.S., Groffman, P.M., Likens, G.E, Belt, K.T, Stack, W.P., Kelly, V.R., Band, L.E & Fisher, G.T. (2005) Increased salinization of fresh water in the norteastern United States. *PNAS*. **102**, 38 pp 13517-13520

Kegley , S.E.& Andrews, J. (1998). *The Chemistry of Water*. University Science Books, California.

Laumer, C. (2012). Pers comm. Email received 20/6/2012.

Moore, J. & Gibson, R. (1973). A new genus of freshwater haplonemertean fron New Zealand. *Zoological Journal of the Linnean Society.* **52,** 133-144.

Oherel, R.L & Register, K.M. (2006). *Volunteer Estuary Monitoring: A Methods Manual* (2nd Edition) EPA, Washington.

Pichette, C., Zhang, H. & Sauve, S. (2008). Using diffuse gradients in thin-films for in *situ* monitioring of dissolved phosphate enmissions from freshwater aquaculture. *Aquaculture.***286**. 3-4 pp198-202.

Quigg , S., and Lowe, C.N. (2011a) *Confirming the existence of the Croston worm (Prostoma jenningsi).* September 2011. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2011b*) Confirming the existence of the Croston worm (Prostoma jenningsi) Stage 2. Winter 2011.* Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2012) *Confirming the existence of the Croston worm (Prostoma jenningsi) Stage 3. Spring 2012*. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Smith, D.G. (2001). *Pennak's freshwater Invertebrates Of The United States Porifea to Crustacea.* (4th Edition) John Wiley & Sons, Inc. United States .

Soucek, D.J., & Dickinson, A. (2012). Acute Toxicity of Nitrate and Nitrite to Sensitive Freshwater Insects, Mollusks, and a Crustacean. *Archive of Environmental Contamination Toxicology.* **62.** pp 233–242.

Sundberg, P. & Gibson, R. (2008) Global diversity of Nemerteans (Nemertea). in freshwater. *Hydrobiologia* **595,** pp 61-66.

Confirming the existence of the

‘Croston Worm’ (*Prostoma jenningsi*)

Stage 3: Spring/Summer 2012

The Clay ‘Ole, Bretherton

A report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside

The research detailed in this report was undertaken by Siobhan Quigg and Dr Chris Lowe from the School of Built and Natural Environment, University of Central Lancashire. The project was funded by Natural England.

Summary

The existence of the fresh water nemertean *Prostoma jenningsi,* more commonly known as the 'Croston Worm', has not been confirmed since 1999. The key actions of the project sought to confirm the existence of the species through systematically surveying the single pond location in which it is known to have existed. As a follow up to the initial study that took place during summer 2011, and stage 2 of the project carried out during winter 2011, stage 3 of the project sought to conduct further surveys of the pond using previous knowledge and recommendations made in stages 1 and 2. An in depth review of nemertean ecology was carried out. The use of the Gerking Box sampler was reviewed. Water and sediment chemistry were analysed and the search was widened to include a similar pond in the area, as recommended by stage 2 of the study and in line with findings from previous research. The study was unable to confirm the continued existence of the species at the Clay 'Ole site; however a greater insight into the ecology of freshwater nemerteans was gained, as was knowledge regarding seasonal variations that occur at the site. Recommendations were made regarding future sampling attempts.

Recommendations

This report makes several recommendations related to future studies:

- Further sampling events are required to account for seasonal changes, ecological life-cycles and any possible monthly sampling anomalies.
- The survey should be further widened to include additional similar pond locations in the surrounding area.
- Historical information regarding flood events and ecological changes to the Clay 'Ole should be sought.

Contents

1.0 Introduction

The freshwater nemertean *Prostoma jenningsi* (the Croston Worm) is a UK BAP Species thought to be endemic to Lancashire. It is listed in the British Red Data book as Insufficiently Known. The species has been found solely at The Clay 'Ole, Bretherton (SD485198); however, its existence has not been confirmed at this location since 1999. The key actions of the project set out to systematically survey the pond and establish the presence, and if possible estimate population density, of the species. Should the species be found, it would then be a priority to secure further populations where suitable conditions exist.

1.1 *Prostoma jenningsi*

Prostoma jenningsi

Class: Enlopa

Order: Hoplonemertea

Family: Terastemmatidae

Genus:*Prostoma*

Species:*Prostomajenningsi*

P. jenningsi was first recovered from samples collected at the study site during July 1969 as part of a study by Johnstone O. Young of Liverpool University, whilst he was thought to be researching freshwater Triclads (Gibson, pers comm., 2011). Young recognised *P. jenningsi* to be of the phylum nemertean; its identification was later officially confirmed with the help of Gibson in 1971 and preserved specimens are held at the Natural History Museum. *P. jenningsi* is thought to be predominately associated with marginal vegetation, particularly beds of *Phragmites* within the middle bay of the Clay 'Ole site, where it feeds on oligochatesetae, particularly Naididae (for more in- depth background of the species, study site and current project, please see phase 1 report – Quigg & Lowe, 2011a).

1.2 Phase three study

Phase three of the project sought to continue sampling for P. *jenningsi* and to fulfil the recommendations made in the phase two report; to re-survey the pond to account for seasonal changes, ecological life-cycles and possible monthly sampling anomalies; to attempt a further sampling event using the modified Gerking box sampler during more favourable conditions, with a view to gaining a more representative comparison between the employed sampling methods. This may be beneficial both

to the study and the research of aquatic phytophilous macro-invertebrates in hard emergent littoral macrophyte beds (for more details please see phase one and two reports - Quigg & Lowe, 2011a; 2011b). The study also sought to investigate other similar ponds within the vicinity. In addition, it was considered that a further review of the literature surrounding the ecology of *P. jenningsi* and similar species would be beneficial.

2.0 Nemertean Ecology

2.1 Nemertea

The phylum Nemertea is made up of approximately 1,150 nominal species, distributed between 250 genera (Gibson, 1995). Nemertean worms can be defined as unsegmented, bilaterally symmetrical, acoelomate animals with a gut, possessing separate mouth and anus, a blood vascular system and a characteristic eversible proboscis situated dorsal to the gut in an enclosed tubular cavity, the rhynchocoel (Gibson, 1972). The proboscis is a shared characteristic of the taxon and is used primarily in prey capture. Nemertean species range in length from a few millimetres to about 30 metres, with a width that rarely exceeds a few millimetres (Turbevile, 2002).

Nemerteans are thought to occupy a broad range of habitats, the majority being found in marine or estuarine environments ranging from the tropics to the polar seas, from the benthos to the pelagial, and the intertidal zone to the deep sea (Turbevile, 2002; Yanfang & Shichun, 2006). With the exception of the true pelagic species, that float inertly or swim slowly, nemerteans are benthic in habitat, living beneath embedded boulders and rocks, within algae, burrowing into sands, mud and gravels, with a few forms living in tubes or inhabiting empty burrows of polychaetes or amphipods (Gibson, 1972). There are a number of known terrestrial forms and a small number recorded in freshwater environments (Turbevile, 2002).

Nemertean-based research has been carried out for over a century, with records indicating that species are dispersed over a wide range of habitat types; however, many aspects of their biology and ecology remain essentially, if not completely, unknown (Gibson, 1982; 1998). Ecological studies relating to nemerteans have largely focussed on their feeding behaviour, or prey interactions, supplemented by laboratory experimentation (for a summary of the research on nemertean diet, feeding behaviour and feeding ecology see McDermott & Roe, 1985). In addition, research has primarily focused on marine and estuarine species. Little is known about the tolerance of nemerteans to environmental factors; any insights into this area have often accumulated as a result of investigations with different objectives (e.g. Gibson, 1972, Yanfang & Shichun, 2006). Research has been carried out on the effects of salinity,

temperature and pH (e.g. Yanfang & Shichun, 2006). In addition, community studies (marine/estuarine) have been undertaken that considered the roles of nemerteans (McDermott & Roe, 1985, Wilson 1991). It is not known how transferable results from these studies are to freshwater species. Laboratory experiments have suggested that suctoral hoplonemerteans can exercise a potentially significant effect on benthic communities and that nemerteans may not be important prey for epi-benthic predators and thus their populations may be regulated by other factors (McDermott, 1993). A study by Cook and Herrmann (1997) regarding feeding behaviour and habitat of a population of *Prostoma graecense,* however, only recorded *P. graecense* in locations where they appeared to be at the top of the food chain. Populations were not recorded in locations where predators such as fish and dragon fly larvae were present.

2.2 Freshwater Nemerteans

Very few freshwater nemerteans have been recorded, with only 22 freshwater species having been identified, representing less than 2% of the total number recorded (Turbevile, 2002). Most freshwater species belong to monotypic genera with their distribution being fairly sporadic and, in most cases species are known only from single localities (Gibson, 1982; Gibson & Moore, 1976; Sundberg & Gibson, 2008). The freshwater genus Prostoma, currently thought to contain 11 species, is an exception to the freshwater form, as it is known to be widely distributed on a global basis, although locally sporadic in occurrence. (It is important to note, however, this known occurrence may be largely reflective of sampling efforts, rather than actual species distribution.) Two particular species,*P. eilhardi* and *P. graecense* have been reported to range from Europe to Africa with *P. eilhardiI* also occurring in Brazil and possibly Argentina and Uruguay, and *P. graecense's* range extending to the British Isles, Japan, America, Australia and Tasmania. The original localities of these species are not known, but possible causes that have led to their widespread distribution include the importation and exportation of freshwater vegetation or being carried on the feet of water birds (Gibson, 1972; Sundberg & Gibson, 2008, see also Coe, 1959).

Moore & Gibson (1973) suggest that *Prostoma* may have evolved from marine ancestors, their origins being suggested from the known habitats of near relatives (See *figure 1*).

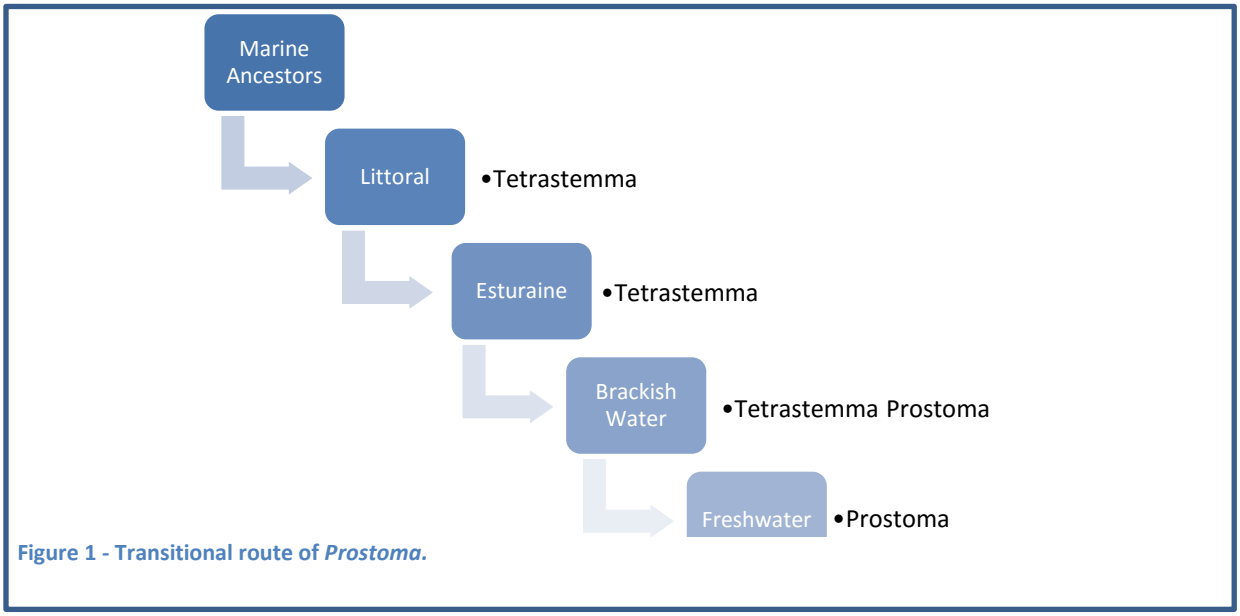


Figure 1 - Transitional route of *Prostoma*.

Evidence of this deviation can be seen as the occurrence of *P. graecense* in brackish regions of the Gulf of Finland, and the tolerance to seawater immersion shown by terrestrial nemerteans *Geonemertes Agricola* (Crozier, 1917) and *G. nightingaleensis* (Brinkmann, 1947; Gibson 1972).

Freshwater species are thought to be predatory worms 10-40mm in length and of pink colouration, although differences in colour from sandy yellow to red, and even green, have been reported, and in most cases colouration tends to vary according to the maturity of the animal. *Prostoma* in particular have been recorded feeding on oligochaete worms, but are also known to feed on crustaceans, nematodes, tubellarians, midge larvae and other small invertebrates. Feeding is thought to be more intense at night (Thorp 1991; McDermott & Roe, 1985; Cook & Herrmann, 1997).

Habitat requirements for freshwater nemerteans are poorly understood (Cook & Herrmann, 1997).Species are thought to be free-living benthic animals confined to small permanent weed dominated ponds and sluggish backwaters of streams and rivers. They often exhibit limited local distribution, being found to exist in one small habitat, when nearby areas appear to virtually identical; this is evident in the case of *P. jenningsi* where the species is believed to have only been found in one particular reed bed at the Clay 'Ole site. When present, they may usually be found amongst submerged vegetation, and particularly on the lower surface of floating leaves (Williams, 1980; Thorp, 1991; Cook & Hermann, 1997).

Although little is known about freshwater nemerteans, some details have been recorded for the North American species *Prostoma rubrum* (since reclassified as *P. Graecense,* due to vague species description – see Gibson & Moore, 1976) (Child 1901; Poluhowich 1968). This species was found commonly amongst

filamentous algae but also occurred regularly upon other aquatic plants. Individuals appeared to be restricted to the pond marsh-interface where an abundance of organic matter was noted. Temperature was reported to vary from 2-19˚C with a reasonably constant acidity of pH 5. *Prostoma* were reported to be abundant within the pond's margins throughout the year; however, evidence of seasonal migration to deeper waters has also been reported (Gibson, 1972). Thorp (1991) suggests that *Prostoma* are clearly associated with littoral habitats, and Kolasa (1977) found a significant number of *Prostoma* species to be associated with filamentous algae in lakes.

However, *P. graecense* possesses a worldwide, though sporadic, distribution as mentioned above, reported to be found in freshwater streams, rivers and ponds (Gibson, 1982; 1995). Specimens recovered in Japan were found in rice-fields and ponds, especially chalybeate water (Iwata, 1954). In Tasmania populations were found on decaying leaves and stems of reeds and rushes growing in what are reported to be somewhat stagnant areas of a freshwater creek and they were also reported to have been found in empty cells in plant tissue where eggs had been deposited (Gibson & Moore, 1971). In addition, Cook & Herrmann (1997) recorded populations to be present within spring seepage near Bogg's Creek, Colorado, where they reported populations to be sparse, with some sample sites lacking any nemerteans. One single nemertean was also recovered from the Arkansas River. Specimens were found within very small habitats and thought to exist within patches of moss. No specimens were recovered within the summer months; it was considered that elevated summer temperatures may have caused the nemerteans to retreat into crevices (NB *P. jenningsi* was thought to retreat into deeper waters within the summer months – see Quigg & Lowe, 2011a).

Furthermore Laumer (2012, pers comm. – see appendix 1) suggested that *Prostomas* seem to have slightly restricted thermal preferences. Throughout his study he found them to occur most often in springs and seeps (see also Cook & Hermann (1997) – see appendix 2 for American locality), in cool, well-oxygenated, flowing reaches of creeks where leaves or aquatic macrophytes have accumulated, and in standing water, mainly in the early spring and autumn, when it grows quite cool (Laumer, 2012, pers comm.) Please see appendix 1 for full correspondence with Chris Laumer and Cook and Hermann (1997) paper regarding observation, feeding behaviour and habitat of *P. gracense*.

3.0 Study Methods

Drawing on the recommendations made in phases one and two of the Project (see Quigg & Lowe 2011a; 2011b), it was considered that a continuation of the previously employed net sampling technique would be most appropriate for the survey in order to maintain consistency and allow comparisons to be drawn with previous results. In addition, the modified Gerking box sampler would also be used (providing

conditions were favourable) in order to collect more quantitative data, and for comparisons to be drawn between sampling methods. The study was widened to include a similar pond in the area. Chemical analysis of water and sediment samples was also carried out for ponds sampled in order to ascertain the conditions in which *P. jenningsi* may be found, and also to compare the water chemistry at the Clay 'Ole with historical data collected in 2003. In addition, comparisons could be drawn between the Clay 'Ole and similar ponds in the area.

3.1 Clay 'Ole

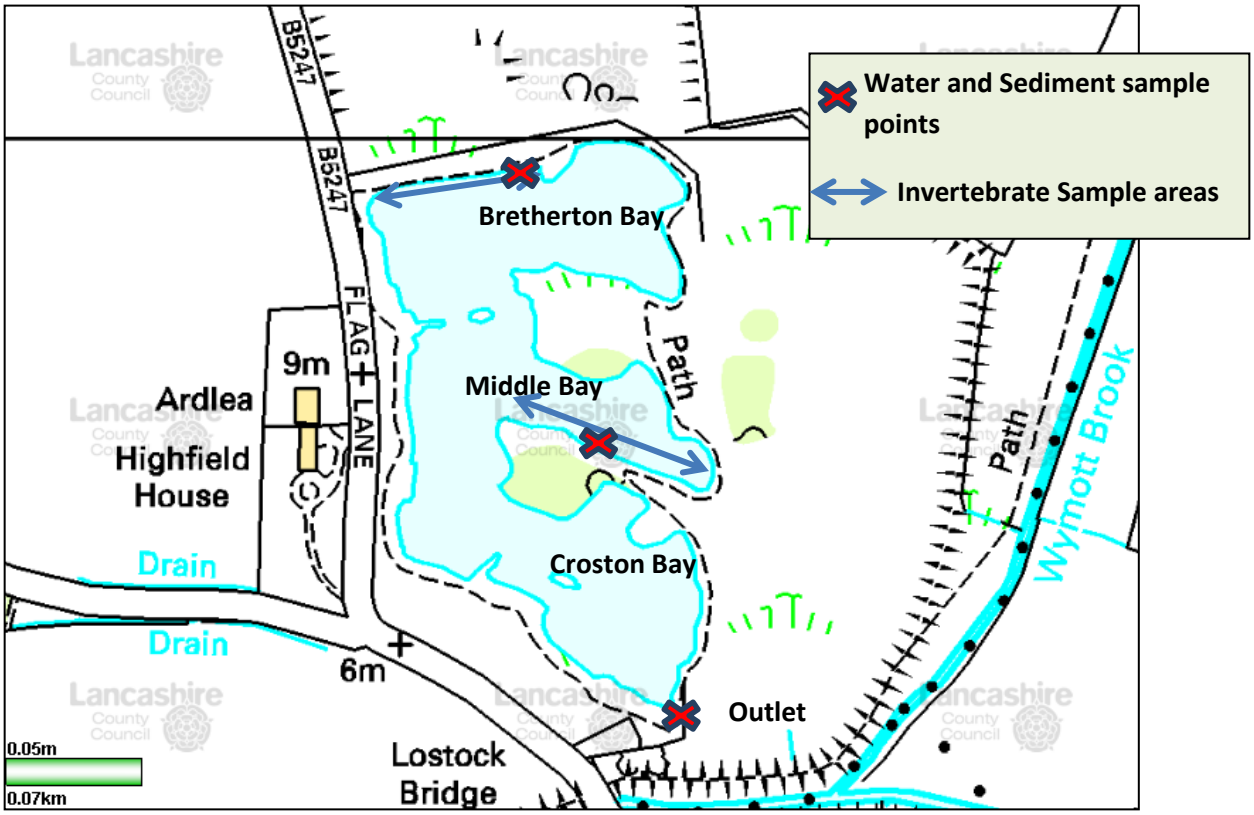


Figure 2 -The Clay 'Ole (source Mario).

The study primarily focused on the stretch of pond located within the Middle Bay of the Clay 'Ole site, in which previous studies have concentrated and the species is known to have been located (see *figure* 2; see also Quigg and Lowe (2011a) for a full outline of the site). Ten replicates were taken at 10 metre intervals using the net sampling technique, between SD 4853419851 and SD 4863819821. In addition replicates were taken using the modified Gerking box sampler. Furthermore, net sampling was also carried out along a stretch of *Phragmite* stands within the Bretherton Bay of the Clay 'Ole (SD 484711996

to SD 4855019983 – see *figure 2*) as in phase one of the project. Garden shears were used to cut a path through the vegetation to the water's edge in order to sample. Where it was not possible to gain access at a ten-metre point, the nearest available access point was used. Water and sediment samples were also taken from points A, B and C – see *figure 2*. Point A is within the Bretherton Bay of the pond where water drains into the pond through drainage pipes from the surrounding land. Point B is within the middle bay of the pond and is the area in which *P. jenningsi* was previously located and point C is within the Croston Bay of the pond where the water overflows and drains out into the River Lostock. The points were chosen to account for any variations that may occur within the site. At each point 3 water samples were taken.

3.2 Ulnes Walton Pond

Figure 3 - Ulnes Walton Pond

In addition to sampling at the Clay 'Ole site, samples were also taken from a similar pond at Ulnes Walton (see figure 4 for location in relation to the Clay 'Ole). The Ulnes Walton pond is situated upstream of the Clay 'Ole close to the River Wymott on the site of a former clay extraction site. The surrounding area is made up of mature trees and scrub**.** Garth and Wymott Prisons are situated adjacent to the site, with a railway line running nearby. The area in general is overgrown, although access to the pond is provided by a path around the perimeter of the pond. The site is privately owned by the Worden estate and under

the management of SITA, but is regularly accessed by members of the public for fishing/recreational purposes. Although much smaller than the Clay 'Ole, the pond shares similar characteristics, exhibiting stands of *phragmites*, *Junctus* and other macrophytic vegetation around its margins (see *figure 3*). Litter and other debris are prevalent at the site, particularly at clearings from which the samples were taken. At the Clay 'Ole, water drains into the pond from the surrounding land but at Ulnes Walton drains out of the pond into adjacent overflow ponds (see figure 5). Four locations were identified for sampling A, B, C and D as seen in figure 5. These points were chosen to provide a good representation of the pond and due to their ease of access. At points A, B, C and D aquatic invertebrate samples were taken using the net sampling technique as outlined below. Water and sediment samples (n = 3 replicates) were also collected at sampling points A, C and D.

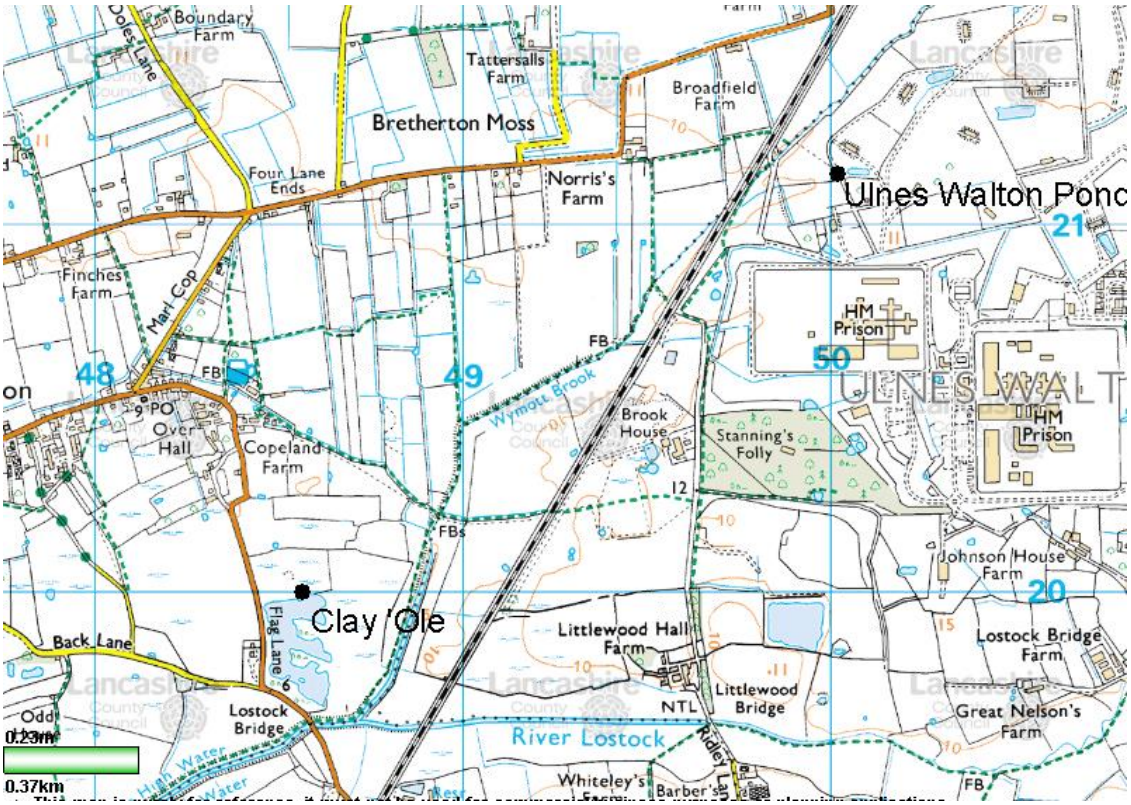


Figure 4 - Map to show the proximity of the Clay 'Ole to Ulnes Walton Pond

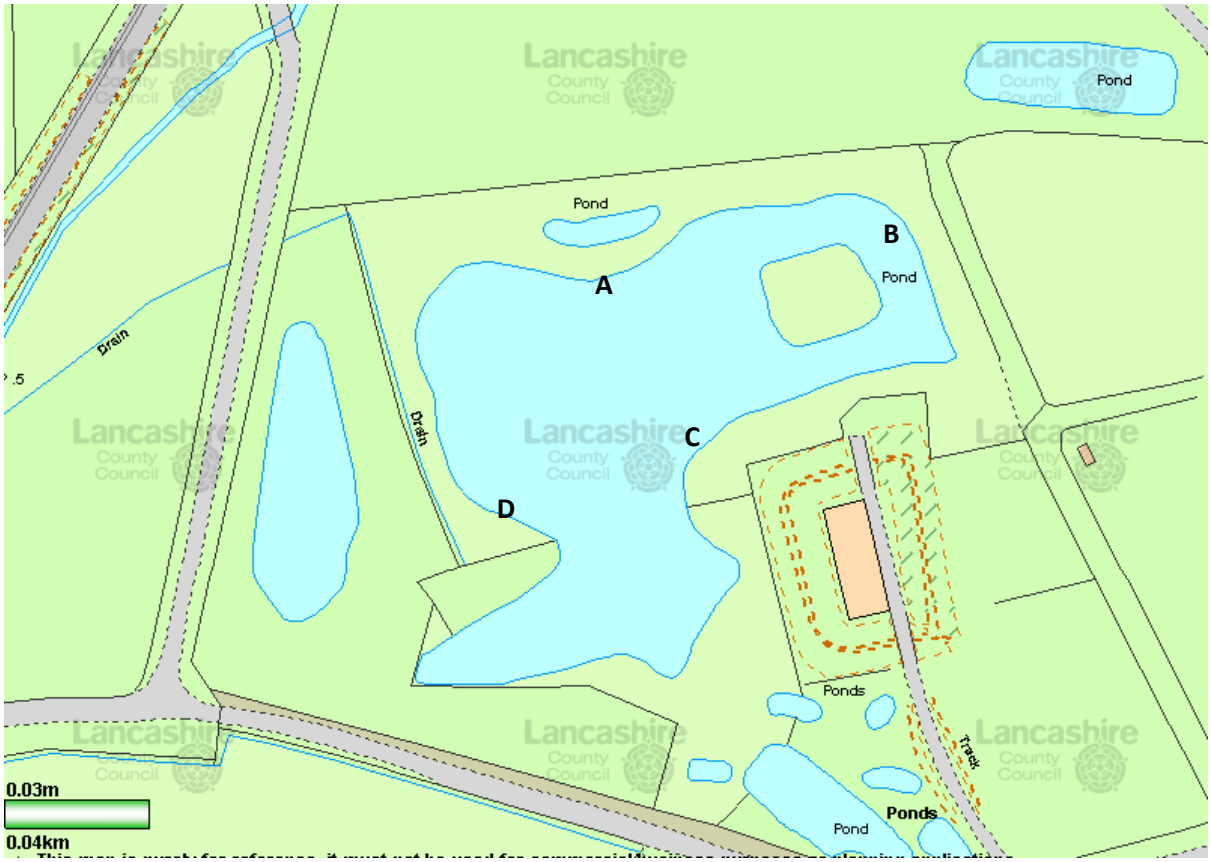


Figure 5 - Ulnes Walton Pond (source Mario)

3.3 Sampling techniques

3.3.1 Net sampling technique

As with Gibson & Young's study (1976), and during stages one and two of the current project, a five-minute sampling time per replicate was adopted. During this time a standard (1mm mesh) hand net was used in a jabbing/shuffling motion throughout the littoral zone and substrata between the reeds. The contents of the net were emptied into 3.4-litre, rectangular, air-tight plastic container. The sampling time included the time taken to empty the net's contents into the container – this occurred approximately 3 to 5 times per replicate. On occasions, when the container became full before the five-minute time period had been reached, sampling ceased. Once sampling was complete, samples were returned to the laboratory for processing.

3.3.2 Modified Gerking box sampler

The box sampler was placed in the marginal macrophyte bed areas of the littoral zones in the middle bay of the Clay 'Ole (*see figure 2*). At each sampling site, where necessary, the upper, emerged part of the reeds were first cut off to allow positioning of the sampling device. The device was then submerged into the substratum with the cutter blade retracted and the poles embedded into the substrate. Where necessary, reeds were cut away around the bottom of the box frame to allow it to sit flat on the substrate. Once in place, the sliding cutter was closed. The sampler was then removed from the water

and the contents poured into 3.4-litre, air-tight plastic containers to be returned to the laboratory for processing. The device was cleaned between each sampling episode using a watering can (and the residue from this process added to the sample).

3.3.3 Laboratory analysis

In the laboratory, larger pieces of debris and roots floating at the surface of the samples were removed from the containers, any organisms present on them having carefully been removed. The sealed containers were then left to stand overnight at room temperature, allowing the oxygen in the water to become depleted, thus bringing organisms near to, on or up to, the surface film, as outlined by Gibson and Young (1976). Any 'worm-like' organisms were then removed from the container and preserved in 4% formalin for further investigation and identification under the microscope. Initial collection was limited to a one-hour period per container, due to the abundance of specimens per sample. It was considered that this would give a good representative sample, whilst adhering to the time constraints involved with processing samples of freshwater macro-invertebrates. However, care was taken not to exclude any potential specimens of *P. jenningsi*. Any specimens considered not to be *P. Jenningsi* were excluded from the results.

N.B. Samples taken by the net were processed in the same way as those taken by the box sampler.

3.4 Water Testing

Water samples were taken in clean airtight plastic bottles. Each bottle was rinsed out with pond water then filled with a water sample. Three replicates were taken per sampling point. Samples were taken from shallow areas around the pond's margins. Water was tested for pH (p Hep+ meter by HANNA calibrated to pH 4 and 7), Total Dissolved Solids (H1914d Dissolved Oxygen Meter by HANNA) Dissolved Oxygen and temperature (Hi 98311 Di STEC/TDS meter by HANNA) on site; the samples were then returned to the laboratory for analysis.

3.4.1 Water sample laboratory analysis

On return to the laboratory, water samples were stored in the refrigerator until processing. A Chromeleon Dionex was used to test for nitratres, phosphates and chloride. In addition, potassium and sodium content was determined by Atomic Absorption Spectrometry (A.A.S.) & Flame Photometer.

3.5 Sediment Samples.

Sediment samples were collected using a small plastic scoop, trying to avoid any debris or leaf litter, and placed in sealable plastic bags. Three replicates were taken per sampling point. Samples were returned to the laboratory for analysis.

3.5.1 Laboratory analysis

Sediment samples were left to air dry over a week; this was necessary, given the moisture content. Samples were then tested for organic matter (loss on ignition).

Procedure

1. Sediment samples (<2mm fraction) were oven dried at 105 ^{o}C to a constant weight in order to remove any moisture
2. Approximately 10g of sediment was placed in a crucible and heated to 550^{o}C for 3 hours in a furnace.
3. After cooling, the crucibles were reweighed to calculate the percentage loss in weight to infer organic matter content. The calculation used was:

$$\frac{Weight\ loss\ (g)}{Oven\ dry\ weight} \times 100$$

4.0 Results

4.1 Invertebrate Survey

4.1.1 Clay 'Ole

After careful observation of all samples, and further study under the microscope, no samples of *P. jenningsi* were recovered for the study period using either sampling technique. Despite this, a wide variety of invertebrates were recovered from the Clay 'Ole site. Samples were found to contain hoglouse, water mites, water beetles, waterboatmen, hydra, chrionomidae, mollusca, numerous leech species, bivalves, numerous flatworm species and other oligochaetes including naididae. Tadpoles were also present in abundance.

The graphs below indicate the abundance of 'worm-like' invertebrates (as outlined above) that were extracted from the samples and preserved for investigation, thus giving an indication of conditions found at the sampling sites. It is important to point out *Prostoma* is often found when sampling for flatworms (Gilbert, pers comm. 2011).

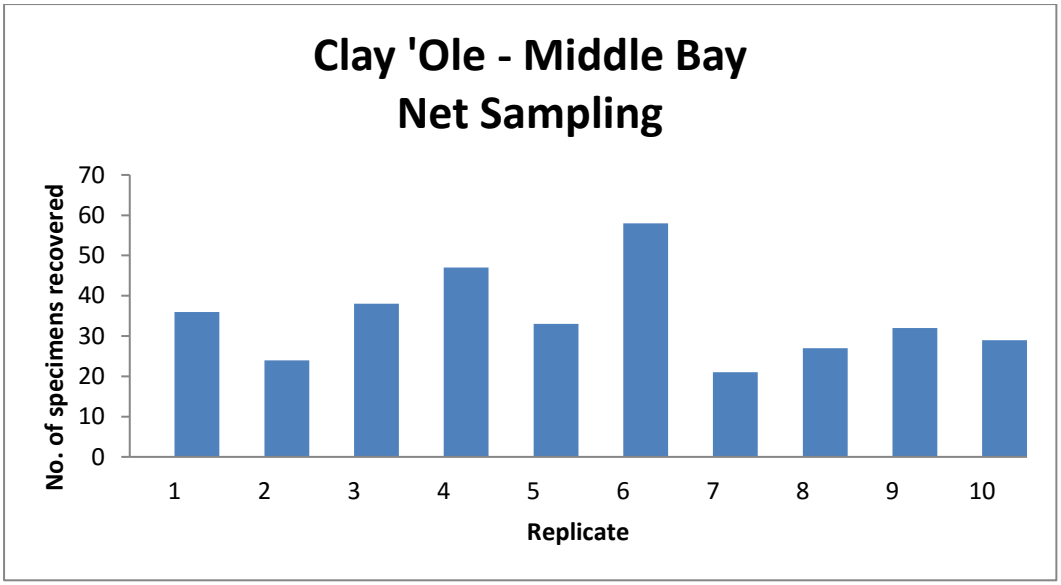


Figure 6 - Graph to show the relative abundance of worm-like organisms collected from the Middle Bay of the Clay 'Ole using a sweep net technique during spring 2012.

Figure 6- shows the number of 'worm-like' organisms recovered using a sweep net technique over ten replicates within the Middle Bay (the area in which *P. jenningsi* has been previously found) of the Clay 'Ole site during phase three of the project, spring 2012. It can be seen that the fewest specimens were recovered from replicate 7, with the most abundant being replicate 6.

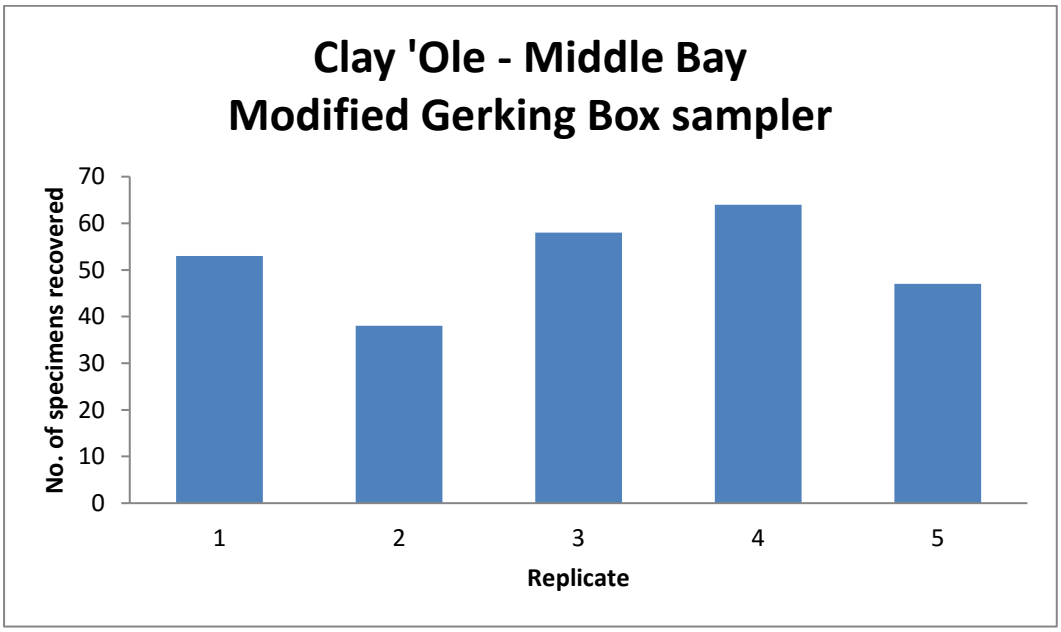


Figure 7 - Graph to show the relative abundance of worm-like organisms collected using the modified Gerking box sampler during spring 2012.

Figure 7 displays the number of 'worm-like' organisms recovered using the modified Gerking box sampling device. It can be seen that although there are variations between the replicates, overall the

device yielded results that are comparable to those recovered by the net sampling method (see *figure 6*).

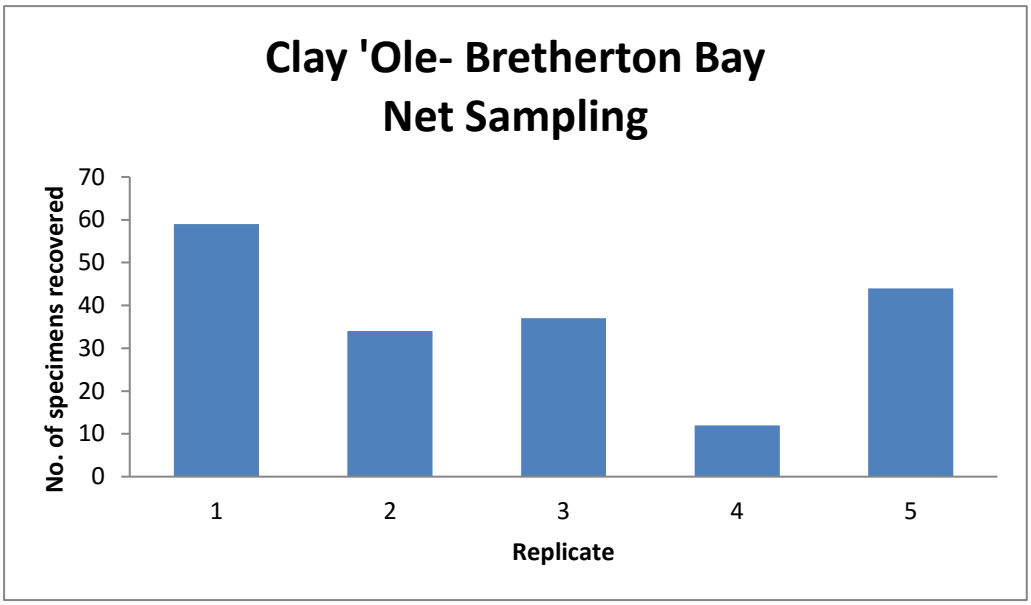


Figure 8 - Graph to show relative abundance of worm-like organisms collected from Bretherton bay using a sweep net technique during sping 2012.

Figure 8 displays the relative abundance of 'worm-like' organisms collected using the sweep net technique (n= 5 replicates) in Bretherton Bay of the Clay' Ole site.

4.1.2 Ulnes Walton

No specimens of *P. jenningsi* were recovered at the Ulnes Walton Pond using the net sampling technique. Despite this, the pond supported a variety of freshwater invertebrates including tadpoles, hoglouse, water mites, water beetles, water scorpion, chrionomidae, mollusca, numerous leech species, Damsel fly nymph, a number of flatworm species; other oligochaetes including lumbriculid and naididae were present in all samples collected.

The graphs below indicate the abundance of 'worm-like' organisms (as outlined above) that were extracted from the samples and preserved for investigation, thus giving an indication of conditions found at the sampling sites.

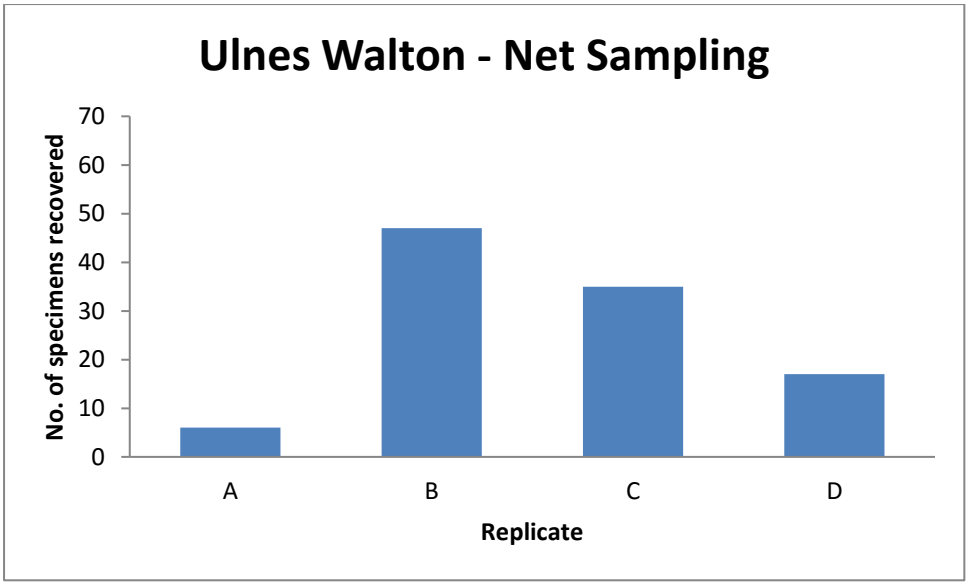


Figure 9- Graph to show relative abundance of worm-like organisms collected using a sweep net technique during spring 2012.

Figure 9 shows the relative abundance of 'worm-like' organisms collected using a sweep net technique over 4 replicates at the Ulnes Walton pond. Replicate A recovered very few specimens.

4.1.3 – Mean numbers of 'worm-like' organisms recorded

Table 1 - Table to show the mean relative abundance of 'worm - like' organisms recovered.

Mean values of relative abundance recorded			
Clay 'Ole - Middle Bay (net sample)	Clay 'Ole – Middle Bay (modified Gerking box sample)	Clay 'Ole – Bretherton Bay (net sample)	Ulnes Walton (net sample)
29.8	52	37.2	26.3

Table 1 displays the mean relative abundance of 'worm-like' organisms recovered over the phase 3 study period. It can be seen the highest numbers recovered were in the Middle Bay of the Clay 'Ole using the modified Gerking box sampler.

4.2 Water Chemistry

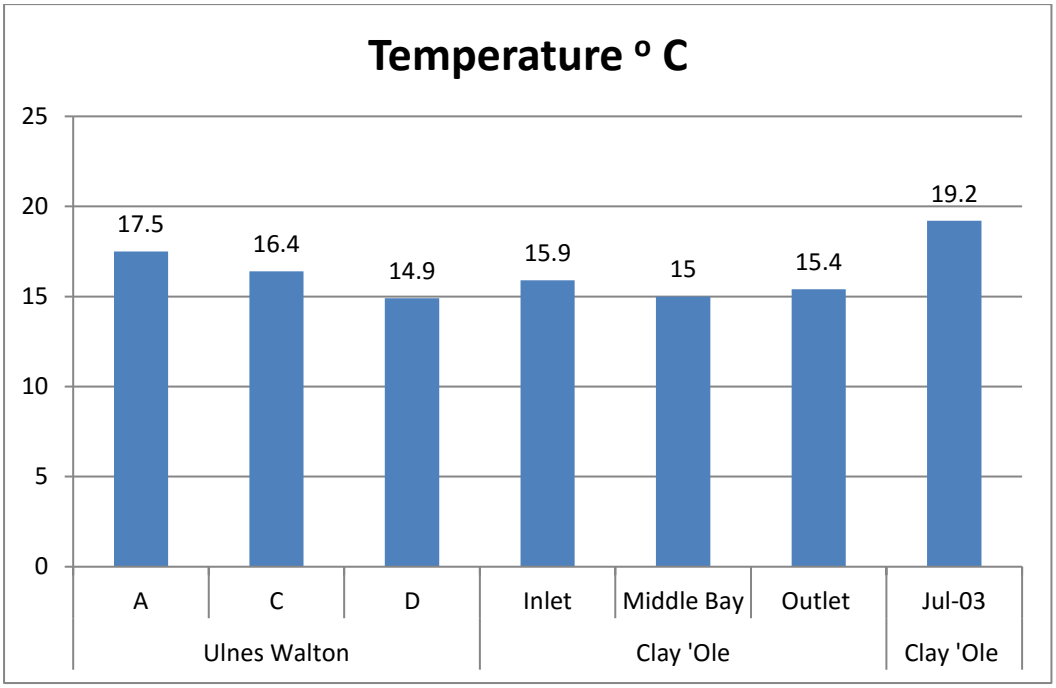


Figure 10 - graph to show water temperature recorded over the sample sites.

Figure 10 shows water temperature recorded over the sample sites. Temperatures range between 15°C and 19.2 °C.

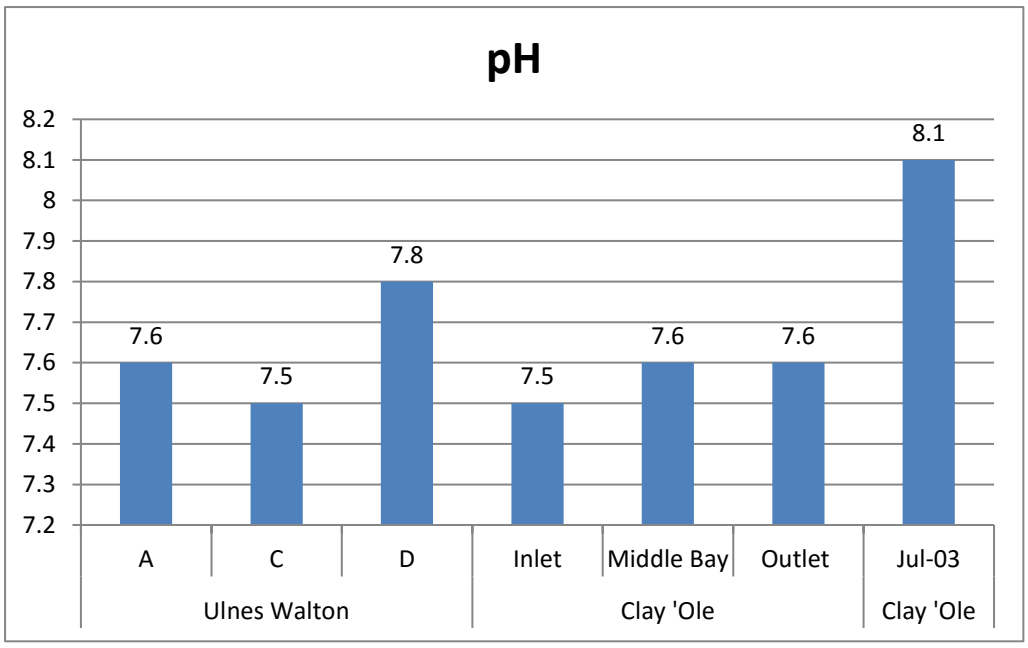


Figure 11 - Graph to show pH readings taken over sample sites; spring 2012 and July 2003.

Figure 11 shows pH levels recorded at the survey sites in addition to data recorded at the Clay 'Ole in July 2003. It can be seen that pH levels are relatively similar, with the exception of that recorded at the Clay 'Ole 2003, where levels were slightly higher.

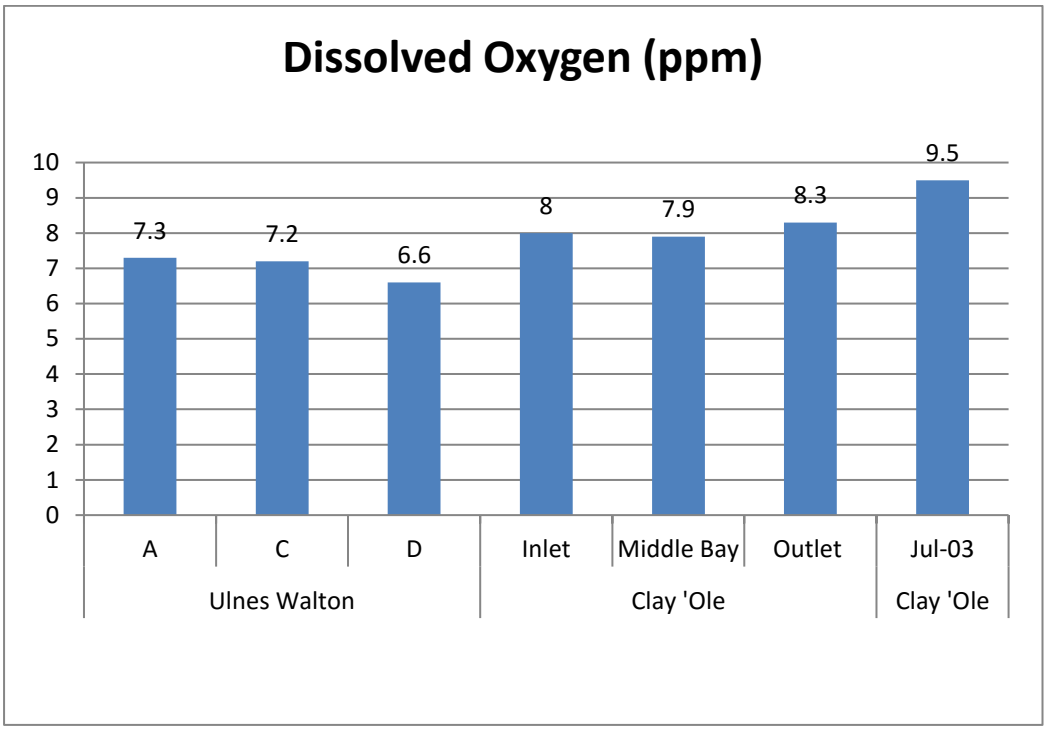


Figure 12 - Graph to show Dissolved Oxygen Content taken over sample sites

Table 2 - Table to show mean values and standard deviation of dissolved oxygen content recorded for Ulnes Walton and the Clay 'Ole.

Dissolved Oxygen (ppm)	Mean Value	Standard Deviation
Ulnes Walton	7.03	0.38
Clay 'Ole	8.07	0.20

Figure 12 shows levels of dissolved oxygen (ppm) taken at survey sites during phase 3 project and July 2003. It can be seen that levels were slightly higher during July 2003. Table 2 displays mean values and standard deviation.

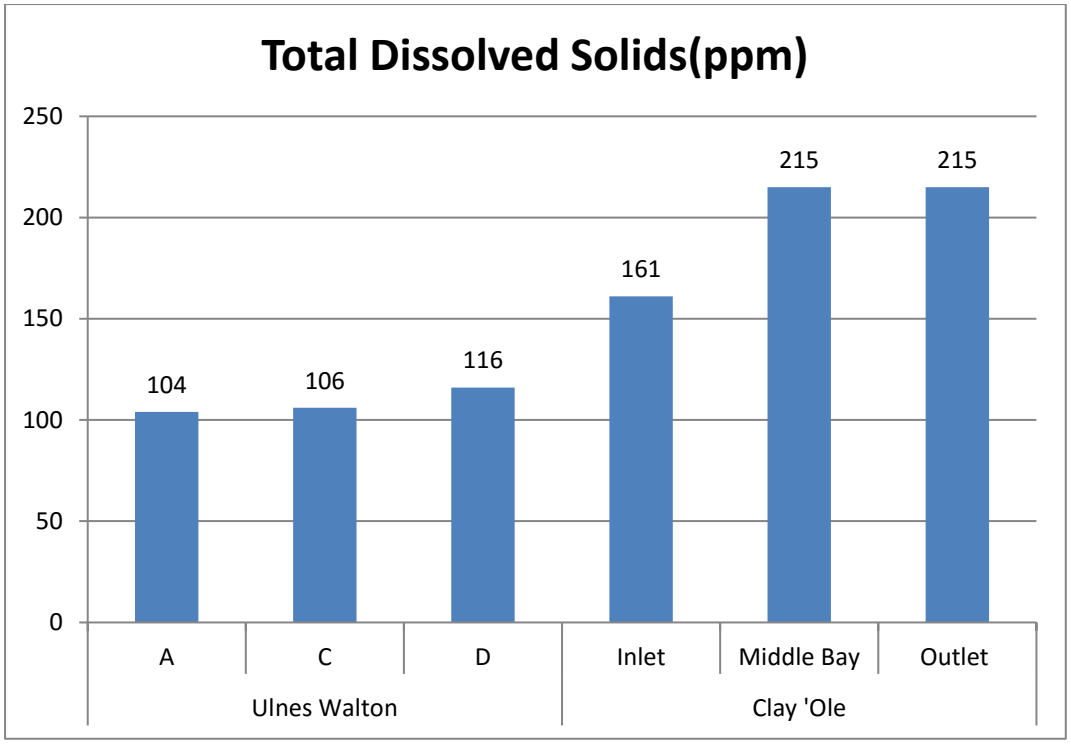


Figure 13 shows the levels of total dissolved solids recorded at the sample sites. It can be seen that levels recorded at the Clay 'Ole, particularly the Middle Bay and the Outlet, are higher than those recorded at the Ulnes Walton pond. No information was available for the Clay 'Ole July 2003.

Table 3 - Table to show mean values and standard deviation of total dissolved solid levels recorded for Ulnes Walton and the Clay 'Ole.

TDS (ppm)	Mean Value	Standard Deviation
Ulnes Walton	108.7	6.42
Clay 'Ole	197.0	31.2

Figure 13 shows the levels of total dissolved solids recorded at the sample sites. It can be seen that levels recorded at the Clay 'Ole, in particular at the Middle Bay and the outlet, are higher than those

recorded at the Ulnes Walton pond. No information was available for the Clay 'Ole July 2003. Table 3 displays mean values and standard deviation.

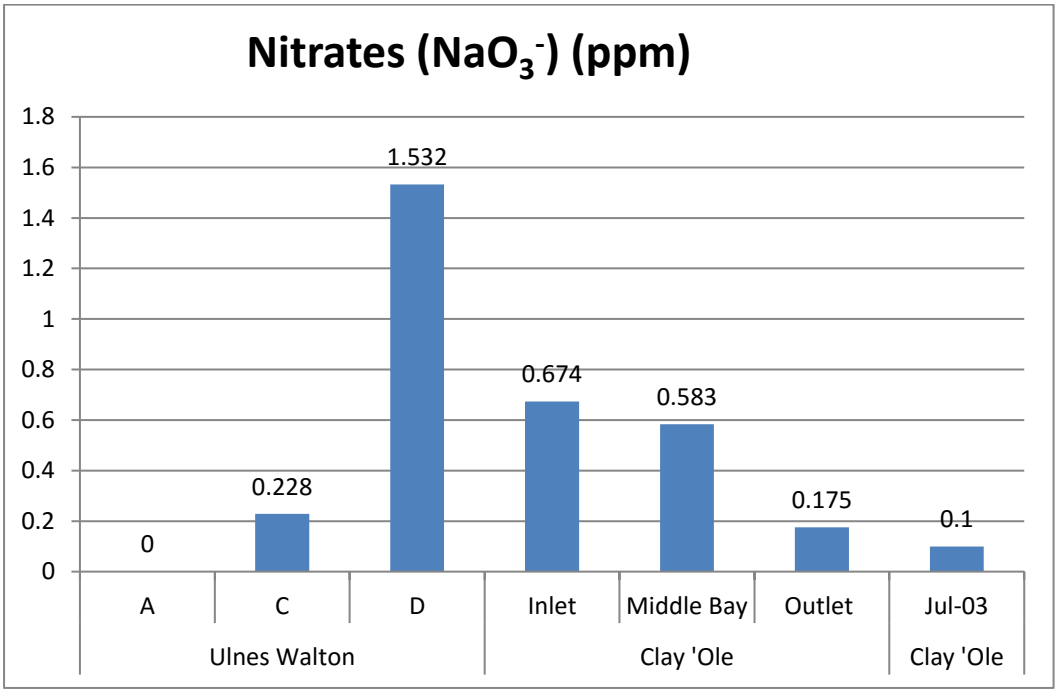


Figure 14 - Graph to show Nitrate levels recorded at sample sites; spring 2012 and July 2003.

Table 4 - Table to show mean value and standard deviation of nitrate levels recorded for Ulnes Walton and the Clay 'Ole.

Nitrate Nitrogen (NO_3^-)	Mean Value	Standard Deviation
Ulnes Walton	0.59	0.83
Clay 'Ole	0.48	0.27

Figure 14 shows trace nitrate levels recorded at Ulnes Walton and the Clay 'Ole sites. It can be seen that the reading for replicate D is higher than other sampling points.

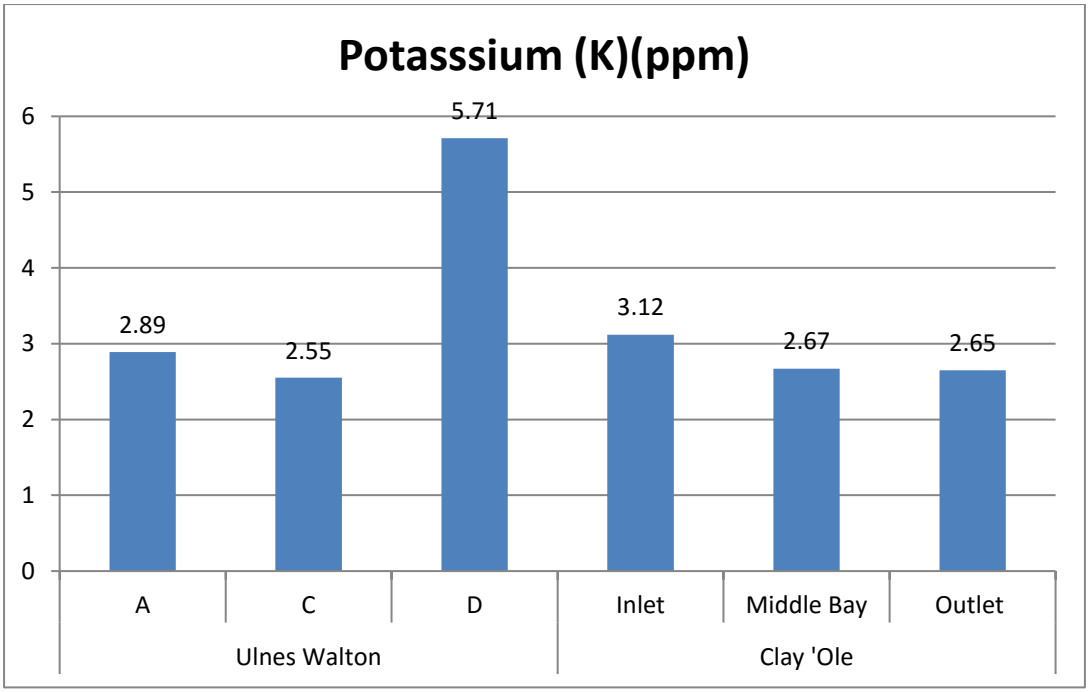


Figure 15 - Graph to show potassium levels (ppm) recorded over sample sites; spring 2012

Table 5 - Table to show mean value and standard deviation of potassium levels recorded for Ulnes Walton and the Clay 'Ole.

Potassium (K) (ppm)	Mean Value	Standard Deviation
Ulnes Walton	3.72	1.73
Clay 'Ole	2.81	0.27

Figure 15 shows levels of potassium recorded at Ulnes Walton and the Clay 'Ole site. It can be seen that the reading taken at point D, Ulnes Walton, is higher than other recordings.

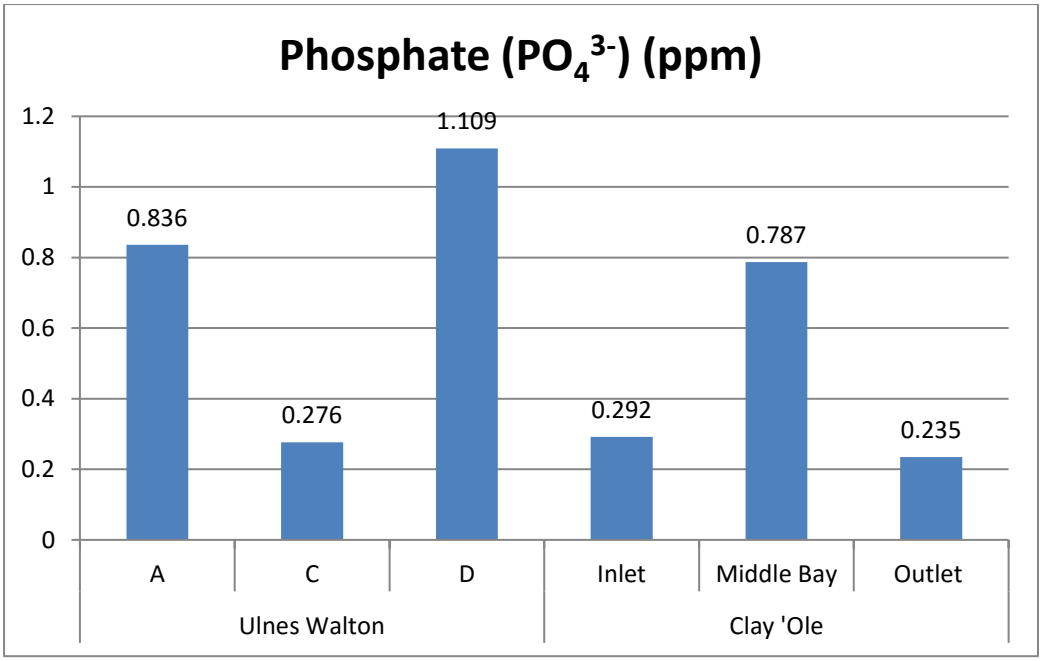


Figure 16 - Graph to show phosphate levels (ppm) over sample sites; spring 2012

Table 6 - Table to show mean values and standard deviation of phosphate levels recorded for Ulnes Walton and the Clay 'Ole.

Phosphate (PO_4^{3-}) (ppm)	Mean Value	Standard Deviation
Ulnes Walton	0.74	0.42
Clay 'Ole	0.44	0.30

Figure 16 shows that trace levels of phosphates can be seen at both Ulnes Walton and the Clay 'Ole. A measurement for phosphate was not recorded for the Clay 'Ole in 2003. It can be seen that phosphate levels are higher at the Ulnes Walton pond. Mean values and standard deviation are displayed in table 6.

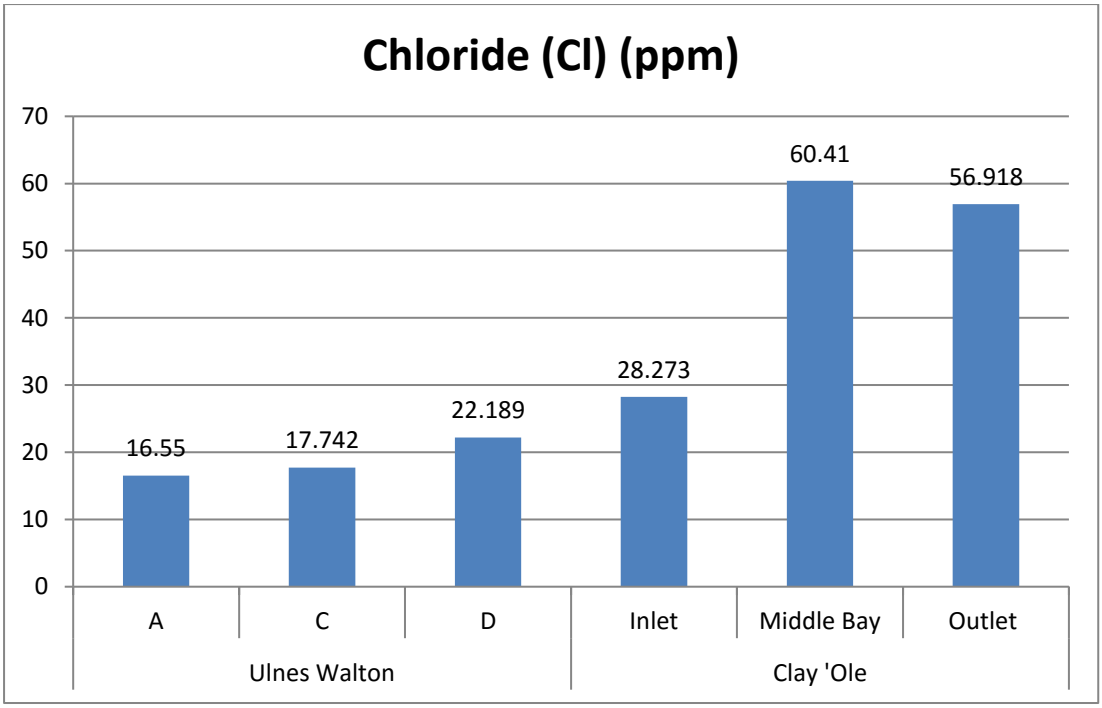


Figure 17 - Graph to show chloride levels recorded (ppm) over sample sites; spring 2012

Table 7 - Table to show mean values and standard deviation of chloride levels recorded for Ulnes Walton and the Clay 'Ole.

Chloride (Cl)	Mean Value	Standard Deviation
Ulnes Walton	18.83	2.97
Clay 'Ole	48.53	17.63

Figure 17 shows chloride levels recorded at Ulnes Walton and the Clay 'Ole. It can be seen that chloride readings are higher within the Middle Bay and outlet point of the Clay 'Ole site than in samples recorded elsewhere. Table 7 displays mean values and standard deviation.

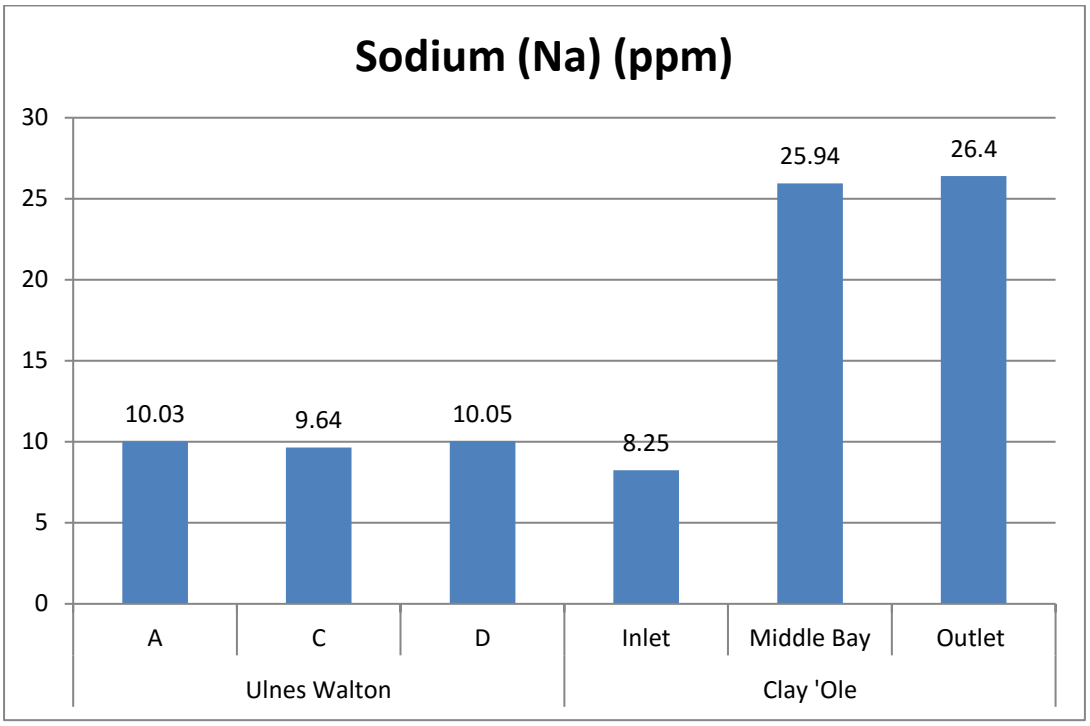


Figure 18 - Graph to show sodium level (ppm) recorded over sample ponds; spring 2012.

Table 8 - Table to show mean values and standard deviation of sodium levels recorded for Ulnes Walton and the Clay 'Ole.

Sodium (Na) (ppm)	Mean Value	Standard Deviation
Ulnes Walton	9.90	0.23
Clay 'Ole	20.19	10.35

Figure 18 shows levels of sodium recorded at Ulnes Walton and the Clay 'Ole. It can be seen that sodium readings are higher within the Middle Bay and outlet point of the Clay 'Ole site. Mean values and standard deviation can be seen in table 8.

4.3 Sediment

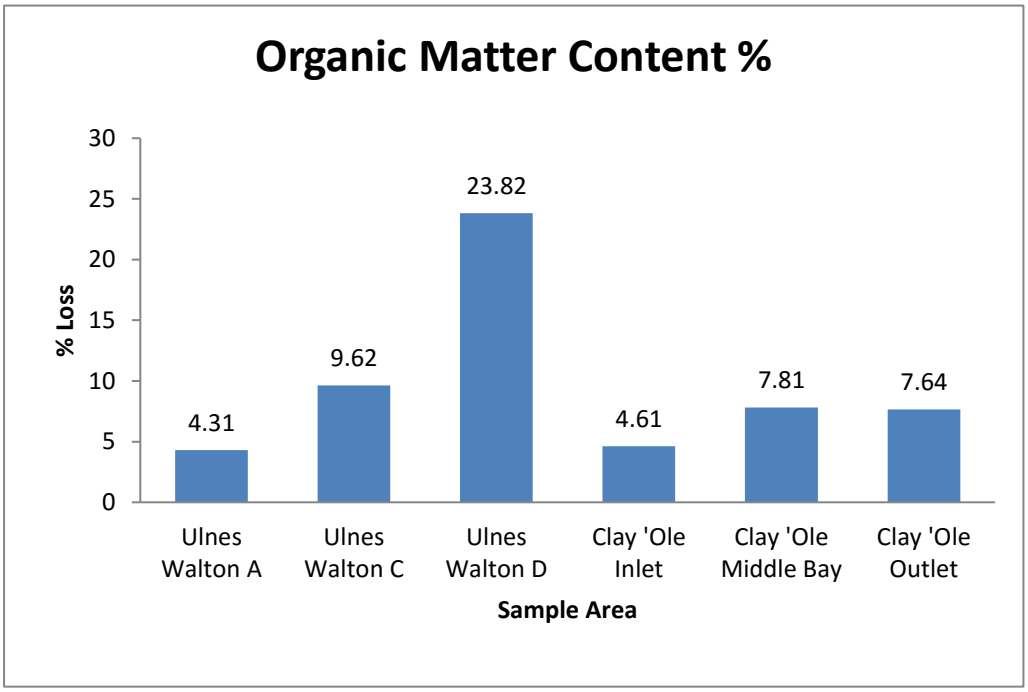


Figure 8 - Graph to show percentage organic matter content in sediment from samples taken across survey ponds.

Table 9 - Table to show the mean values and standard deviation for organic matter content (%) recorded for Ulnes Walton and the Clay 'Ole.

Organic Matter Content (%)	Mean Value	Standard Deviation
Ulnes Walton	12.58	10.09
Clay 'Ole	6.89	1.69

Figure 19 shows the organic matter content (%) by loss on ignition from the survey sites. It can be seen that Ulnes Walton D displayed grater levels than the other sample points. Table 5 shows the mean values and standard deviation for results recorded for organic matter content (% loss on ignition).

NB. Throughout the results it must be noted that the number of replicates is restricted to 3 per sample pond.

5.0 Discussion

5.1 Net sampling

As with phases one and two of the project, the net sampling method employed for the study was not quantitative and samples taken can only be considered representative of each point. It is possible that any specimens of *P. jennings*i present at the site have simply been missed by the net (see Quigg & Lowe 2011b; O'Connor *et al,* 2004). When comparing results of relative abundance to phases one and two of the study, overall far fewer specimens were recovered, particularly in comparison to summer 2011, but also in relation to samples taken over the winter months (see Quigg & Lowe 2011b). Although freshwater invertebrates occur throughout the year and seasonal variation is expected, greatest species richness and abundance would be anticipated in the spring months. Variations are thought to occur due to factors such as food supply, intra-specific competition and temperature, (Thorpe & Covich, 2009). It is surprising that species abundance is so low, particularly in relation to numbers recorded in summer 2011. It could be suggested that the presence of tadpoles may have had an effect on the species abundance as they have not been present during any previous sampling events; further to this, studies suggest that tadpoles may not merely function as filter feeding omnivores, as in some cases tadpoles are major predators of macro-invertebrates in ponds, particularly soft-bodied sedentary benthic invertebrates. It is, however, considered that this predation is more common in temporary ponds and subject to the relative abundances of acceptable food items, although there is relatively little research in this area (Petranka & Kennedy, 1999; Altig et al, 2007).

5.2 Modified Gerking Box Sampler

As recommended by phase two of the Project, the modified Gerking box sampler was once again employed as a sampling method to test the suitability of the device and allow comparisons with the net sampling method, in addition to the collection of more quantitative data. During phase two of the project, difficulties had been encountered whilst sampling, due to adverse weather conditions and dangers involved with the nature of the pond, in addition to dense reed growth (Quigg & Lowe 2011b). Throughout the current phase of the project, the sampling device was considerably more effective than during the previous sampling event. Sampling proved to be easier due to weather conditions being more favourable and water levels lower. In addition, the emergent reeds were far easier to slice through with the retractable cutting device; however, it was still necessary to use shears to cut away some of the submerged reeds in order to allow the device to sit flush to the substrate.

It can be seen in the results (figure 6 and table 1) that the modified Gerking box sampler was more efficient in capturing specimens relevant to the study than the net sampling technique, thus corroborating results from other research (see Syrcha & Adamek, 2010). It is important to note that the

net sampling technique covered a much greater area than that of the box sampler; should the net samples have been restricted to the same sampling area, they would probably have yielded even fewer organisms. The modified Gerking box sampler should therefore be considered a viable sampling method, one which is capable of collecting quantitative data. Should specimens of *P. jenningsi* be recovered in future sampling events, the modified Gerking box sampler could be used (given favourable conditions) to establish relative abundance of the species.

5.3 Water Chemistry

5.3.1 pH

……

……. *11* that all pH readings from both the Ulnes Walton pond and the Clay 'Ole fall within the 'normal' range for freshwater. The variation in the reading taken at the Clay 'Ole during July 2003 may be accounted for by seasonal variation, particularly as the reading was taken in the summer months, where all other readings were taken in the spring.

5.3.2 Dissolved Oxygen

Dissolved oxygen is an important requirement for the survival of species living within aquatic environments. Overall, the concentration of dissolved oxygen in water is broad, with tolerance to concentrations varying from species to species (Thorp and Covich, 2010). The specific oxygen requirement of *P. jenningsi* is unclear.

5.3.3 Total Dissolved Solids

Freshwater fauna are clearly distinguished by occurrence in waters that, while dilute, have a wide range of dissolved salts. Commonly the lower limit for TDS in which freshwater fauna exists is in the region of 10 mg/L with the upper limit being as high as 1000 mg/L (see figure 20 below), although the species that exist within certain parameters vary (Smith, 2001).

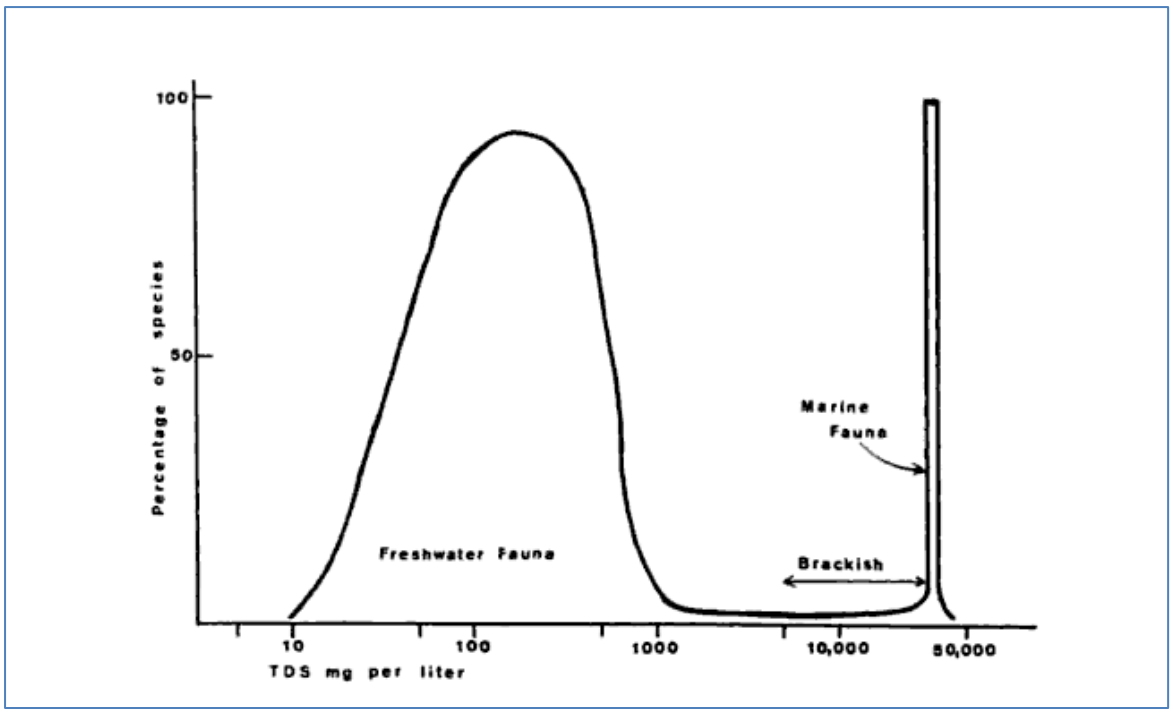


Figure 9 - Salt content and environmental preferences of the aquatic faunas. NB Figure does not show limits of tolerance of faunas

Values recorded for total dissolved solids (TDS) fall comfortably in the range expected for freshwater fauna. It can be seen in *figure 13* that levels of TDS are elevated within the Middle Bay and Croston Bay of the Clay 'Ole sit;, this result is reflected in results recorded for sodium and chloride - see *figures 17 and 18* and further discussion below.

5.3.4 Nitrate

Nitrates are present in natural freshwater ecosystems as normal biological degradation products of proteins and nucleic acids. High concentrations of NO_3^- can be found in surface waters as a consequence of freshwater pollution. Nitrogen compounds can enter freshwater ecosystems through various means; point sources include aquaculture operations, livestock and agricultural operations, industrial waste and sewage effluence, with non-point sources also related to agriculture, through means such as fertilization and manure, and urbanisation, through runoff from septic systems etc. (Camargo & Ward, 1995; Soucek & Dickinson, 2012). It can be seen in *figure 14* that nitrate levels are very low across the sample sites. Point D at the Ulnes Walton pond displays a slightly raised reading as it does for potassium - see *figure 15* and below. Levels at the Clay 'Ole are slightly higher at the Inlet point and the Middle Bay in comparison with the Outlet point. They are also higher than the levels recorded during July 2003. These results are possibly influenced by the factors mentioned above.

5.3.5 Potassium

Freshwaters usually contain less than 10 mg/L of potassium, and this is often only in traces. Large quantities of potassium salts in freshwaters are known to be toxic to many freshwater invertebrates (Smith, 2001). It can be seen in *figure 15* that potassium levels over all samples fall below 10mg/L and are therefore within the normal range. Levels are slightly raised at sample point D within the Ulnes Walton Pond, possibly due to a point source of pollution.

5.3.6 Phosphates

Phosphorous occurs in natural waters almost exclusively as phosphates, playing a significant role in the eutrophication of surface waters. The main impact of raised phosphorus concentrations in rivers or lakes is to promote plant growth which may then cause detrimental effects on ecosystem quality and functioning. Phosphates can be introduced to freshwaters from sources such as fertilizers and other agricultural operations, wastewaters, farm waste products and the degradation of organic matter (Pichette *et al*, 2009). It can be seen that levels of phosphates recorded across all sample areas are low, falling below 1.2 ppm. Again levels recorded at point D within the Ulnes Walton pond are marginally higher as seen in *figure 16*.

5.3.7 Sodium and Chloride

Sodium ion (Na+) is the metal ion with the lowest toxicity for aquatic organisms; however, chronic concentrations of chloride as low as 250 ppm have been recognised as harmful to freshwater life (Kaushal *et al,* 2005). It can be seen in *figures 17 and 18* that sodium and chloride levels remain at low levels across the survey points; however, although still remaining within the 'normal' range, they are noticeably higher at the Middle Bay and outlet (within the Croston Bay) of the Clay 'Ole site. As there is no previous data surrounding concentrations of sodium and chloride levels at the Clay 'Ole, allowing for comparisons to be drawn, it is unclear as to whether such levels are a 'normal' occurrence. A salinity level of 5-8% is assumed to be the upper limit for freshwater invertebrates, with information concerning the lower limits being scarce. Oligochaetes and crustaceans are thought to be most tolerant to changes in water salinity both through substantial increase and decrease (Berezina, 2003). It could be suggested that the salinity of the Clay 'Ole is slightly raised due to the possible influx of brackish water from the nearby river Lostock. It has been established that the river Lostock at this point remains within the tidal reach, and that the area is prone to flooding (see phase one report). During this study period the nearby river Lostock is known to have flooded on two occasions, with one storm event being the most severe in the area for the last 25 years. Due to the lack of research surrounding *P.jenningsi*, it is unclear as to how sensitive the species would be to such changes to its environment. Although previous reports have

discussed that over the years the surrounding area in which the Clay 'Ole is located has been prone to flooding, no further data has been gathered surrounding the frequency and severity of such events.

5.4 Sediment

5.4.1 Organic matter content

Organic matter content provides a minor but important fraction of freshwater sediments. It originates from the complex mixture of lipids, carbohydrates, proteins and other organic components produced by organisms that have lived in and around an ecosystem. (It can provide information that is important to interpretation of both natural and human induced changes in local and regional ecosystems (Paul & Meyer, 2008). It can be seen in *figure 19* that the % organic matter content across all sampling areas remains low, with the exception of site D at the Ulnes Walton pond where levels exceed 20%.

5.5 Prostoma jenningsi?

The current phase of the study has been unable to prove the existence of *P. jenningsi* at the Clay 'Ole site. As with Gibson's last sampling attempt (date unknown), no specimens have been recovered (Gibson, 2011 pers com). The result is disappointing, as previous research suggests that during studies in which *P.jenningsi* has been recovered, the highest numbers were recovered during the winter/spring (Gibson & Young, 1976). Although the species has not been recovered, conclusions cannot be drawn regarding the species continued existence. As with the previous sampling event, the weather conditions may have had an effect. Prior to sampling, temperatures reached seasonal highs, then continued to fluctuate; weather conditions were changeable with heavy rainstorm events and the wettest April to July recorded since records began. Recorded relative abundance of 'worm-like' organisms was lower than in phases 1 and 2, a result that is surprising, given that greatest invertebrate abundance usually occurs over the spring months.

It is possible that the population of *P. jenningsi* may be present in another area of the Clay 'Ole pond. Although the reed bed in Bretherton bay has also been investigated and a number of samples taken from fishing pegs during phase one of the project, the species may exist elsewhere around the pond margins as suggested by Thorp (1991); species often exist in a small part of one locality where other parts appear equally as suitable. Further to this Laumer (2012, pers comm) suggests it is possible that the population is still in this pond, but has simply been sampled in the wrong microhabitats.

Although there are a number of explanations available to support the possible continued existence of *P. jenningsi* at the Clay 'Ole site, the likelihood that the species may no longer be present should also be considered. Causes for the species to no longer exist in the pond could include changes in land and water management practices, inter-specific competition and predation as outlined in phase 2 report (see Quigg & Lowe, 2011,b).

In addition, changes in water quality/salinity may have had an effect on the species. Given that the species may have been introduced to the Clay 'Ole from the river Lostock, it is possible that over time the salinity of the water has increased/decreased thus making conditions less favourable. Laumer (2012, pers comm.) suggests that if *P .jenningsi's* really is a distinct species it must have a longer history than that of the circa 60 years in which the Clay 'Ole has been present; thus it may be a case of a species with pronounced dispersal ability but rather restricted ecological requirements. Through his own personal observations Laumer (2012, pers comm.) suggests that "*Prostoma* seem to have restricted thermal preferences, favouring cool well oxygenated flowing reaches of creeks where leaves or aquatic macrophytes have accumulated, and in standing water, mainly in the early spring and autumn, when it grows quite cool."

During the sampling period it has been shown that the River Lostock is prone to flooding; such floods could alter the salinity balance in the Clay 'Ole and thus have an impact on *P.jenningsi.* The frequency of such flood events could have changed between the present and the last confirmation of the existence of *P. jenningsi* and have influenced the status of this species at the Clay 'Ole site.

6.0 Conclusions and recommendations

The study has been unable to prove the existence of *P.jenningsi* at the Clay 'Ole site; however, neither has it been disproved. The possibility that the population of *P.jenningsi* has become locally extinct at the site should be considered; however this cannot be concluded, due to the limited period over which the project has been executed. Given the results surrounding freshwater invertebrates present in the pond over all sampling periods, and with the addition of water chemistry analyses, it is clear that the pond is 'healthy', with no obvious signs of pollution. When drawing comparisons between the Ulnes Walton Pond and the Clay 'Ole, the pond, and its surrounding area, is maintained to a much higher standard (given that it is managed as an angling club and is a site of biological heritage – see Quigg & Lowe, 2012,b for possible issues surrounding site management). Questions could however be raised surrounding the increased levels of sodium and chloride within the Middle Bay and Croston Bay (the bay in which the outlet exists). Concern could also be raised relating to the reduced number of 'worm-like' organisms recorded for the site during this stage; however, it is likely that this could be down to a sampling anomaly, and possible predation by tadpoles. In addition to possible explanations for the species to no longer exist, as outlined in stage two of the project (Quigg & Lowe 2011,b), the possibility of flood events affecting the pond has arisen.

In order to draw conclusions surrounding the continued existence of *P.jenningsi* it may be beneficial to investigate historical flood events in the area to gain a greater insight into the impacts such floods may

have had on possible populations of *P.jenningsi* and the Clay 'Ole as a whole. Further investigation into the salinity of water within the Clay 'Ole and the nearby river Lostock may also be valuable.

A further sampling event would be advisable given the low numbers of 'worm like' invertebrates recovered during stage three of the study, thus ruling out any sampling anomalies or accounting for seasonal fluctuations. Should a further sampling event occur over the summer months, it would allow for direct comparisons to be made with data gathered during phase one of the survey. In addition, it may be advantageous to widen the survey area in the Clay 'Ole, should the species have migrated to another area. Furthermore, given that the Clay 'Ole is a man-made site, dating back to circa 1955, the existence of *P.jenningsi* within the pond can only be through introduction (for more details and a comprehensive site history, please see Quigg & Lowe, 2011a). Due to the ecological nature of the project, it would be advisable to widen the survey to include similar sites in the surrounding area. As discussed by Quigg & Lowe (2011a), it is possible that the species may have been introduced to the Clay 'Ole site through a number of different means (see also Sundberg & Gibson, 2008) and probable that the species may occur elsewhere in the local vicinity. This suggestion is supported by advice given by Laumer (2012, pers comm.), who suggests that it may be easier to find specimens of *P. jenningsi* in a neighbouring pond that is similar today to how the Clay 'Ole may have been when it was first discovered. Information regarding flood events and changes to the Clay 'Ole should be sought.

Drawing on the knowledge gained so far, any additional information and data obtained would allow for more substantial conclusions to be drawn surrounding the continued existence of the species within the Clay 'Ole Site and, if applicable, the surrounding area.

References

Altig, R., Whiles, M.R. & Taylor, C.L. (2007). What do tadpoles really eat? Assessing the trophic status of an understudied and imperilled group of consumers in freshwater habitats. *Freshwater Biology.* **52** pp 386-395.

Berizina, N.A. (2003). Tolerance of freshwater invertebrates to changes in water salinity. *Russiun Journal of Ecology.* **34,** 4, pp 261 -266.

Brinkmann, A. & Christophersen, E. (1947). A New Land Nemerteran from the Tristan Da Cunha Group, and a Survey [of] the Genus Geonemertes in: **3,** 15.

Camargo, J.A. & Ward, J.V. (1995). Nitrate (NO_3-N) Toxicity to Aquatic Life: A Proposal Of Safe Concentrations For Two Species Of Neartic Freshwater Invertebrates. *Chemosphere* **13,** 5 pp 3211-3216.

Child, C.M. (1901). The habits and natural history of *Stichostemma. American Naturalist.* **35,** pp129 – 327.

Coe, W. (1959). Nemertea. Pp366-367, in *Freshwater Biology* (2nd edition) Wiley & Sons, New York.

Cook, J.L. & Herrmann, S.J. (1997) New Record of Prostoma graecense (Nemertea: Hoplonemertea) from Southern Colorado, with Observations on its Feeding Behaviour and Habitat. *Southwestern Association of Naturalists*. **42,** 2 pp 162-169.

Crozier, J.W. (1917). Fusion of "Rhinophores" in Chromodoris. *The American Naturalist.* **51,** 612, pp 756 -758.

Gibson, R. (1972). *Nemerteans.* Hutchinson & Co, London.

Gibson, R. (1982). *British Nemerteans*. Cambridge University Press, Cambridge.

Gibson, R. (1995). Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. *Journal of Natural History.* **29,** pp 271-562.

Gibson, R. (1998) Epilogue – one hundred years of nemertean research:Burger(1895) to the present in beneath. In Norenburg, J.L & Roe, P. (eds),(1998). *Fourth International Conference on Nemertean Biology*. Kluwer Academic Publishers, Belgium. *Hydrobiologia* **368:** 301–310.

Gibson, R. (2011). Pers comm. –Telephone conversation. 22/08/2011.

Gibson, R. & Moore, J. (1971). Occurrence of the freshwater hoplonemertean *Prostoma graecense* (Böhmig) in Tasmania. *Freshwater Biology.* **1,** pp 193-195.

Gibson, R. & Moore, J. (1976). Freshwater Nemerteans. *Zoological Journal of the Linnean Society.* **58.** pp 177-218.

Gibson, R. & Young, J.O. (1971). *Prostoma jenningsi* sp. Nov., a new British freshwater hoplonemertean. *Freshwater Biuology.***1** pp 121 -127.

Gibson, R. & Young, J.O. (1976) Ecological observations on a population of the freshwater hoplonemertean *Prostoma jeninngsi. Archives of Hydrobiology.* **78.** 1. Pp 42-50.

Iwata, F. (1954). The Fauna of Akkeshi Bay XX. Nemertini in Hokkaido. *Journal of the Faculty of Science Hokkaido University Series VI.* **12** (1-2) pp 1-39.

Kaushal, S.S., Groffman, P.M., Likens, G.E, Belt, K.T, Stack, W.P., Kelly, V.R., Band, L.E & Fisher, G.T. (2005) Increased salinization of fresh water in the norteastern United States. *PNAS*. **102**, 38 pp 13517-13520

Kolasa, J. (1977). *Fatworms: Turbellaria and Nemertea* in Thorp , J.H. (1991) *Ecology and Classification of North American Freshwater Invertebrates.* Academic Press, California.

Laumer, C. (2012). Pers comm. Email received 20/6/2012.

McDermott, J.J. (1993). Nemertea inhabiting the *Haploopls* (Amphipoda) community of the northern Øresund with special reference to the biology of *Nipponemertes pulcher* (Hoplonemertea). *Hydrobiologia.* **266,** pp 15-28.

McDermott , J.J & Roe, P. (1985). Food feeding behaviour and feeding Ecology of Nemerteans. *American Zoological.* **25,** pp 113 – 125.

Moore, J. & Gibson, R. (1973). A new genus of freshwater haplonemertean fron New Zealand. *Zoological Journal of the Linnean Society.* **52,** 133-144.

O'Connor, A., Bradish, S., Reed, T., Moran, J.,Regan, E., Visser, M.,Gormally, M, & Skeffington, S. (2004). A comparison of the efficiency of pond-net and box sampling methods in turloughs – Irish ephemeral aquatic systems. *Hydrobiologia.* **100,** 1 pp 153-168.

Paul, M.J. & Meyer, J.L. (2008) Sreams in the Urban Landscape. *Urban Ecology.* **3**, pp207 -23.1

Petranka, J.W. & Kennedy, C.A. (1999). Pond tadpoles with generalized morphology: is it time to reconsider their functional roles in aquatic communities? *Oecologia.* **120**, pp 621-631.

Pichette, C., Zhang, H. & Sauve, S. (2008). Using diffuse gradients in thin-films for in *situ* monitioring of dissolved phosphate enmissions from freshwater aquaculture. *Aquaculture.***286**. 3-4 pp198-202.

Poluhowich, J.J. (1968). Notes on the freshwater nemertean Prostoma rubrum.*T urtox News* **46** pp 2-6.

Quigg , S., and Lowe, C.N. (2011a) Confirming the existence of the Croston worm (*Prostoma jenningsi*). September 2011. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Quigg , S., and Lowe, C.N. (2011b) Confirming the existence of the Croston worm (*Prostoma jenningsi*) Stage 2. Winter 2011. Unpublished report prepared on behalf of the Wildlife Trust for Lancashire, Manchester and North Merseyside.

Smith, D.G. (2001). *Pennak's freshwater Invertebrates Of The United States Porifea to Crustacea.* (4th Edition) John Wiley & Sons, Inc. United States .

Soucek, D.J., & Dickinson, A. (2012). Acute Toxicity of Nitrate and Nitrite to Sensitive Freshwater Insects, Mollusks, and a Crustacean. *Archive of Environmental Contamination Toxicology.* **62.** pp 233–242.

Sundberg, P. & Gibson, R. (2008) Global diversity of Nemerteans (Nemertea) in freshwater. Hydrobiologia **595,** pp 61-66.

Sychra, J. & Adamek, Z. (2010) Sampling Efficiency of Gerking Sampler and Sweep Net in Pond Emergent Littoral Macrophyte beds – a Pilot Study. *Turkish Journal of Fisheries and Aquatic Sciences.* **10,** pp161-167.

Thorp , J.H. (1991) *Ecology and Classification of North American Freshwater Invertebrates.* Academic Press, California.

Thorp, J.H. & Covich, A.P. (2009) *Ecology and Classification of North American Freshwater Invertebrates*. Academic Press, London.

Turbevile, J.M.(2002). Progress in Nemertean Biology: Development and Phylogeny. *Integral and Comparative Biology.* **42,** pp692-703.

Williams, W.D. (1980) *Australian freshwater life*. Macmillian Publishers, Australia

Wilson, W.H. (1991) Competition and Predation in Marine Soft-Sediment Communities. *Annual Review Ecological Systems.***21,** pp221-241.

Yanfang, Z. & Shichun, S. (2006) Effects of salinity, temperature and pH on the survival of the nemertean *Procephalothrix stimulus* Iwata (1952). *Journal of Experimental Biology and Ecology*. **328,** pp 168 – 176.

Appendix 1

From: Christopher Laumer [claumer@fasmail.harvard.edu]
Sent: 30 June 2012 14:01
To: Siobhan Mary Quigg
Subject: Re: Freshwater nemertean - Prostoma jenningsi

Dear Siobhan,

Apologies for my long delay in response. Your message came just as I was leaving for a conference & long field season in continental Europe, and I've only just now had a few moments to revisit my inbox.

Very interesting to hear about your work on *Prostoma jenningsi*! Jon probably told you about our project on the dispersal and population genetics of various *Prostoma*s we've been finding throughout the world. Flatworms are really more my field of expertise, but over the past 4 years I've been encountering a number of freshwater nemerteans as well, so maybe I can offer some advice.

There's unfortunately, as you probably have become aware, relatively little ecological work done on freshwater nemerteans. And indeed, others have noticed that their appearance can be very sporadic, with previously abundant populations apparently disappearing. (My own supervisor's first paper was on a new population of *Prostoma* in Spain, and he tells me that nobody's seen them in this locality since then.)

I can't really remark on ecological factors that may have contributed to your negative observations. Any speculation about habitat change, competition with flatworms, etc. would be just that - speculation. And of course, absence of evidence is not the same as evidence of absence - so it's possible that the population is still in this pond, but has simply been sampled in the wrong microhabitats or at the wrong times of year. Can you tell me - have you been successful in finding *Prostomas* from other places? Maybe it would be easier to find the species in a neighboring pond similar today to how this clay pit might have looked in the late sixties? My suspicion is that *Prostoma jenningsi* must be only *apparently* endemic to Croston: if it really is a distinct species, it must have a much longer history than the 60 year old pond in which it was originally found. It may just be a case of a species with pronounced dispersal ability but rather restricted ecological requirements.

For future reference: from my own collections I notice - although, this is very much only a personal impression, not a result from any thorough quantitative study - that *Prostomas* seem to have slightly restricted thermal preferences. I find them most often in springs and seeps (see the attached paper on an american locality), in cool, well-oxygenated, flowing reaches of creeks where leaves or aquatic macrophytes have accumulated, and in standing water, mainly in the early spring and autumn, when it grows quite cool. If you are using an oxygen-depletion technique to find them (I always do), consider making samples in spring or autumn, focusing on cool water, well-oxygenated habitats with abundant macrophytes.

I hope this is helpful. Please don't hesitate to write again if you have further questions.

Yours,

Christopher Laumer

PS - If you do end up finding new populations of *Prostoma* in the next few months, I should say we'd be very interested in having specimens preserved in 100% ethanol for genetic analysis! Particularly if you're able to confirm the species as *P. jenningsi*.

PPS - You say you have been finding flatworms in abundance - have you been able to identify these? The group I particularly study is a freshwater taxon of microturbellarians, called "Prorhynchidae", somewhat externally resembling nemerteans (in fact they were described as such originally!). If you have seen these in your collections, I'd be really very interested to know more!

Appendix 2

Column1	Column2	Column3	Column4	Column5	Column6	Column7	Column8	Column9	Column10	Column11	Column12	Column13	Column14	Column15	Column16	Column17	Column18	Column19	Column20	Column21	Column22	Column23	Column24	Column25	Column26	Column27	Column28
CL_3_CO1																											
CL_4_CO1	0.002																										
CL__6_CO1	0.002	0.004																									
CL__7_CO1	0.002	0.004	0.004																								
CL_8_CO1	0.000	0.002	0.002	0.002																							
CL_10_CO1	0.000	0.002	0.002	0.002	0.000																						
CL_11_CO1	0.000	0.002	0.002	0.002	0.000	0.000																					
CL_13_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002																				
CL_18_CO1	0.002	0.004	0.000	0.004	0.002	0.002	0.002	0.004																			
CL_20_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004																		
CL_26_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002																	
TL__4_CO1	0.004	0.007	0.007	0.007	0.004	0.004	0.004	0.007	0.007	0.007	0.004																
TL_7_CO1	0.007	0.009	0.009	0.009	0.007	0.007	0.007	0.009	0.009	0.009	0.007	0.002															
TL_8_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009														
TL_10_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002													
TL_13_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002												
TL_15_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004											
TL_14_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.004	0.004	0.002	0.004	0.004										
TL_16_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.000	0.004									
TL_22_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.000	0.004	0.004	0.004								
TL_24_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002							
TL_23_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002						
TL_29_CO1	0.007	0.009	0.009	0.009	0.007	0.007	0.007	0.009	0.009	0.009	0.007	0.011	0.013	0.009	0.007	0.004	0.009	0.009	0.009	0.004	0.007	0.009					
ME__1_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007				
ME_2_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000			
ME_3_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000		
ME__4_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	
ME__5_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_6_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_7_CO1	0.004	0.007	0.007	0.007	0.004	0.004	0.004	0.007	0.007	0.007	0.004	0.009	0.011	0.007	0.004	0.007	0.007	0.007	0.007	0.007	0.004	0.007	0.011	0.004	0.004	0.004	0.004
ME__8_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
ME__9_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_10_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_11_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_12_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_13_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_14_CO1	0.002	0.000	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
ME_16_CO1	0.002	0.000	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
ME_17_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
ME_27_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
ME_29_CO1	0.004	0.007	0.007	0.007	0.004	0.004	0.004	0.007	0.007	0.007	0.004	0.009	0.011	0.007	0.004	0.007	0.007	0.007	0.007	0.007	0.004	0.007	0.011	0.004	0.004	0.004	0.004
RL_3_CO1	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
RL__7_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
RL__8_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
RL_11_CO1	0.013	0.015	0.015	0.015	0.013	0.013	0.013	0.015	0.015	0.015	0.013	0.018	0.020	0.015	0.013	0.015	0.015	0.015	0.015	0.015	0.013	0.015	0.020	0.013	0.013	0.013	0.013
RL_13_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
RL_14_CO1	0.007	0.009	0.009	0.009	0.007	0.007	0.007	0.009	0.009	0.009	0.007	0.011	0.013	0.009	0.007	0.004	0.009	0.009	0.009	0.004	0.007	0.009	0.009	0.007	0.007	0.007	0.007
RL__9_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
RL_10_CO1	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.004	0.007	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.000	0.002	0.007	0.000	0.000	0.000	0.000
Prostoma_graecense_JX017298.1_(Lake_Ohrid)	0.004	0.002	0.007	0.007	0.004	0.004	0.004	0.007	0.007	0.007	0.004	0.009	0.009	0.007	0.004	0.007	0.007	0.004	0.007	0.007	0.004	0.007	0.011	0.004	0.004	0.004	0.004
Prostoma_gracecese_EU489490.1	0.002	0.000	0.004	0.004	0.002	0.002	0.002	0.004	0.004	0.004	0.002	0.007	0.009	0.004	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.009	0.002	0.002	0.002	0.002
Prostoma_sp_HQ848594.1	0.011	0.009	0.013	0.013	0.011	0.011	0.011	0.013	0.013	0.013	0.011	0.015	0.018	0.013	0.011	0.013	0.013	0.013	0.013	0.013	0.011	0.013	0.018	0.011	0.011	0.011	0.011
Prostoma_sp._BOLD:AAN8900_voucher_HQ938796.1	0.040	0.038	0.042	0.042	0.040	0.040	0.040	0.042	0.042	0.042	0.040	0.044	0.046	0.042	0.040	0.042	0.042	0.042	0.042	0.042	0.040	0.042	0.046	0.040	0.040	0.040	0.040
Prostoma_sp._BOLD:AAN8900_voucher_HQ939311.1	0.040	0.038	0.042	0.042	0.040	0.040	0.040	0.042	0.042	0.042	0.040	0.044	0.046	0.042	0.040	0.042	0.042	0.042	0.042	0.042	0.040	0.042	0.046	0.040	0.040	0.040	0.040
Prostoma_sp._BOLDCFWIE357-10.COI-5P	0.247	0.247	0.249	0.249	0.247	0.247	0.247	0.249	0.249	0.249	0.247	0.252	0.252	0.247	0.247	0.245	0.247	0.247	0.247	0.245	0.247	0.249	0.249	0.247	0.247	0.247	0.247
Emplectonema_gracile_NC_016952.1	0.166	0.163	0.166	0.163	0.166	0.166	0.166	0.168	0.166	0.168	0.166	0.170	0.172	0.166	0.166	0.163	0.166	0.168	0.166	0.163	0.166	0.168	0.168	0.166	0.166	0.166	0.166

Column29	Column30	Column31	Column32	Column33	Column34	Column35	Column36	Column37	Column38	Column39	Column40	Column41	Column42	Column43	Column44	Column45	Column46	Column47	Column48	Column49	Column50	Column51	Column52	Column53	Column54	Column55	Column56	Column57
0.000																												
0.004	0.004																											
0.002	0.002	0.007																										
0.000	0.000	0.004	0.002																									
0.000	0.000	0.004	0.002	0.000																								
0.000	0.000	0.004	0.002	0.000	0.000																							
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000																					
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000																				
0.002	0.002	0.007	0.004	0.002	0.002	0.002	0.002	0.002	0.002																			
0.002	0.002	0.007	0.004	0.002	0.002	0.002	0.002	0.002	0.000																			
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002																		
0.002	0.002	0.007	0.004	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002																	
0.004	0.004	0.009	0.007	0.004	0.004	0.004	0.004	0.004	0.007	0.007	0.004	0.007																
0.002	0.002	0.007	0.004	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002	0.004	0.007															
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.004	0.002														
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.004	0.002	0.000													
0.013	0.013	0.018	0.015	0.013	0.013	0.013	0.013	0.013	0.015	0.015	0.013	0.015	0.018	0.015	0.013	0.013												
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.004	0.002	0.000	0.000	0.013											
0.007	0.007	0.011	0.009	0.007	0.007	0.007	0.007	0.007	0.009	0.009	0.007	0.009	0.011	0.009	0.007	0.007	0.020	0.007										
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.004	0.002	0.000	0.000	0.013	0.000	0.007									
0.000	0.000	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.004	0.002	0.000	0.000	0.013	0.000	0.007	0.000								
0.004	0.004	0.009	0.007	0.004	0.004	0.004	0.004	0.004	0.002	0.002	0.004	0.007	0.009	0.007	0.004	0.004	0.018	0.004	0.011	0.004	0.004							
0.002	0.002	0.007	0.004	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.002	0.004	0.007	0.004	0.002	0.002	0.015	0.002	0.009	0.002	0.002	0.002	0.000					
0.011	0.011	0.015	0.013	0.011	0.011	0.011	0.011	0.011	0.009	0.009	0.011	0.013	0.015	0.013	0.011	0.011	0.024	0.011	0.018	0.011	0.011	0.011	0.009	0.009				
0.040	0.040	0.044	0.038	0.040	0.040	0.040	0.040	0.040	0.038	0.038	0.040	0.042	0.044	0.042	0.040	0.040	0.053	0.040	0.046	0.040	0.040	0.040	0.038	0.038	0.038			
0.040	0.040	0.044	0.038	0.040	0.040	0.040	0.040	0.040	0.038	0.038	0.040	0.042	0.044	0.042	0.040	0.040	0.053	0.040	0.046	0.040	0.040	0.040	0.038	0.038	0.038	0.000		
0.247	0.247	0.252	0.249	0.247	0.247	0.247	0.247	0.247	0.247	0.247	0.247	0.247	0.252	0.249	0.247	0.247	0.258	0.247	0.249	0.247	0.247	0.247	0.247	0.247	0.247	0.265	0.265	
0.166	0.166	0.170	0.163	0.166	0.166	0.166	0.166	0.166	0.163	0.163	0.166	0.168	0.170	0.163	0.166	0.166	0.179	0.166	0.163	0.166	0.166	0.166	0.163	0.163	0.159	0.166	0.166	0.307