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An analysis of the meroplankton assemblages of Station L4 and the development and application of molecular techniques to aid taxonomic resolution.

James Mark Highfield

ABSTRACT

Zooplankton data from the 1988-2007 Station L4 time-series were used to determine inter-and intra-annual patterns of meroplankton community change at Station L4, Plymouth, UK. Abundances were calculated for five groups: Cirripedia, Decapoda, Polychaeta, Echinodermata and Bivalvia. Analyses showed that, while there is some annual variability, seasonal variation accounts for the major changes in the meroplanktonic community composition throughout the time-series. Cirripedia were the only group to show any significant change in abundance over the time-series. Further sampling at the study site produced data at a finer taxonomic resolution allowing for the analyses of the seasonal cycles of abundance of previously unresolved families and genera from the 1988-2007 time-series. A similar pattern was shown to that seen in the earlier time-series with no evidence of major changes in the meroplanktonic community being found. Comparison with historical data taken from Lebour (1947) showed little evidence of major variation in meroplanktonic species composition. The limitations of traditional methods of taxonomic resolution were highlighted during these analyses and led to the investigation of molecular techniques as a viable aid to identification. Bivalve larval samples were identified to species in many cases using PCR and sequencing reactions focussing on the 18S rRNA gene. Larvae of *Phaxas pellucidus* were shown to be the most common, and further analysis revealed the presence of two groups of species within the data over the time sampled, comprising the larvae of several hard-substrata species. Development of an RFLP technique focussing on the

mtCOI gene, allowed for the successful discrimination of porcellanid larvae to species that were unable to be resolved morphologically. Long-term data-sets play an important role in revealing the long-term patterns of community composition and abundance of meroplanktonic larvae and should be continued to identify those patterns not evident over the time-period studied. Molecular techniques were valuable in aiding the taxonomic resolution of meroplanktonic larvae, allowing previously unknown patterns of species diversity and richness to be ascertained.

An analysis of the meroplankton assemblages of Station L4 and the development and application of molecular techniques to aid taxonomic resolution.

By

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LIST OF ABBREVIATIONS.

A	Adenine
ANOSIM	Analysis of Similarities
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumen
C	Cytosine
°C	Degrees centrigrade
cm	Centimetres
COI	Cytochrome-c oxidase subunit 1 gene
CTD	Conductivity, Temperature, Depth
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
DW	Durbin-Watson
EDTA	Ethylenediaminotetra-acetic acid
G	Guanine
ISH	*In-situ* hybridisation
LCR	Ligase Chain Reaction
LsurRNA	Large sub-unit ribosomal Ribonucleic acid
M	Molar
m	Metres
mg	Milligrams
mL	Millilitres
mm	Millimetres

mM	Millimolar
m. s^{-1}	Metres per second
MDS	Multi-Dimensional Scaling
min(s)	Minute(s)
mt	Mitochondrial
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
ng	Nanograms
$N.m^{-3}$	Numbers per cubic metre
NTC	No Template Control
PCR	Polymerase Chain Reaction
pmol	Picomolar
RCF	Relative Centrifugal Force
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RV	Research Vessel
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SIMPER	Similarity percentages
SIMPROF	Similarity profile
sp.	Species
SSCP	Single-strand conformation polymorphism
SST	Sea Surface Temperature
STST	Selective tidal-stream transport
T	Thymine

TAE	Tris base, Acetic acid, and EDTA
µg	Micrograms
µL	Microlitres
µm	Micrometres
UK	United Kingdom
UV	Ultraviolet
w/v	Weight per volume ratio
WoRMS	World Register of Marine Species

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AUTHOR'S DECLARATION

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CHAPTER 1. GENERAL INTRODUCTION.

1.1. MEROPLANKTON.

1.1.1. Meroplankton life-histories.

Many marine invertebrate species have complicated and intricate life-cycles in which earlier stages often differ considerably from their adult counterparts in terms of morphology, diet, habitat, and ecology (Thorson, 1950; Pechenik, 1999; Pradillon *et al.*, 2007). Many marine invertebrates spend their juvenile and adult lives in the benthos, but release gametes or larvae into the water column. Their larvae are planktonic, growing and developing through one or more larval stages (Eckman, 1996) and allowing potential dispersal over hundreds or thousands of miles (Thorson, 1950; Belgrano *et al.*, 1995a; Livi, 2006). Onset of a rapid and extensive metamorphosis is triggered by, in most cases, contact with certain physical or chemical cues that correspond with a favourable environment for juvenile development (Thorson, 1950; Giese & Pearse, 1974; Starr *et al.*, 1990; Young, 1995; Pechenik, 1999; Pradillon *et al.*, 2007). These planktonic larvae of marine benthic invertebrates comprise the majority of the meroplankton, which includes a variety of taxa and forms such as the planktotrophic and lecithotrophic larvae of both benthic and nektonic species, planktonic eggs, and medusae (Marcus & Boero, 1998; Pechenik, 1999). Meroplanktonic organisms are what can be considered temporary plankton in that they only spend a portion of their life-cycles in the plankton.

The survival and successful development of meroplanktonic larvae into adults is affected by numerous factors including adult fecundity and fertilisation success as typified by the creation of embryos and larvae, growth and larval stage duration, behaviour, dispersal (Bhaud, 2000) and settlement (Eckman, 1996). Larval mortality is

also influenced by multiple factors, including predation, inter- and intra-specific competition for food or space, disease, parasites, and various physiological stresses such as temperature (Kirby *et al.*, 2008), and salinity (Eckman, 1996; Todd, 1998). A warmer sea surface temperature will shorten larval development time (Hoegh-Guldberg & Pearse, 1995) and therefore increase survival where food supply is not limiting (Reitzel *et al.*, 2004).

Between 55-85% of all benthic marine invertebrate species produce long-lived planktotrophic larvae while 5% and 10% produce short-lived planktotrophic and lecithotrophic larvae respectively (Thorson, 1950). Planktotrophic larvae have a varying planktonic lifespan, from weeks to months (Thorson, 1950; Belgrano *et al.*, 1995b; Pedersen *et al.*, 2008), and a relatively low energetic production cost per larva (Thorson, 1950; Pedersen *et al.*, 2008). As a result of this low energy cost, those species that release planktotrophic larvae are able to produce a larger number of small eggs in comparison with lecithotrophic species, which produce fewer larger eggs (Thorson, 1946; 1950; Levin *et al.*, 1987; Levin *et al.*, 1997); these hatch to become larvae that only exist for hours to days in the water column. Therefore, planktotrophic larvae are very common in the life histories of many benthic species (Morgan, 1995) and, as a result of their longer residence time, have a higher dispersal potential and can travel over longer distances thus encountering a greater range of potential habitats for settlement than lecithotrophic larvae (Livi, 2006). This potential, while considered by some to be the dominant factor in determining the success of a species with a planktonic larval stage (Bhaud, 2000), does result, however, in a higher mortality rate as a consequence of predation whilst in the water column (Thorson, 1950; Levin *et al.*, 1997; Pechenik, 1999). The evolution of a highly dispersive larval stage has several advantages to the success of benthic invertebrate species (Luttikhuizen *et al.*, 2003). It

reduces competition for food among siblings and between parents and offspring, increases the likelihood of recolonisation following local extinctions (Barnay *et al.*, 2003), allows a large geographical range for a species, reduces the likelihood of inbreeding, and facilitates a greater species persistence in geological time (Pechenik, 1999). The transition to the benthos (or settlement) is a vital part of the life cycle of meroplanktonic organisms, and can play an important role in structuring local populations (Underwood & Fairweather, 1989).

The nourishment of meroplanktonic larvae is directly dependent upon the existing plankton community and the release of larvae is often timed to correspond with phytoplankton blooms (Barnes, 1962) in order to maximise the exposure to an abundant food supply (Thorson, 1946). This synchronicity often leads to meroplanktonic larvae becoming the dominant members of the coastal zooplankton community during the reproductive season of benthic organisms (Thorson, 1946; Williams & Collins, 1986; Martin *et al.*, 1996). In the Bay of Blanes in the NW Mediterranean, for example, meroplanktonic larvae generally account for around 13% of the total zooplankton throughout the year, but at certain times they can contribute up to 60% of community biomass (Andreu & Duarte, 1996; Martin *et al.*, 1997). The potential impact of the meroplanktonic larvae on microbial holoplankton communities has been demonstrated with studies of the grazing effects of polychaete larvae on heterotrophic nanoflagellates revealing that such an impact could be similar to that of normal zooplankton grazing pressure (Martin *et al.*, 1996). The results achieved strongly support the theory that meroplanktonic larvae have an important trophic role both in the direct control of microbial holoplanktonic populations, and as a source of cascading effects in the microbial planktonic food webs.

Meroplanktonic larvae initially feed as herbivores before turning omnivorous and ultimately carnivorous as they develop, e.g. decapod zoea metamorphosing into megalopa (Williams & Collins, 1986). This is likely to be due to their changing energy demands as they grow and, as their mouthparts develop, they are able to consume larger and more robust food items (Cox & Bruce, 2003; Cox & Johnston, 2003). The relationship between the spring phytoplankton bloom and the spawning of meroplanktonic larvae has been studied extensively and examples of both direct and indirect induction of spawning in benthic species as a consequence of either blooms or those environmental conditions that trigger blooms have been reported. For example, certain species of barnacle and spider crab spawn upon direct contact with phytoplankton cells (Starr *et al.*, 1991; 1993; Starr *et al.*, 1994; Andreu & Duarte, 1996) and the Green Sea Urchin, *Stronglycentrotus droebachiensis* (Müller), and the Blue Mussel, *Mytilus edulis* (Linnaeus), both undergo spawning upon detection of a heat-stable metabolite released by certain phytoplankton species (Starr *et al.*, 1990; 1992).

In order to better understand the particular importance of larval and benthic dynamics to the spatial and temporal distribution, abundance, and population structure of any given species, it is essential to consider both pelagic larval and adult benthic ecology (Eckman, 1996). There is a vast library of research that has been carried out on adult benthic organisms due to the relative ease in which they can be studied, whereas there is a distinct lack of development in our understanding of the corresponding planktonic larval phases due to the difficulties associated with actually studying them (Eckman, 1996). The research conducted on meroplanktonic larvae has also been skewed. There has been a much larger focus on the larvae of decapod crustaceans, especially those living in coastal or estuarine environments than the larvae of other phyla. This is partly due to the size of the larvae and the relative ease of identifying them using

morphological characteristics (Young, 1995). Recent studies have attempted to examine the larvae of numerous bivalve species (Hubert & Hedgecock, 2004, Webb et al, 2006, Taylor et al, 2007, Sawada, 2008) through the use of molecular tools.

1.1.2. Larval transport, dispersal, and distribution in the water column.

The role of dispersal is fundamental in determining population structure in the marine environment (Weersing & Toonen, 2009). Most meroplanktonic larvae may occupy the pelagic environment for a few days or weeks, although some may remain planktonic for several months. When compared with other planktonic organisms, some meroplanktonic larvae such as the megalopal stages of decapods have strong swimming abilities (Chia *et al.*, 1984; Luckenbach & Orth, 1992; Fernandez *et al.*, 1994). They do, however, have to rely on oceanographic processes such as tides and currents for large-scale transport (Butman, 1987; Shanks, 1995) as their swimming capabilities are often exceeded by tidal stream velocities (Lee *et al.*, 2004) and therefore they are unable to swim large distances towards the shore. Such processes however, are variable and can deliver larvae to near-shore habitats, or too far offshore. This can lead to the loss of larvae and contribute substantially to spatial and temporal variation in larval supply (Roughgarden *et al.*, 1986; Lee *et al.*, 2005).

Larval dispersal is affected by both the biological properties of the larva and by a series of physical and chemical processes (Thiébaut *et al.*, 1998). Hydrodynamic processes such as local tidal streams, currents, and turbulence all have an impact on larval mortality (Pedersen *et al.*, 2008). The effects of wind upon larval dispersal can be numerous as wind-driven currents can alter the normal direction of larval transport across a given area. This would cause larvae to be carried away from favourable

habitats for settlement, which can lead to poor recruitment for a given year in a particular area (Belgrano *et al.*, 1995a). Multiple spawning events can, however, overcome these effects and many species employ this strategy (Thiébaut *et al.*, 1998).

While it appears that larvae drift passively with the prevailing transporting processes, it has been shown that they demonstrate the ability to regulate their vertical position in the water column and influence their final destination (Metaxas, 2001; Cox & Bruce, 2003). Upon reaching coastal areas, where tides and other hydrographical processes are more pronounced, it is of particular importance, in terms of local-scale distribution, for larvae to exhibit behaviour which synchronises with transport processes (Young, 1995). Selective tidal-stream transport (STST) occurs where horizontal transport is achieved by migrating vertically in and out of the water column in synchronisation with the direction of tidal currents. This is one mechanism that is utilised frequently by invertebrates and fishes to allow them to return to shallow coastal areas or estuaries (Forward & Tankersley, 2001). Those larvae that have developed offshore need to move up in the water column during flood tides in order to move closer to the shore and, to ameliorate the effects of ebb-tide currents, they must migrate to the bottom. This has been demonstrated in studies on *Callinectes sapidus* (Rathbun) (Forward *et al.*, 2003a; Forward *et al.*, 2003b; Lee *et al.*, 2005).

Experimental and descriptive studies, both in the field and the laboratory, have shown that invertebrate larvae exhibit spatial distribution patterns in accordance with physical features in the water column. These include haloclines (well defined vertical layers of differing salinity), thermoclines (vertical layers of differing temperature), pycnoclines (vertical layers of differing water density) and fronts (separate regions of warm and cold water) (Metaxas, 2001). Biological features in the water column such as areas of

increased concentration of chlorophyll *a*, and their effects on the distributional patterns of invertebrate larvae, however, have received little attention either in the field or in the laboratory. It is known that an increase in phytoplankton can lead to an increase in meroplankton abundance (Kirby *et al.*, 2008). There is little field evidence, however, to suggest that spatial aggregations of invertebrate larvae may coincide with areas of increased concentration of phytoplankton, mainly near pycnoclines and fronts (Metaxas, 2001), but it has already been demonstrated that benthic organisms time the release of larvae and reproductive products to coincide with phytoplankton blooms (Barnes, 1962; Martin *et al.*, 1996).

For marine benthic invertebrates with meroplanktonic life cycles, the importance of the role played by larval supply in determining the spatial distribution of adult populations has been well established (Gaines *et al.*, 1985; Metaxas, 2001). It is also thought that the factors affecting the supply and successful settlement of meroplanktonic larvae to the benthos are very important in determining both population and community structure and may even supersede the effects of post-settlement processes such as predation, competition, and physical disturbance (Todd, 1998). Although there have been considerable advances in the understanding of planktonic larval stages since the 1970s, the relative importance of those factors that determine survival and dispersal while larvae are present in the plankton remains poorly documented (Young, 1995).

1.2. LARVAL IDENTIFICATION.

1.2.1. Morphological identification

The identification of the larvae of benthic invertebrates is often a labour intensive process (Garland & Zimmer, 2002). There are many difficulties associated with the use of morphological characteristics examined by light microscopy (Livi, 2006; Sawada *et al.*, 2008; Wight *et al.*, 2009). The scarcity of distinct morphological taxonomic characters among larvae often hinders their identification to species level. This is particularly prevalent in the examination of bivalve larvae as the early larval stages of numerous different species often exhibit very similar morphological features regardless of whether they are distantly related or not (Livi, 2006). Environmental variables and phenotypic plasticity observed during their developmental stages can also reduce the level of taxonomic resolution possible (Medeiros-Bergen *et al.*, 1995; Webb *et al.*, 2006; Sawada *et al.*, 2008).

Until recently, it has only been possible to identify species with any degree of reliability via direct microscopic observation and this method is only suitable for those species with obvious morphological differences between their larvae and developmental stages (Garland & Zimmer, 2002; Webb *et al.*, 2006). It has often been the case with many of the youngest larval stages to either group them into supra-specific assemblages or to ignore them completely, which can severely reduce the suitability of any data for assessing patterns of abundance or seasonality within a given species or assemblage (Garland & Zimmer, 2002).

In order for definitive identification of larval forms to be accomplished using morphological characters, a detailed examination of those characters is required. This is often problematic given that many of the most relevant taxonomic keys were produced in the 1920s, 1930s, and 1940s (Lebour, 1927; 1928a; b; 1930; 1931; 1936; 1937a; b; 1938; 1940; 1943; 1944; 1947) and tend to focus mainly on those groups that can be identified more easily than others, such as crustaceans, whereas there are very few published descriptions on bivalve larvae for instance (Garland & Zimmer, 2002). The accuracy of morphological analysis is often dependant on the level of expertise shown by the taxonomist. Loss of both accuracy and precision can often result when several taxonomists make identifications on a given sample or samples from a given region, as well as a degree of subjectivity being adopted (Garland & Zimmer, 2002). The investigation of larval supply patterns is important in understanding the ecology of benthic communities as a whole (Underwood & Fairweather, 1989), but since this fact was revealed, little progress has been made in the identification of larvae to a higher taxonomic resolution (Webb *et al.*, 2006).

1.2.2. Molecular identification.

In recent years, the use of molecular methods in taxonomy has been feted as a possible solution for overcoming the difficulties in larval identification. This approach has provided considerable advances in the taxonomic field and has been mirrored by a decline in the use of conventional morphological examination and thus its associated skill base (Webb *et al.*, 2006). Molecular tools can facilitate the identification of larvae, especially bivalves, to species level regardless of their developmental stage thus alleviating at least a portion of the ambiguity and subjectivity associated with traditional microscope-based taxonomy (Garland & Zimmer, 2002). They are considered by some

to be the only solution in the provision of sustainable identification capability (Hebert *et al.*, 2003a). However, the technology required for molecular techniques is more expensive and less accessible than a light microscope. Molecular techniques, as is the case with morphological methods, also require specimens that are directly sampled from the field to be reasonably undamaged in order to provide sufficient genetic material for analysis (Garland & Zimmer, 2002).

Polymerase Chain Reaction (PCR) has been at the forefront of these advances and forms the basis of numerous techniques that are employed today in the identification of marine larvae. These include: PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Corte-real *et al.*, 1994), multiplex PCR (Hare *et al.*, 2000; Larsen *et al.*, 2005; Pan *et al.*, 2008), quantitative PCR (Vadopalas *et al.*, 2006; Wight *et al.*, 2009), PCR Single Strand Conformation Polymorphism (SSCP) (Livi, 2006), DNA barcoding (Hebert *et al.*, 2003a; Webb *et al.*, 2006), and microsatellite analysis (Hubert & Hedgecock, 2004). Other methods have been reported that do not utilise PCR including the use of species-specific oligonucleotide probes (Bell & Grassle, 1998; Le Goff-Vitry *et al.*, 2007b), and in-situ hybridisation (Le Goff-Vitry *et al.*, 2007a; Pradillon *et al.*, 2007).

The use of species-specific primers during PCR has served to amplify species-specific size products from the mitochondrial Cytochrome oxidase I gene of individual larvae in order to distinguish between different marine bivalve species (Hare *et al.*, 2000). Through the application of one or more of the methods listed above, it has been possible to identify the meroplanktonic larvae of a wide range of benthic organisms including: penaeid shrimps (Palumbi, 1991; Nunan *et al.*, 2000), various bivalve species (Garland & Zimmer, 2002; Hubert & Hedgecock, 2004; Larsen *et al.*, 2005; Webb *et al.*, 2006;

Sawada *et al.*, 2008), porcellanid crabs (Werding *et al.*, 2001), several polychaete species e.g *Lagis koreni* (Malmgren) (Jolly *et al.*, 2004), thoracican barnacles (Harris *et al.*, 2000), and various brachyuran crab species (Schubart *et al.*, 2001; Pan *et al.*, 2008).

Three genes are generally used for the identification of bivalves using molecular methods: the mitochondrial ribosomal gene, 16S (Svedberg units) rRNA (Livi, 2006), the small subunit nuclear ribsomal 18S rRNA (Meyer *et al.*, 2010), and the mitochondrial Cytochrome-c oxidase subunit I DNA (mtCOI) (Bell & Grassle, 1998; Hare *et al.*, 2000; Garland & Zimmer, 2002). Small subunit nuclear rRNA is suitable for phylogenetic analyses for a number of reasons: it is (a) universal, (b) conservative i.e. in terms of the similarity and length of the nucleic acid sequence, (c) reasonably simple to sequence, (d) functionally constant, and (e) of a sufficient size to yield usable data for analysis (Kim & Abele, 1990). The structure of nuclear rRNA sequences is suitable for use in phylogenetic studies as their highly conserved flanking regions allow for the use of universal primers and, due to their repetitive arrangement within the genome, allow for the provision of plentiful quantities of template DNA for PCR purposes (Meyer *et al.*, 2010).

Despite being less conserved and displaying higher mutation rates than nuclear DNA, mitochondrial DNA is used extensively as it is easy to amplify (Galtier *et al.*, 2009) due to its appearance in multiple copies within a cell, and is highly variable in natural populations due to the higher mutation rate (Hare *et al.*, 2000; Garland & Zimmer, 2002; Livi, 2006). While mitochondrial DNA possesses remarkably stable gene order and content, variation can occur in the non-coding or “control” regions and this makes mtDNA useful for phylogenetic studies of species and populations, while nuclear DNA is more suitable for studies of genera and families (Garland & Zimmer, 2002).

1.2.2.1 Mitochondrial 16S rRNA.

The mitochondrial ribosomal 16S rRNA gene is often used in phylogenetic and taxonomic investigation. It has been used to examine phylogenetic relationships in: several decapod species (Schubart *et al.*, 2001; Porter *et al.*, 2005), thoracican barnacles (Perez-Losada *et al.*, 2004), several oyster species including *Crassostrea gigas* (Thunberg) (O'Foighil *et al.*, 1995), and used extensively on calanoid copepods (Lindeque *et al.*, 1999; Lindeque *et al.*, 2006).

There has been mixed success when using the 16S gene for phylogenetic analysis. Studies on the 16S mitochondrial ribosomal gene in certain bivalves have reported the construction of oligonucleotide probes for use as primers in PCR. It was shown that these probes did not distinguish between different species in the same family (O'Foighil *et al.*, 1995). Subsequent two-step PCR-restriction fragment length polymorphism (RFLP) analysis for species-specific discrimination was used in these studies and allowed some restriction fragments to be obtained, thus indicating a difference of at least one base pair in the targeted species (Garland & Zimmer, 2002). It has been suggested that, as a result of this one base pair difference, the Ligase chain reaction (LCR) could lead to more successful results. LCR uses two primers that are only ligated together when they occur adjacently and only requires a single base pair difference in order to distinguish between two species (Garland & Zimmer, 2002).

1.2.2.2. Small subunit nuclear 18S rRNA.

The 18S rDNA gene is one of the most important molecular markers, especially for use in random target PCR in environmental biodiversity screening (Meyer *et al.*, 2010). It

displays a high level of conservation and is represented by multiple copies that are repeated in tandem in the nuclear genome (Livi, 2006). It exhibits a high degree of homogeneity within species and this can be explained by gene conversion and unequal crossing over, which is an exchange of genetic material between homologous chromosomes (Creighton & McClintock, 1931). The 18S gene is a remarkably useful marker for examining phylogenetic relationships at higher taxonomic levels due to the high level of conservation that it possesses (Kim & Abele, 1990). 16S and COI have a higher variability than 18S and this can be a limiting factor during the identification of priming sites over a wide taxonomic range; therefore 18S has the advantage in certain phylogenetic studies and DNA bar-coding (Hare *et al.*, 2000).

An example of the use of the 18S rRNA gene was reported by Bell & Grassle (1998). Probes were developed for use as primers and targeted a particular sequence within nuclear 18S rRNA gene. This sequence was specific for bivalves of the family Mactridae and was used to discriminate between *Spisula solidissima* (Dillwyn) and *Mulinia lateralis* (Say). This was accomplished by a two-step PCR- RFLP that led to the production of fragments of varying length that were then used to differentiate between the two species (Garland & Zimmer, 2002). The 18S rRNA gene has been used to differentiate between numerous molluscan species (Rice *et al.*, 1993; Winnepenninckx *et al.*, 1996; Adamkewicz *et al.*, 1997; Giribet & Wheeler, 2002; Larsen *et al.*, 2005; Livi, 2006; Taylor *et al.*, 2007; Meyer *et al.*, 2010), thoracican barnacles (Harris *et al.*, 2000), polychaetes (Colgan *et al.*, 2006)`, and decapod species (Kim & Abele, 1990).

Recent studies have developed an *in situ* hybridisation (ISH) technique that is non-destructive to the species in question (Le Goff-Vitry, 2007a). This tehnique utilised

oligonucleotide probes specific to the 18S ribosomal RNA gene in order for marine larvae to be identified. As it leaves the larvae intact, it allows for the description of larvae possessing previously unknown morphology. It displays nearly 100% efficiency as it requires only one mismatch between the rRNA sequences of the target and non-target species in order to discriminate at the species level (Pradillon et al., 2007).

1.2.2.3. Mitochondrial Cytochrome-c oxidase subunit I DNA (mtCOI).

The mitochondrial Cytochrome-c oxidase subunit I DNA (mtCOI) has been shown to be useful at in facilitating species resolution (Simon-Bouhet et al., 2006). The mitochondrial genome as a whole acts like a single, non-recombining locus as its inheritance is maternal, i.e. clonal, which allows for the representation and analysis of within-species variation to be vastly simplified (Galtier *et al.*, 2009). The mtCOI gene is typified by a series of variable regions flanked by highly conserved sections e.g. the control region interspersed with ribosomal DNA in which PCR primers can be designed (Galtier *et al.*, 2009).

The COI gene has two important advantages for its use as a molecular marker: (a) the universal primers for COI are remarkably robust (Folmer *et al.*, 1994; Hebert *et al.*, 2003a) in that their 5' ends can be recovered in most animal phyla, and (b) they possess a greater range of phylogenetic signal than any other mitochondrial gene (Hebert *et al.*, 2003a). This has made the COI gene more useful for broad taxonomic studies than other mitochondrial genes such as 12S and 16S, which are hindered by insertions and deletions that can complicate sequence alignments (Herbert *et al.*, 2003a). Such is the potential of the COI gene in its use as a tool for taxonomic identification that it is acting

as the core of a global bio-identification system for animals known as the Barcode of Life (Hebert *et al.*, 2003a).

Hare *et al*, (2000) developed a single-step multiplex PCR identification assay that was used to differentiate between five species of bivalve, both accurately and efficiently, based on the size of COI products. They eschewed the use of DNA extractions and restriction digestions and developed primers in order to amplify species-specific size products from mtCOI of individual larvae (Garland & Zimmer, 2002). This assay was found to be very efficient as it uses a single molecular step to discriminate multiple target species and, as such, can be automated to process large numbers of larvae (Hare *et al.*, 2000).

For studies involving decapods, mtCOI and the large subunit ribosomal RNA (LsurRNA) genes are more commonly used than all other mitochondrial genes. The mtCOI gene has been used to identify many species of decapod such as *Liocarcinus depurator* (Linnaeus) (Pan *et al.*, 2008), *Marsupenaeus japonicus* (Bate) (Yamauchi, 2005), numerous species of bivalve (Phillips *et al.*, 2008), polychaetes (Jolly *et al.*, 2004), and echinoderms (Webb *et al.*, 2006). This is thought to be due the early availability of the previously mentioned "universal" primers developed by Folmer in 1994 (Yamauchi, 2005).

1.2.2.4. Other genes.

Numerous studies have used other genes when attempting to differentiate between marine species. Mussel species in particular are often analysed using the nuclear Me15/16 DNA marker (Hilbish. *et al.*, 2002; Śmietanka *et al.*, 2004; Coghlan &

Gosling, 2007; Gosling *et al.*, 2008; Kijewski *et al.*, 2009). Other genes have been examined for use in identifying polychaete families. For example, along with existing 18S rRNA data, four additional gene sequences were studied: histone H3, snU2 RNA, and the D1 and D9-10 expansion regions of 28S rRNA. This allowed the creation of a data set that included 38 new sequences from 21 species and covered a wide range of polychaete diversity (Colgan *et al.*, 2006). As previously mentioned, the 18S rRNA nuclear ribosomal gene and the mitochondrial coding gene, mtCOI, are often used in examining bivalve classification, but some studies (Giribet & Wheeler, 2002) have also focused on a further nuclear ribosomal gene: 28S rRNA.

1.3. TEMPORAL DISTRIBUTION OF LARVAE IN AND AROUND PLYMOUTH SOUND.

Prior to investigation of the current patterns of species composition and abundance of the meroplankton community of the Plymouth Sound area, it is important to to examine previous records to gain an accurate picture of the historical meroplankton community that allows for any comparison to be made. The following section represents a summary of meroplanktonic larvae recorded in and around Plymouth Sound and their occurrences throughout the year. The term “inshore plankton” refers to the area of Plymouth Sound within the confines of the breakwater and “outside” refers to the waters beyond the breakwater. The summary is taken from observations by Marie Lebour (Lebour, 1927; 1928a; b; 1930; 1931; 1936; 1937a; b; 1938; 1940; 1943; 1944; 1947) and the Plymouth Marine Fauna (Marine Biological Association of the United Kingdom, 1957). This data was converted into an abundance scale of 1-3 to allow comparison with current data and

kite diagrams showing the taxa which were comparable were constructed (Fig. 1.1. & 1.2).

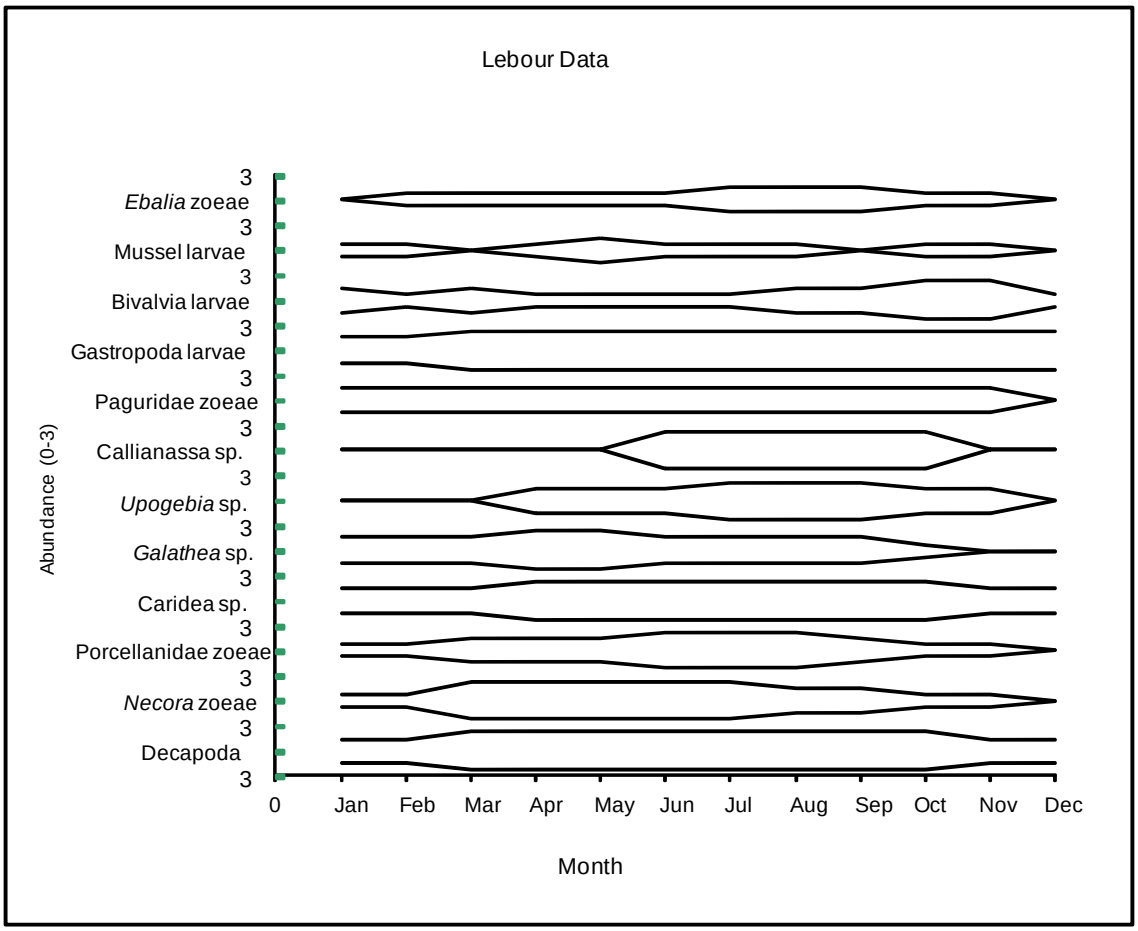

Figure. 1.1. Kite diagrams showing the average seasonal abundance of the major comparable taxa derived from the historical data taken from Lebour (1947). Abundance scale is 0-3 with 0 = Absent, 1 = Rare, 2 = Common, and 3 = Abundant.

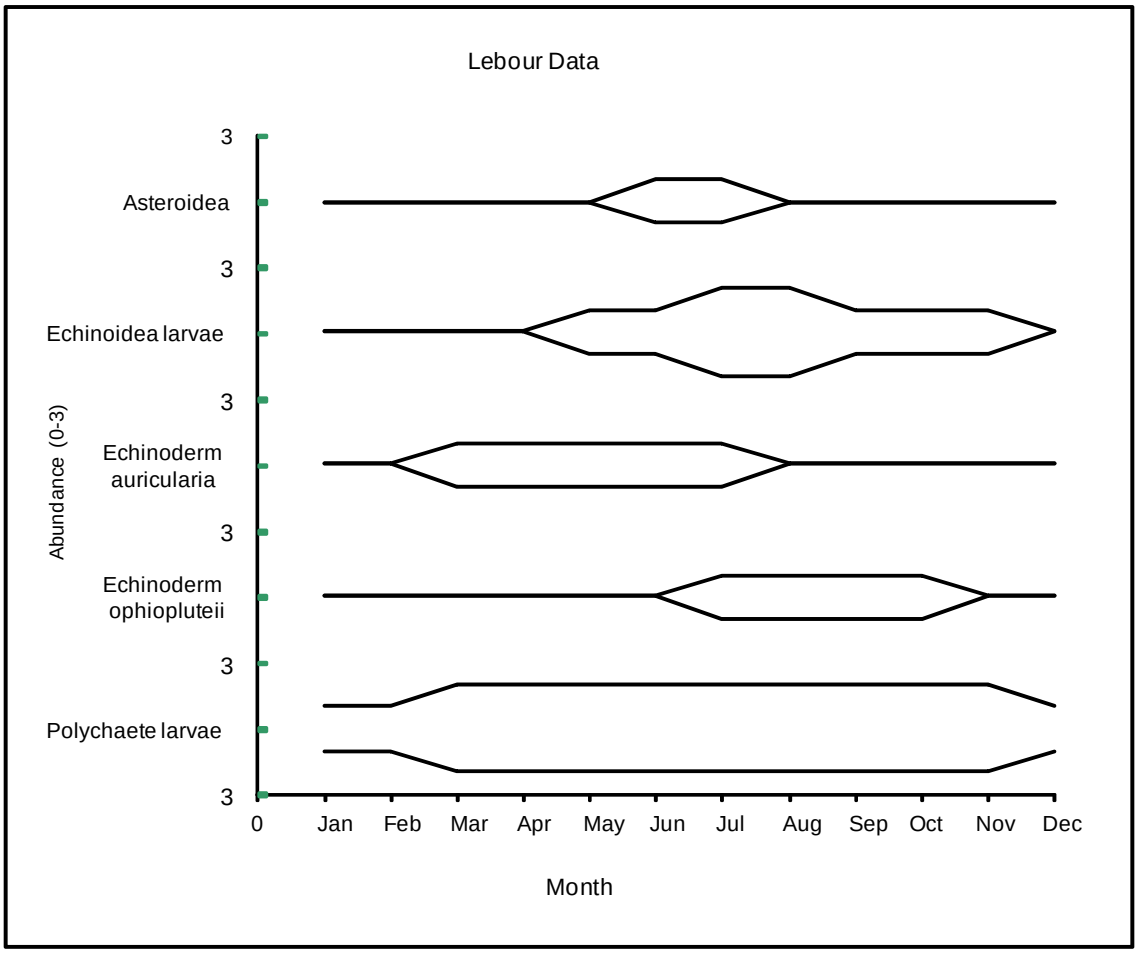

Figure. 1.2. Kite diagrams showing the average seasonal abundance of the major comparable taxa derived from the historical data taken from Lebour (1947). Abundance scale is 0-3 with 0 = Absent, 1 = Rare, 2 = Common, and 3 = Abundant.

1.3.1. Crustacea.

The Shore Crab, *Carcinus maenas* (Pennant), is known to breed throughout the year with larvae recorded in the plankton in every month with the largest numbers being from January to September and the fewest numbers from October to December. The Harbour Crab, *Liocarcinus depurator* (Linnaeus), is known to breed in all months with there being few larvae in January and February before becoming numerous from March through to September and then dwindling markedly in October to December. They were, however, less abundant inshore than the zoeae of *C. maenas*. The Dwarf

Swimming Crab, *Liocarcinus pusillus* (Leach), has been recorded in the plankton from January to July. However, records are, in all likelihood, incomplete, owing to the similarities between small portunid zoeae causing difficulties in identification and resulting in many unidentified specimens not being recorded.

The Velvet Swimming Crab, *Necora puber* (Linnaeus), is already known to breed predominantly in spring and summer with records showing larvae present in the plankton from January to November. However, those in autumn and winter are rare. The largest numbers of zoeae occurred from March to July. The Flying Crab, *Liocarcinus holsatus* (Fabricius), has been recorded in spring and summer with zoeae being recorded from May to June, usually in small numbers. The Marbled Swimming Crab, *Liocarcinus marmoreus* (Leach), exhibited similar patterns with larvae also being recorded in spring and summer. The zoeae have been recorded from March to June and in October, again usually in small numbers. Larvae of Pennant's Swimming Crab, *Portumnus latipes* (Pennant), were found occasionally in the inshore plankton from June to September.

The Edible Crab, *Cancer pagurus* (Linnaeus), is known to breed in spring and summer with its larvae being present in the inshore plankton from April to November. They occurred in their highest numbers in May and July but were occasionally found in November. The Circular Crab, *Atelecyclus rotundatus* (Olivi) usually occurred in the plankton from April to August. Records show that it could be present as early as February and as late as November, but it generally occurred from March to September with the largest numbers being from May to July. The Masked Crab, *Corystes cassivelaunus* (Pennant), is one of the earliest zoeae in the plankton with records showing that it occurred from January to June. It was never particularly numerous close

inshore however, but could occur in very large numbers in the outside waters. The Long- and Broad-clawed Porcelain Crabs, *Pisidia longicornis* (Linnaeus) and *Porcellana platycheles* (Pennant), were both seen in the inshore plankton in the Sound. *P. longicornis* occurred every month except December, gradually increasing through the spring before becoming most abundant during the summer and then decreasing in the autumn and winter. *P. platycheles* has a more restricted season than that of *P. longicornis* and occurred from April to September and were found in its highest numbers in June.

The larvae of the genus *Xantho* were not often distinguished to the species level and were rarely seen in the plankton, occurring in the summer months only. The zoeae of the Hairy Crab, *Pilumnus hirtellus* (Linnaeus), occurred in the inshore plankton from May to November with the largest numbers being recorded in July, although they were never very abundant. The Polished Crab, *Thia scultellata* (Fabricius), is rare and only the adult is found in outside waters. Zoeae were recorded in the inshore plankton during August and September. The zoeae of the Angular Crab, *Goneplax rhomboides* (Linnaeus) were seen in the inshore plankton from June to September with the highest numbers being recorded in July. They are never very numerous. While they are almost certainly the Pennant's Nut Crab, *Ebalia tuberosa* (Pennant), the zoeae of *Ebalia* species are not often distinguished from one another. They occurred in the inshore plankton from February to November with the highest numbers recorded from July to September, although rarely in large numbers.

Five species of spider crab have been recorded in and around Plymouth Sound. The Common Spider Crab, *Maja squinado* (Herbst), generally occurs from April to October, although, its zoeae were rarely seen in the inshore plankton. The Strawberry Crab,

Eurynome aspersa (Pennant), was more abundant outside the Sound rather than in the inshore plankton between July and October. The zoeae of the Contracted Crab, *Hyas coarctatus* (Leach), were generally seen in the spring, albeit rarely. Species of *Inachus*, which were in all likelihood, the Scorpion Spider Crab, *Inachus dorsettensis* (Pennant), were only identified to genus and occurred from May to October. They were much more numerous outside Plymouth Sound than in the inshore plankton. As with *Inachus*, species of *Macropodia* were not identified to species, although they were most likely predominantly those of the Long-legged Spider Crab, *Macropodia rostrata* (Linnaeus). Their zoeae were found in every month throughout the year in low numbers.

Three species of squat lobster have been observed: *Galathea squamifera* (Leach), *Galathea strigosa* (Linnaeus), and *Munida rugosa* (Fabricius). The zoeae of *G. squamifera* were among some of the earliest larvae in the inshore plankton and occurred inshore from January to October, being most numerous in April and May which is usually the maximum month for breeding. *G. strigosa* were found in the inshore plankton from January to October, and, like *G. squamifera*, were most common between April and May. They were, however, less common than *G. squamifera* and probably occurred in higher numbers outside Plymouth Sound. The larvae of *M. rugosa* were more commonly found in the waters outside of the Sound but did occasionally occur in the inshore plankton.

Pagurid larvae were not often identified to species in the observations made by Lebour, but were probably the Common Hermit Crab, *Pagurus bernhardus* (Linnaeus), *Pagurus prideauxi* (Leach), and *Anapagurus laevis* (Bell). They were found from January to November in the inshore plankton although never in large numbers. The larvae of the Common Lobster, *Homarus gammarus* (Linnaeus), were usually infrequent, generally

occurring singly and were found in the inshore plankton from June to September. The phyllosoma larvae of the European Spiny Lobster, *Palinurus elephas* (Fabricius), were found abundantly in the plankton outside of Plymouth Sound from February to September but only occurred infrequently in the inshore waters from June to August.

Those species of Thalassinidea whose larvae were found in Plymouth Sound and the surrounding areas are: *Axius stirhynchus* (Leach), *Jaxea nocturna* (Nardo), the Burrowing Mud Shrimp *Callianassa subterranea* (Montagu), *Upogebia deltaura* (Leach), and *Upogebia stellata* (Montagu). *A. stirhynchus* occurred from May to October but was most numerous in July. The larvae of *J. nocturna* were usually found outside the Sound in the summer months and were rarely seen in the inshore plankton. *C. subterranea* was very common in the summer and early autumn and was found inshore between June and October. The larvae of *U. deltaura* and *U. stellata* were known to be very common in spring and summer. *U. stellata*, however, breeds earlier than the former and can be found in the inshore plankton from April to November, being most common from July to September.

There are a wide variety of caridean species whose larvae can be found in the plankton in and around Plymouth Sound throughout the course of the year. The larvae of *Athanas nitescens* (Montagu) can be found in the plankton from June to October and were most common in July through to September. *Alpheus ruber* (Milne-Edwards) occurred fairly frequently in the inshore plankton from June to October, albeit in small numbers. The larvae of the Brown Shrimp, *Crangon crangon* (Linnaeus), were present in the plankton from January to November and were especially common from April to August before decreasing in the autumn. *Crangon allmanni* (Kinahan) on the other hand, is predominantly found outside Plymouth Sound and has only been recorded very

infrequently in the inshore waters. Its larvae were usually found earlier in the year between February and April.

Three species of *Philocheras* have been recorded: *P. fasciatus* (Risso), *P. trispinosus* (Hailstone), and *P. bispinosus* (Hailstone). The larvae of *P. fasciatus* have been recorded in small numbers in the inshore plankton from May to September. *P. trispinosus* is aspecies found outside the Plymouth breakwater and its larvae occurred in spring and summer. *P. bispinosus* could be found inshore from March to October. The largest numbers of the Chameleon Prawn, *Hippolyte varians* (Leach), were recorded in September and October, although the larvae were present in the inshore plankton throughout the year. While the species of *Eualus* were not distinguished, they are thought to include *Eualus cranchii* (Leach) and *Eualus occultus* (Lebour), and occurred from January to November in the inshore plankton. They were most numerous from August to October.

The larvae of *Caridion steveni* (Lebour) occurred in small numbers in the plankton from April to June and in October. The Pink Shrimp, *Pandalus montagui* (Leach), occurred commonly in the outside waters in spring and summer and in the inshore plankton from February to October. Larvae of *Pandalina brevirostris* (Rathke) were very common in spring and summer and occurred from January to October in the inshore waters with the largest numbers being in July. *Processa edulis crassipes* (Nouvel & Holthuis) and *Processa canaliculata* (Leach) were both common in large numbers in the outside plankton from April to September with *P. edulis crassipes* extending into October and being more numerous within the inshore plankton.

There are two species of *Palaemon* whose larvae were found within Plymouth Sound: the Common Prawn, *Palaemon serratus* (Pennant), and *Palaemon elegans* (Rathke). *P. serratus* was found from January to October, and was most abundant in June to August. *P. elegans*, however, occurred in lower numbers from May to September. Finally, *Nyctiphanes couchii* (Bell), a euphasiid, was generally found outside the Breakwater only. Its nauplii occurred in small numbers in May and June, and its calyptopis and early furcilia were found in January, March to June, and September to November.

A summary of the occurrences of crustacean larvae in the plankton in and around Plymouth Sound can be found in Appendix 1.

1.3.2. Mollusca.

There are many difficulties inherent in identifying mollusc larvae due to their similarity so that, especially in the case of bivalve larvae, it is often almost impossible to recognise individual species (Lebour, 1947). Gastropod and bivalve larvae occur throughout the year in the inshore waters but are generally more abundant in the spring and summer months. However, it has been noted that a large number of bivalve larvae appear in the autumn. The following is a summary of those species that have been recorded within Plymouth Sound taken from the observations derived from laboratory culture and field sampling by Lebour and those of the Plymouth Marine Fauna (Lebour, 1947, Marine Biological Association of the United Kingdom, 1957).

Larvae of the Common Limpet, *Patella vulgata* (Linnaeus), predominantly occurred during the autumn and winter months from September to April with numbers reaching their maximum in December and January. The Blue-rayed Limpet, *Helcion pellucidum*

(Linnaeus), never occurred in large numbers and was generally found from August to February. The egg capsules of the Common Periwinkle, *Littorina littorea* (Linnaeus), were very common in the inshore plankton throughout the year and February and March seemed to comprise the principal breeding season. However, while the maximum numbers were generally found in March, they were sometimes plentiful from January to June and from August to December. The larvae were frequently abundant in most months. The Small Periwinkle, *Melarhaphe neritoides* (Linnaeus), is considered to be a winter and early spring breeder with its egg capsules being found from December to April and also in October.

Rissoid larvae, of which *Rissoa parva* (da Costa) are predominant, are not often distinguished from one another. They occurred throughout the year, usually in large numbers, in the inshore plankton and were most abundant from April to December before decreasing from December to January. *Tornus subcarinatus* (Montagu) occurred in the plankton in November to December and only rarely. The larvae of the Arctic Cowrie, *Trivia arctica* (Pulteney), have been recorded from autumn to early spring but never in summer. They occurred in the inshore plankton from January to May, with May being considered exceptionally late. The Spotted Cowrie, *Trivia monacha* (da Costa), on the other hand, was known to breed in late spring and early summer and occurred from April to September, thus possibly overlapping with *T. arctica.* However, it never occurred in the winter. *Lamellaria perspicua* (Linnaeus) was most common in June and July but occurred throughout the year, although never in large numbers. *Simnia patula* (Pennant) is a summer breeder and usually occurred in the waters outside the Sound but could be found in the inshore plankton from June to September. The Necklace Shell, *Polinices catena* (da Costa), is also a summer breeder and could be found inshore from March to October and was most common in June and July.

The larvae of the Needle Whelk, *Bittium reticulatum* (da Costa), were generally only found in July and August. *Triphora perversa* (Linnaeus) usually occurred in the outside waters in spring, summer and autumn although it can be found occasionally inside Plymouth Sound. It never occurred in large numbers and was recorded from July to October. *Cerithiopsis tubercularis* (Montagu) and *Cerithiopsis barleei* (Jeffreys) were both recorded in the inshore plankton. *C. tubercularis* was found in small numbers from June to October in both the inshore and outside waters, whereas *C. barleei* was found predominantly in August. Species of *Caecum*, in all likelihood *Caecum imperforatum* (Kanmacher), were present from July to January and occurred in their largest numbers in November. The Netted Dog Whelk, *Nassarius reticulatus* (Linnaeus), is thought to breed in spring and early summer and its larvae were present from March to October. They were most common from March to June. The larvae of the Thick-lipped Dog Whelk, *Nassarius incrassatus* (Ström), were usually found outside the Breakwater throughout the year especially in spring and summer. They generally occurred in the inshore plankton from April to October and were most abundant from May to August. They were not as common as those of *N. reticulatus*.

Larvae probably belonging to *Haedropleura septangularis* (Montagu) occurred quite frequently in the inshore plankton from May to September with July seeing the highest numbers. *Mangelia nebula* (Montagu) is known to breed in the summer and larvae were found in both the inshore and the outside shallow-water plankton, albeit in small numbers, from July to November. *Comarmondia gracilis* (Montagu) were fairly abundant in the outside waters in spring and summer from May to September and occurred most frequently in July and August. The larvae of *Raphitoma linearis* (Montagu) were reasonably common, both inshore and outside, in spring and summer and were most abundant in July.

Indeterminate bivalve larvae occurred throughout the year, often in large numbers. As has already been mentioned, a vast amount of these larvae occurred in late summer or autumn, especially in September. The larvae of the *Anomia* group have been recorded almost throughout the year, with the possible exception of March and April, and generally comprise a large fraction of the autumn outburst. These larvae were generally thought to belong to *Heteranomia squamula* (Linnaeus), which reaches the height of its breeding in late summer and early autumn and was particularly numerous from August to October.

The Common Mussel, *Mytilus edulis* (Linneaus), breeds in spring and nearly all of the records of mytilid larvae in spring and early summer probably referred to *M. edulis*, but the larval stages of many mytilid species are so similar that it is impossible to be sure of this. The largest numbers were found in May but were present in almost every month except March, September, and December. Another species group that are hard to distinguish from one another are those belonging to the family Pectinidae, which of the 13 known British species, 6 have been recorded from the Plymouth area (Marine Biological Association of the United Kingdom, 1957). These larvae were recorded in their largest numbers in September and October.

The larvae of the Gaping File Shell, *Lima hians* (Gmelin), were already known to occur most frequently in the plankton in late summer and early autumn. While larvae have been recorded during most months in the year, they were found in the largest numbers in October and November. However, the species has been absent from the meroplankton community in recent years. The larvae of *Kellia suborbicularis* (Montagu) are some of the largest and most conspicuous of the lamellibranchs and were common in the

plankton, particularly in spring and summer. *K. suborbicularis* breeds throughout the year and was especially numerous in August and September. Larvae of the genus *Mysella* were found in the inshore plankton in February and from August to October. While not being found in as large a number as in the outside waters, they were most numerous in September. As with mytilids, it is difficult to separate the early stages of different species of *Ensis* (razor shells), although due to the presence of a red pigment behind the siphon in *Phaxas pellucidus* (Pennant), it can be distinguished after the early stages. It breeds in autumn and winter and was common in December. Generally, the plankton in early spring were characterised by the larvae of *Ensis siliqua* (Linnaeus), although larvae of all of the *Ensis* species occurred in the inshore plankton throughout the year.

Larvae of the Wrinkled rock borer, *Hiatella arctica* (Linnaeus), were found in the inshore plankton in every month except December and January and were particularly abundant in September and October and *Teredo* sp. carry larvae throughout the year. Opistobranchs such as *Limacina retroversa* (Fleming), *Clione* sp., and *Doto* sp. were usually found in the plankton during the spring and summer months.

A tabular summary of the occurrence of molluscan larvae in the plankton in and around Plymouth Sound according to the historical data (Lebour, 1947; Marine Biological Association of the United Kingdom, 1957) can be seen in Appendix 1.

1.3.3. Polychaeta.

Many different species of polychaete larvae could be found in the inshore plankton in any month, but generally occurred in the largest numbers in the spring and summer. The most common species recorded included: *Loimia medusa* (Savigny), *Lanice conchilega* (Pallas), *Magelona mirabilis* (Cox & Johnston), and *Poecilochaetus serpens* (Allen), although numerous syllids, nereids, spionids, and sabellarians were often very abundant (Lebour, 1947). The following is a summary of those polychaete species known to occur in the inshore plankton in Plymouth Sound as taken from Lebour (1947). Indeterminate polynoid larvae were found to occur in small numbers in March, April, July, August, and October. Unidentified syllid larvae were recorded in the inshore plankton in June and July, but were not common. Adult males and females with eggs (epitokes) of the genus *Autolytus* were found in almost every month except December and February and were most abundant in the summer.

Unidentified nereid eggs and larvae were found in the largest numbers in the inshore plankton in September and November, although they have been recorded in every other month except June and August. Indeterminate spionid eggs and larvae were found in every month in the inshore plankton except May and September and were most numerous in March and April. Three species from the genus *Magelona* were recorded in the inshore plankton. *M. mirabilis*, one of the most common larvae of the inshore plankton, was found in every month except February and was one of the most abundant larvae in spring and summer, especially from May to July. The other two species could not be identified with any degree of certainty and were found to occur less frequently from April to October.

The larvae of *P. serpens* were most numerous in the spring and summer months, with May seeing the greatest numbers. They can, however, be found in the inshore plankton from February to November. The indeterminate larvae of *Chaetopterus, Arenicola,* and *Pectinaria* sp. were found in small numbers within Plymouth Sound. *Chaetopterus* occurs from August to November, *Arenicola* was seen from March to May and in November, whilst *Pectinaria* has been recorded from April to August and in December. Another of the most common annelid larvae in the inshore plankton, *L. conchilega*, was known to occur every month except January. It was most abundant from April to October and was rarer in the intervening months. *Loimia medusa* was common from January to June in the inshore waters and was most frequent from March to May.

A summary of the seasonal occurrences of polychaete larvae in the plankton in Plymouth Sound and its surrounding waters can be found in Appendix 1.

1.3.4. Echinodermata.

There are a few species of echinoderm whose larvae are known to occur within Plymouth Sound. The most common by far were the ophiopluteii of *Ophiothrix fragilis* (Abildgaard) which were found from February to November and were most abundant from June to September, often in large numbers. The ophiopluteii of other unidentified ophuiroid species occurred occasionally in the inshore plankton from July to October, with August experiencing the highest numbers, although they were never particularly numerous. Unidentified echinopluteus larvae, most likely belonging to *Echinocardium* and *Echinocyamus*, were fairly common in the inshore waters and occurred from May to November. They were most common in the summer, especially in July and August. The

bipinnaria larvae of *Asterias* and the auricularia larvae of unidentified holothurians were found in the plankton in spring and early summer, albeit very rarely.

1.3.5. Other Larvae.

The larvae of various species of Nemertea, Bryozoa, Enteropneusta, and Phoronida were also found in the inshore plankton in the spring and summer months. A table showing the seasonal occurrences of echinoderm and other larvae in the plankton in and around Plymouth Sound can be found in Appendix 1.

1.4. BENTHIC-PELAGIC COUPLING.

Benthic-pelagic coupling can be defined as the linkages between benthic and pelagic environments in aquatic systems (Marcus & Boero, 1998) and covers a wide range of areas: the exchange of larvae to and from the benthos (Pan *et al.*, 2011), the triggering of spawning in benthic organisms in response to pelagic cues (Barnes, 1962; Starr *et al.*, 1991), the composition of the plankton in terms of holoplankton and meroplankton (Kirby *et al.*, 2007), food webs incorporating both pelagic and benthic organisms (Graf, 1989; Gaston *et al.*, 1997), control of nutrient export to the water column (Chauvaud *et al.*, 2000; Vidal & Morgui, 2000), the flow of nutrients to the benthos (Cattaneo-Vietti *et al.*, 1999), and changes in the composition and abundance of the benthic and meroplanktonic communities in response to changing physical and chemical conditions such as sea-surface temperature (Kirby *et al.*, 2008).

In some cases, the most important factor, besides recruitment, in structuring benthic communities is the flow of organic matter from the pelagic environment to the seabed, thus demonstrating how important benthic-pelagic coupling is in marine ecosystems (Cattaneo-Vietti *et al.*, 1999). Changes in the pelagic environment are often mirrored in benthic ecosystems and often exhibit a time delay, often as little as eight days (Graf, 1989), although they can take place over a larger time-scale depending on the magnitude of the changes in the pelagos (Buchanan, 1993). The degree to which pelagic ecosystems are reliant upon the benthos has also been documented. Changes in benthic community composition could have serious implications for the trophodynamics of the water column; for example, the increase in abundance of *Echinocardium cordatum* (Pennant) in the North Sea has led to significant changes in the composition of the local meroplankton community (Kirby *et al.*, 2007).

The temporal and spatial variability of communities of *Abra alba* (Wood), *Lagis koreni* (Malmgren), and *Macoma balthica* (Linneaus) within the Seine estuary were examined and sediment composition, which can be quite variable throughout the year, was found to be a driving factor in inducing benthic-pelagic coupling and, thus, determining macrobenthic faunal dynamics (Desroy *et al.*, 2007). Benthic suspension feeders have been shown to be a major factor in linking the sediments and the water column (Kristensen *et al.*, 1991) as they have considerable effects upon the flow of carbon, nutrients, and energy between the benthos and the pelagic region (Thompson & Schaffner, 2001). Nutrient cycling by benthic organisms can play an important role in phytoplankton abundance. By controlling the rate at which biogenic silica is exported to the water column through filtration, biodeposition, and recycling, benthic organisms can determine the specific composition of secondary phytoplankton blooms. This has been demonstrated in the Bay of Brest (Chauvaud *et al.*, 2000).

Changes in phytoplankton composition have been linked to subsequent changes in benthic community composition especially when those benthic communities exhibit annual variability due to the life-cycles of the organisms in question (Buchanan, 1993), thus providing a further examples of benthic-pelagic coupling. The extent to which benthic-pelagic coupling occurs can vary, for instance the effect on phytoplankton communities via consumption by benthic organisms is less in the Gulf of Mexico than was documented previously in estuaries on the east and west coasts of the United States (Gaston *et al.*, 1997).

1.5. AIMS AND OBJECTIVES.

The records on the seasonal abundance and species composition of the meroplankton communities of Plymouth Sound and its surrounding areas provide an in-depth and useful data-set. In some cases, however, these records are 80 years old and very little research has been conducted on these meroplankton assemblages since the Plymouth Marine Fauna (1957) was published. As a result, there is little indication of the current status of the seasonal and annual cycles of larval abundance of these species, which represents a considerable gap in the knowledge of the meroplankton assemblages of the Plymouth Sound area. The degree of taxonomic resolution previously applied to the data-set used in Chapter 4 of this thesis was limited to a higher level of classification. This has restricted the degree of understanding of the complex life-cycles of meroplanktonic organisms that has been possible and it is the intention of this thesis to fill the gaps in knowledge that have arisen as a result. The potential for the use of molecular techniques in enabling larval identification has been discussed and the

investigation detailed in this thesis examines this potential and develops specific methods for this purpose.

The aims of this thesis are: (1) to update the knowledge and understanding of the meroplankton community at Station L4 in the English Channel by examining both the data from the 1988-2007 Station L4 time-series and generating new empirical data from this location, (2) to assess the benefits of increasing the taxonomic resolution of meroplanktonic organisms in terms of the patterns of seasonal abundance and community composition they reveal, and (3) to develop and utilise molecular techniques that allow for the further distinction of meroplanktonic larvae to a lower taxonomic level, and assess their potential and applicability for studies of this nature.

- Chapter 2 describes the generic methods that have been used in this thesis that are not specific to any of the different investigations detailed in the following chapters.

- Chapter 3 details the analysis of the meroplankton data derived from the 1988-2007 Station L4 time-series to determine the seasonal cycles of abundance and species composition of five broad taxonomic groups (Cirripedia, Decapoda, Echinodermata, Bivalva, and Polychaeta). Previous studies of planktonic organisms from this data-set have focussed upon those of holoplanktonic species such as calanoid copepods and the analysis in this chapter provides information on the largely overlooked meroplankton community at Station L4.

- Chapter 4 details the analysis of the 2008-2010 Station L4 survey that serves as a continuation of the previous data-set and examines any possible variation in the patterns observed in Chapter 3, and those patterns revealed by an increase in the

level of taxonomic resolution applied in this investigation. A comparison of the data collected in this study with the historical observations of Lebour assesses the evidence of long-term changes in the meroplankton community not apparent in the 1988-2007 time-series.

- Chapter 5 describes the use and refinement of a molecular technique focussing upon the 18S rRNA gene of bivalve larvae collected from Station L4 in order to increase the level of taxonomic resolution applied. Subsequent analysis of these data discusses insights into the seasonal cycles of abundance and species composition of bivalve larvae at the study site.

- Chapter 6 details the development of a relatively quick and simple molecular technique that allows the larvae of the different British species of the family Porcellanidae to be distinguished to species level without the requirement of the sequencing phase applied to the bivalve larvae detailed in Chapter 5.

- Chapter 7 discusses each of the investigations detailed in this thesis and draws conclusions as to the importance of the role played by meroplankton in coastal pelagic ecosystems. This is followed by consideration of the potential and requirements for future investigation.

CHAPTER 2. MATERIALS AND METHODS.

2.1. INTRODUCTION.

This chapter will describe those general methods whose use is not specific to the studies in any one chapter.

2.2. COLLECTION OF BENTHIC ADULTS FOR GENETIC ANALYSIS.

The benthic sampling effort was conducted using the research vessel Plymouth Quest at five different locations in and around the Plymouth Sound area (Figure 2.1 & Table 2.I). Riverine inputs from the Tamar estuary periodically affect the hydrography of these sites (Smythe *et al*, 2010, Southward *et al.*, 2004) and recent rainfall, wind mixing, and tidal state determine the magnitude of these inputs. All sites are tidally influenced with a maximum surface stream of $0.6ms^{-1}$ at mean spring tide (Pingree, 1980). Station L4 is located 10 km southwest of the Plymouth Breakwater in the UK (Figure 2.1). It has been sampled since the 1920s and intensively studied since around 1988. Various physical, chemical, and biological data are collected (Smyth *et al.*, 2010) with zooplankton and phytoplankton species' composition being of particular note (Aiken *et al.*, 2004; Southward *et al.*, 2004; Eloire *et al.*, 2010; Widdicombe *et al.*, 2010). The depth is approximately 51m and the water is mixed from September to March. A transitional period from mixing to stratification then occurs during the spring, before it becomes stratified during the summer months. Numerous phytoplankton blooms occur during the spring and summer months (Southward *et al.*, 2004), followed by a small autumn bloom (Boalch, 1987; Edwards & Richardson, 2004). These blooms provide a valuable food source for planktotrophic larvae (Holligan & Harbour, 1977).

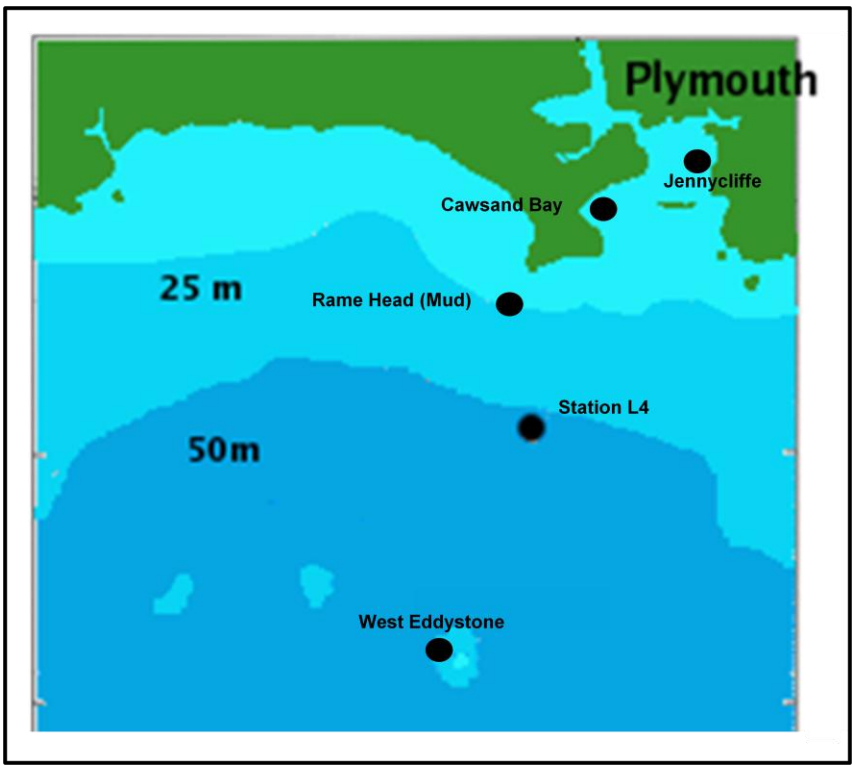

Figure. 2.1. The location of the benthic sampling sites detailed in this chapter (Source: Western Channel Observatory).

Table. 2I. Latitude and longitude of the sampling sites.

SITE	Central Position (sampling within a 200 m radius).
West Eddystone	50° 11'N 04° 16'W
Station L4	50° 13'N 04° 11'W
Cawsand	50° 19'N 04° 11'W
Jennycliffe	50° 20'N 04° 07'W
Rame Head (Mud)	50° 17'N 04° 16'W

The collection methods for each site are described below with the general sampling programme consisting of 0.25m^2 box-cores and 1m-wide Naturalist dredges.

2.2.1. West Eddystone.

A 0.1m^2 Day Grab was intended for use but was not particularly successful as only one grab in six provided any sediment therefore, the decision was taken to switch to box-cores. Five 0.25m^2 box-cores were taken at the West Eddystone site on each sampling trip. Each sample was sieved over a 500 µm mesh by adding a spade-full of sediment at a time to a bucket before elutriating (stirring the sediment to suspend biological material) and pouring off the floating material onto a sieve table and then washing through the sieve. Each portion was elutriated five times to ensure all fauna had floated out. The remaining residue in the bucket was checked on the sieve table before disposal and prior to the next portion being processed. Samples were labelled 1-5 with date and location. Due to the large number of pot lines present in the area, the 1m wide Naturalist dredge was unable to be deployed.

2.2.2. Station L4.

The same protocol as that used at the Eddystone site was used at Station L4 due to the Day grab again being unsuccessful. Five 0.25m^2 box-cores were taken on each sampling trip. In addition, three Naturalist dredges were towed for 5 minutes and were sorted on the sieve table over 4mm mesh. All macrofauna were picked out and labelled with the dredge number (1-3), date and location.

2.2.3. Cawsand.

At the Cawsand site, where the prevailing sediment type was muddy sand/sandy mud, the 0.25m^2 box-corer was not able to penetrate the sediment sufficiently, and so the

decision was taken to use a $0.1m^2$ Day grab instead. Five grab samples were labelled 1-5 with date and location and each one was sieved over a 500 µm mesh. There was no need for elutriation due to the smaller volume of sample and ease with which the sediment passed through the mesh. Three Naturalist dredge tows of 3 minutes were made instead of the 5 minutes used at previous sites due to the large volume of material collected and time constraints involved in processing each sample before the next was taken.

2.2.4. Rame Mud and Jennycliffe.

Due to the similar nature of the sediment at both Rame Head and Jennycliffe, the same protocol was used for each of them. Five $0.25m^2$ box-cores were taken and labelled 1-5 with date and location. The top 20cm was removed and elutriated five times in a bucket before sieving over 500 µm mesh. The residue was then examined before disposal to ensure that all macrofauna had been floated off. The rest of the core was broken up by hand and mixed with water to form a slurry, this was then sieved over a 4mm mesh to find any macrofauna still remaining in the sediment. The material retained by the sieve was then checked on the sieve table. No Naturalist dredges were taken as they tended to fill with mud immediately and could not accurately be towed for any amount of time and could therefore no longer be considered quantitative.

2.2.5. Collection of specimens from the shore at Mountbatten Bay.

It was necessary to visit the rocky shore habitat present at Mountbatten Bay in Plymouth Sound in order to collect specimens of *Porcellana platycheles* (Pennant) and *Galathea squamifera* (Leach) due to their absence from the core and trawl samples

taken from the other five sites. The shore was visited on three occasions and between 15-30 individuals of each species were collected and preserved upon return to the laboratory.

2.2.6. Identification of marine species.

All specimens in this thesis were identified to species where possible using light microscopy. All scientific names are derived from those accepted by the World Register of Marine Species (WoRMS) and can be found at www.marinespecies.org.

2.3. PRESERVATION OF SAMPLES.

Specimens of *Upogebia* sp., *Galathea* sp., *Porcellana platycheles*, and *Pisidia longicornis* (Linneaus) were removed from the samples and were placed in secure containers and stored at -20°C until use. The remainder of each sample was stored in 4% formaldehyde solution.

2.4. DNA EXTRACTION ON ADULT CRUSTACEANS.

In order to successfully extract DNA from small *Upogebia* and Porcellanidae adults stored at -20°C, it was necessary to remove one or two chelipeds from the organism using forceps and a scalpel. This tissue was then placed in a mortar before adding liquid nitrogen and grinding into powder using a pestle. The resultant powder was transferred to a separate 1.5mL microcentrifuge tube for each organism. If the specimen was large

enough then a scalpel was used to remove the muscle tissue from within the cheliped. This tissue was then placed directly into a microcentrifuge tube without the need for liquid nitrogen. Homogenising solution (340 µL) consisting of 400mM Tris-Hydrochloric acid (HCl) solution, 60mM EDTA, 150mM Sodium Chloride (NaCl) and 1% Sodium dodecyl sulphate (SDS), was added with 10 µL of 10mg mL^{-1} RNase A (Qiagen) to each tube and the tissue was homogenised using a Pellet pestle homogeniser (Kontes). This homogenate was then incubated at 37°C for 30 minutes. Proteinase K (8.75µL of 10 mg mL^{-1}) was then added to the solution to give a final concentration of 250 µg mL^{-1} before being further incubated at 37°C for 30 minutes. 100µL of 5M Sodium perchlorate was then added before the solution was shaken at room temperature for 20 minutes using a floor standing shaker. The solution was then incubated at 65°C for a further 20 minutes. Phenol chloroform (500µl) was added to each tube and the tubes were gently inverted 10 times until mixed. The tubes were then spun for 5 minutes in a desk-top centrifuge before the upper aqueous phases were transferred to new tubes. Chloroform (500µl cooled to -20°C) was added and the tubes shaken at room temperature for 20 minutes. The tubes were then spun in a desk-top centrifuge for a further 5 minutes before the upper aqueous phases were collected and transferred to new 1.5mL tubes. 100% ethanol at 2.5 times the volume of the solution was then added and the tubes incubated at -20°C overnight. The tubes were then spun in a desk-top centrifuge for 10 minutes to allow a pellet to form and the supernatant was discarded before the pellets were washed in 70% ethanol. A further spin cycle of 5 minutes in the centrifuge was conducted before the ethanol was discarded and the tubes placed in a miVac Modular DNA Concentrator Vacuum Centrifuge until all of the residual ethanol had evaporated. The remaining pellets were then suspended in 50µL of distilled water overnight in preparation for PCR.

2.5. POLYMERASE CHAIN REACTION (PCR).

For each series of PCR reactions a master mix was made up due to the concentration for 1 sample being too small to pipette accurately. When making a master mix reaction solution for use with a 96-well plate, the reagent volumes were multiplied by the number of samples with a negative and positive control included and then an extra 5 multiples of each reagent were added to account for the inevitable loss of solution during the aliquoting of the mix into a 96-well plate. Below is the standard reaction mix used in the majority of PCR reactions described in this study. Variations have occurred and are documented elsewhere in this report.

- DNA template 10-690 ng
- dNTPs 0.02 mM
- Promega Gotaq Flexi buffer 1x
- $MgCl_2$ 2.5 mM
- Forward Primer 50 pmol
- Reverse Primer 50 pmol
- Gotaq DNA polymerase 1.25 units
- Molecular-grade water (to a total reaction volume of 50µL)

The Polymerase Chain Reaction consisted of 6 stages:

1. An Initial Denaturation phase at 93-94°C for 5 minutes.
2. A Denaturation phase at 93-94°C for 1 minute.
3. An Annealing phase at 50-60°C for 1 minute (depending on primer composition).
4. An Extension phase at 72°C (1 min per kilo-base of DNA)
5. An Elongation stage of 72°C for 5 minutes
6. Samples were held at 4°C until they could be removed from the PCR machine.

The PCR cycle is shown in Figure 2.1. Stages 2-4 were repeated around 40x in order to maximise the amount of DNA amplified.

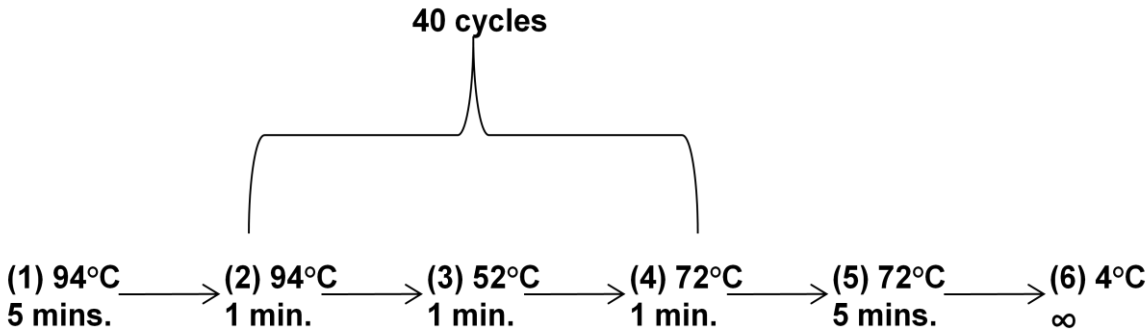

Figure. 2.2. A visual representation of standard PCR reaction.

2.5. CLEAN-UP OF PCR REACTION.

This was conducted using the Promega Wizard® SV Gel and PCR Clean-Up System following the manufacturer's instructions.

2.6. PREPARATION OF STOCK AND WORKING SOLUTIONS OF PRIMERS.

Desiccated primers were rehydrated to a stock solution of 100µM with distilled Millipore water and stored at -20°C. These solutions were then subjected to $^{1}/_{10}$ dilution using distilled Millipore water to produce a working solution of 10µM stored at -20°C until use.

2.7. PREPARATION OF AGAROSE GELS.

Agarose gels were created using agarose powder and TAE buffer. For a small 1% gel, 500 mg agarose were combined with 50 mL TAE buffer in a conical flask and placed in the microwave on full power for 1.5 minutes before 2.5 µL Ethidium bromide was added. The mixture was then poured into a gel plate and left to set for 45 minutes. For larger volumes and different concentrations, the reagent amounts were adjusted accordingly.

2.8. STATISTICAL ANALYSIS.

Nonparametric multivariate analysis and multi-dimensional scaling ordination (MDS) was conducted using the PRIMER statistical package and the R software package was used to produce average seasonal cycles and conduct anomaly analysis on the 1988-2007 Station L4 data.

2.9. LIST OF MATERIALS AND SUPPLIERS.

Table 2.II shows a list of the various reagents used, their concentrations (where applicable), and the suppliers used to source them. It includes all the reagents used during the investigations detailed in this thesis.

Table. 2II. List of reagents and suppliers used during the course of the studies detailed in this thesis.

MATERIAL/ REAGENT	SUPPLIER
RNase A (10 mg mL^{1} working solution/ 100 mg mL^{-1} stock)	Qiagen
dNTPs (2mM working solution/ 10mM stock)	Qiagen
Proteinase K (20 mg mL^{-1})	Sigma-Aldrich
5x Gotaq Flexi Buffer (coloured & colourless)	Promega
Gotaq DNA polymerase	Promega
Magnesium chloride ($MgCl_2$ - 25mM)	Promega
Primers	Eurofins MWG
Liquid nitrogen	BOC Cryospeed
Chloroform: Isoamyl alcohol 24:1	Sigma-Aldrich
Phenol: Chloroform: Isoamyl alcohol 25:24:1 (saturated with 1mM EDTA & 10mM Tris- HCl – pH 8.0)	Sigma-Aldrich
Tris Base	Fisher Scientific
Hydrochloric acid (HCl)	VWR International
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich

Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
100x Purified Bovine serum albumen (BSA - 10 mg mL^{-1})	New England Biolabs
Restriction Enzymes (*Bpm*I, *Sex*AI, & *Avr*II – 2500 & 5000 units mL^{-1})	New England Biolabs
NEBBuffer 3 (10x concentration)	New England Biolabs
5mM Sodium perchlorate ($NaClO_4$)	Sigma-Aldrich
TAE Buffer (50x)	AppliChem
Iberose – High specification agarose for electrophoresis	Web Scientific
Ethidium bromide solution	Sigma-Aldrich
Molecular grade Ethanol	Fisher Scientific
4% Formalin solution (Formaldehyde)	Fisher Scientific

CHAPTER 3. SEASONAL DYNAMICS OF THE MEROPLANKTON ASSEMBLAGE AT STATION L4 DERIVED FROM THE 1988-2007 TIME SERIES.

Aspects of this chapter have been published as:

Highfield, J. M., Eloire, D., Conway, D. V. P., Lindeque, P. K., Attrill, M. J., & Somerfield, P. J. (2010) Seasonal dynamics of meroplankton assemblages at station L4. *Journal of Plankton Research*, **32**, (5) 681-691.

3.1 INTRODUCTION.

Meroplanktonic larvae play an important role in coastal pelagic marine ecosystems. At certain times of the year, usually in conjunction with phytoplankton blooms, they comprise a large percentage of the total zooplankton community and can act as both planktonic predators and a food source for other planktonic predators such as fish (Beaugrand, 2005) and planktivorous zooplankton species thus having a major effect upon community composition. Given this important and vital role that meroplankton play in marine ecosystems, it is important to understand the trends in abundance and seasonality in this group in order to estimate how these trends might change in response to various environmental triggers.

Meroplanktonic larvae have been studied in the Western English Channel at Station L4 (50.25°N 04.217°W), for the duration of a long-term time-series comprising the period 1988-2007. Station L4 has been extensively sampled on a weekly basis since 1988. Although numerous studies have described aspects of zooplankton at Station L4 (Irigoien & Harris, 2003; López-Urrutia *et al.*, 2004; Bonnet *et al.*, 2007; Eloire *et al.*, 2010), the focus has primarily been on holoplankton and very few studies have considered the dynamics of meroplanktonic larvae off Plymouth. The many species-specific records in the Plymouth Marine Fauna (Marine Biological Association of the United Kingdom, 1957) are primarily based on earlier records, such as those of Lebour (1947) who reported presence/absence observations on a monthly basis. In order to address the lack of explicit meroplankton studies, the aim of this investigation was to describe how the composition of the meroplankton community and its contribution, in terms of percentage composition, to total zooplankton varied seasonally and annually at Station L4.

The investigation detailed in this chapter forms part of the Station L4 time-series special edition of the Journal of Plankton Research. This edition marked the 20-year anniversary of the founding of the station L4 weekly zooplankton time-series that commenced on 14/03/1988. The issue contains work reviewing the zooplankton and phytoplankton time-series, fish larvae studies, recent physical, optical, and chemical studies, and the wider oceanographic context of the sampling station (Harris, 2010).

3.2 MATERIALS AND METHODS.

Data for this study are derived from zooplankton samples collected weekly (weather permitting) at Station L4 from March 1988 to December 2007, a total of 862 samples. No samples were collected in August 2000. On each sampling trip 2 replicate vertical WP2 net (mesh size = 200 µm, mouth aperture = 60 cm diameter) hauls were taken from the seabed (51 m) to the surface and stored in 5% formalin. Owing to the large number of organisms in most samples, 2 sub-samples were taken from each haul for identification. Because of the wide range of sizes and abundances of organisms present, distinct sub-samples for small and large organisms were necessary as one sub-sampler size would not adequately sample all of those organisms present in a sample. For sub-sampling, each sample was made up to a known volume e.g. 500 ml, and split using an appropriate method. For smaller organisms sub-samples were taken with a Stempel pipette. Larger (and rare) organisms were counted in sub-samples taken using a Folsom splitter to separate the sample into a half, a quarter, or an eighth depending on the richness of the original. Both hauls were analysed, the average numbers calculated, and counts were converted to numbers per m^3 (John *et al.*, 2001). The full data set can be

found in Appendix 2 and at the Western Channel Observatory website www.westernchannelobservatory.org.uk.

Owing to the difficulties in larval identification and because different analysts, with varying abilities to discriminate larvae, have worked on the samples over the years, this analysis is restricted to variation in five different groups defined at a broad taxonomic level. These groups are: Decapoda; Cirripedia (including nauplii and cyprids); Echinodermata; Bivalvia; and Polychaeta. These groups provide an overall picture of the seasonal changes in the meroplankton assemblages at L4. Data from phytoplankton and physico-chemical measurements collected using methods described by Smyth *et al* (2010) and average weekly sea surface temperatures were used in this investigation and were collected by the crews of the research vessels over the time-series. Average weekly sea surface temperatures °C (SST) were measured using a thermometer and bucket during the years 1988-1998, with the Plymouth Marine Laboratory CTD system during the years 1998-2002, and since 2002 using a SeaBird SBE19+ (Smyth *et al.*, 2010).

During this investigation, the average seasonal cycles of the total zooplankton, total meroplankton, each meroplankton group and SST, were examined using weekly averages, over the entire time series. The abundance of each group was determined for each month and a monthly abundance anomaly for each month of the time series was obtained using the formula:

$$x'_{m,y} = \frac{x_{m,y} - \bar{x}_m}{\sigma(x_m)}$$

where m is the month (m: 1=January, 2=February, …, 12=December) and y is the year, $x'_{m,y}$ is the monthly anomaly for month m in the year y; $x_{m,y}$ is the monthly average

abundance in month *m* in the year *y*; $\bar{x}_m$ is the average abundance and $\sigma(x_m)$ is the standard deviation in month *m* over the entire time series. Thus, a positive anomaly means that the observed value was higher than the overall average for that month, and a negative anomaly equates to the observed value being lower than the overall monthly average (Fig. 3.4). Annual anomalies were obtained by averaging the monthly anomalies for each year of the time series from 1988 to 2007. Gaps occurred in January and February 1988, and August 2000. Prior to estimating annual anomalies by using monthly anomalies, the values for the missing months were interpolated using the overall average for that month over the entire time series.

Trends for the monthly anomalies were obtained by applying a type I linear model. Prior to testing the significance of the trend slope, a Durbin-Watson test was performed to detect autocorrelation in the residuals of the regression analysis (MacKenzie & Koster, 2004). When autocorrelation was present the effective number of degrees of freedom for significance tests was adjusted using the following formula:

$$n_{eff} \approx n_t \frac{1 - r_1}{1 + r_1}$$

where n_{eff} is the effective number of independent values, n_t is the total number of values and r_1 is the lag-1 temporal autocorrelation coefficient (Quenouille, 1952; Hays *et al.*, 1993; Pyper & Peterman, 1998; Santer *et al.*, 2000). A Student *t*-test was used to determine whether the slope of the linear model was significantly different from 0 (Table 3.I).

Variation in community composition in the time series data was analysed using nonparametric multivariate analysis (Clarke, 1993). Data were converted to monthly within-year averages and the Bray-Curtis similarity coefficient (Clarke *et al.*, 2006) was

calculated between every pair of monthly average values. Inter-sample resemblances were ordinated in 2D using non-metric multi-dimensional scaling (MDS). Fourth root transformations were conducted upon the data during statistical analysis. Two-way crossed ANOSIM using Spearman's rank correlation was conducted on the year and month groups within the data to assess the significance level of the differences between these groups.

When analysing seasonal variation, the seasons were defined as follows: spring = March, April, and May, summer = June, July, and August, autumn = September, October, and November, and winter = December, January, and February. Each subsequent referral to different season uses these distinctions.

3.3. RESULTS.

3.3.1. Meroplankton.

Of the five groups studied, Cirripedia were exhibited the largest average abundance with 300.67 N m^{-3} being recorded (Table 3.I). Anomaly analysis (Table 3.I) shows that there was a slight, but significant ($p<0.0002$) increase in the average sea surface temperature over the duration of the time series.

Table 3.I. Statistics of the total zooplankton, total meroplankton, each meroplankton group and the SST. The average abundance, standard deviation and relative contribution for the meroplankton were estimated over the period 1988-2007. DW statistic and DW p-value correspond to the results of the Durbin-Watson test. Trend p-value gives the significance of the slope (Trend slope) of the regression analysis. Highlighted in grey are the non-significant trends (p > 0.1).

Time Series (1988-2007)	Average abundance ($N.m^{-3}$)	SD (N.m-3)	Relative contribution (%)	Monthly Anomalies			
				DW statistic	DW p-value	Trend p-value	Trend slope
Total zooplankton	**3075.61**	**2138.65**	**-**	**2.06**	**0.68**	**0.542**	**-0.0006**
Meroplankton	**536.98**	**928.53**	**17.46**	**2.00**	**0.95**	**0.032**	**0.0020**
Cirripedia	300.67	881.00	9.78	2.01	0.97	0.007	0.0024
Decapoda	14.82	13.58	0.48	2.03	0.82	0.193	0.0012
Polychaeta	29.15	31.82	0.95	2.03	0.82	0.482	-0.0006
Echinodermata	76.30	232.18	2.48	2.01	0.95	0.644	0.0004
Bivalvia	52.19	124.74	1.70	2.04	0.81	0.425	0.0007
SST	**12.80**	**2.78**	**-**	**2.16**	**0.24**	**0.0002**	**0.0033**

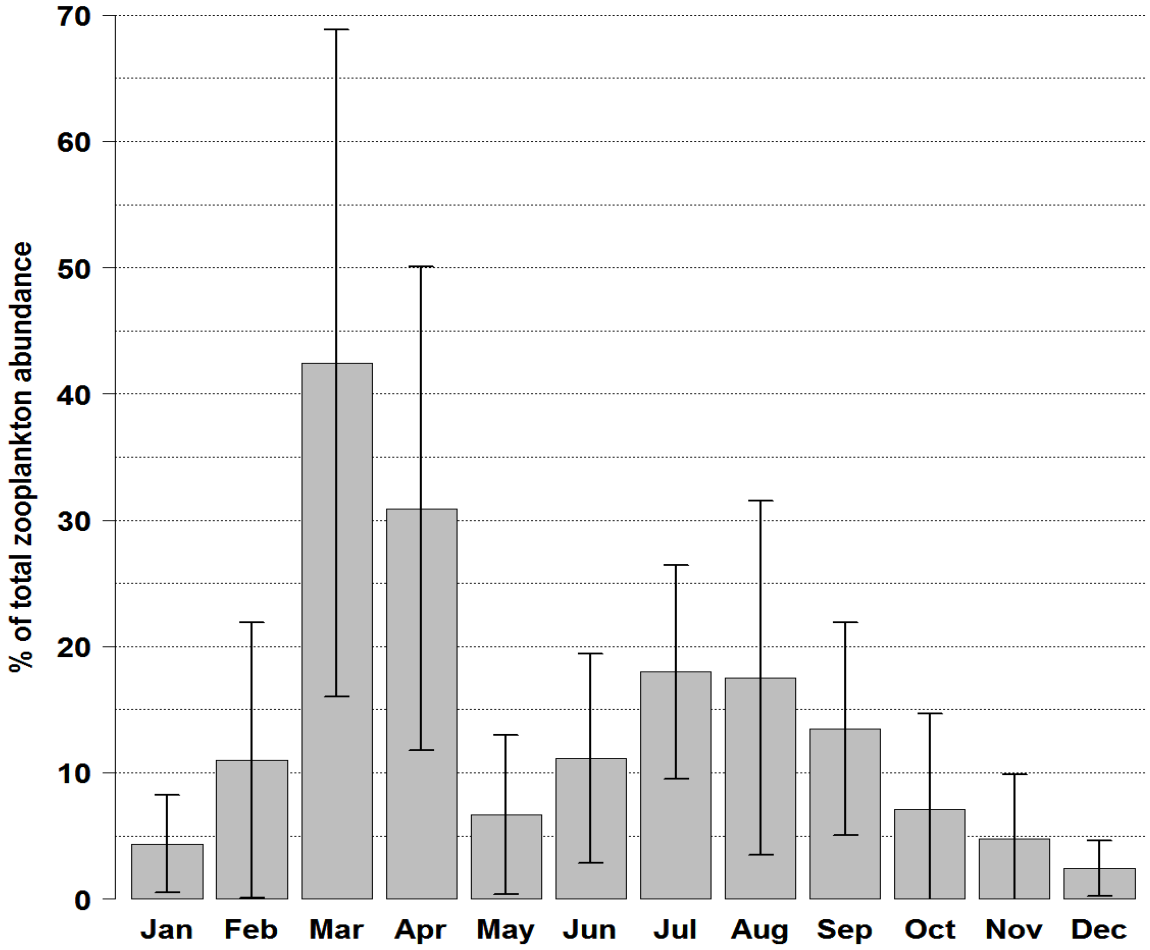

Figure. 3.1. The average monthly percent contribution of meroplanktonic larvae to total zooplankton abundance at Station L4. The error bars represent the range of monthly percentage contributions to zooplankton abundance over the entire time-series.

The average percentage contribution of meroplanktonic larvae to the total zooplankton over the annual cycle varies throughout the year (Fig.3.1) and averages 17.46%. The highest values occur in March where meroplankton may comprise 42.5% of the total zooplankton, with April showing the next largest contribution. The fraction comprising meroplankton increases again in July and August before decreasing in the autumn and winter.

3.3.2. Phytoplankton.

The average seasonal cycle of phytoplankton at Station L4 (Fig. 3.2) is characterised by a large increase in cell concentration that starts in March, and reaches its peak in May (the spring bloom), followed by a series of smaller peaks that occur throughout the summer months before a larger peak occurs in September. This peak is the autumn bloom which is smaller in cell concentration than the spring bloom. Cell concentrations then continue to decrease throughout autumn and winter. The increase in cell concentration that occurs in March is concurrent with the onset of an increase in sea surface temperature.

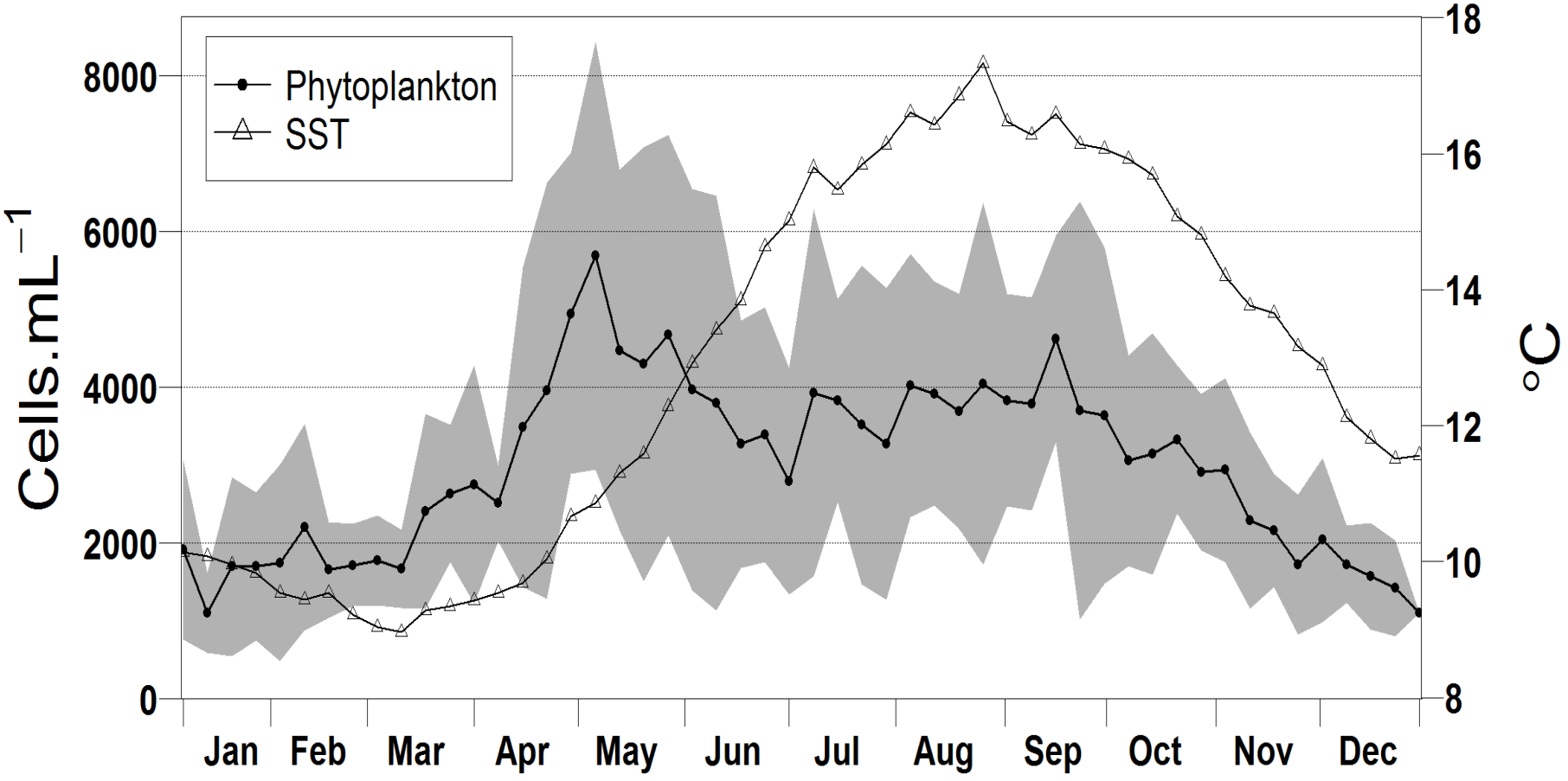

Figure. 3.2. Changes in weekly average abundance (Cells mL^{-1}) of total phytoplankton over the annual cycle at Station L4. Solid line indicates the mean; grey band indicates ±1 SD. Mean weekly sea surface temperature (SST) is also shown.

3.3.3. Raw abundance data.

Plots of the raw abundance data over 20 years for the total zooplankton, total meroplankton, and each of the five groups under study (Fig.3.3) do not show strong inter-annual changes or trends and show that within-year variability is relatively large. Plotting monthly anomalies (Fig. 3.4) fails to clarify any seasonal or multi-annual patterns in the data.

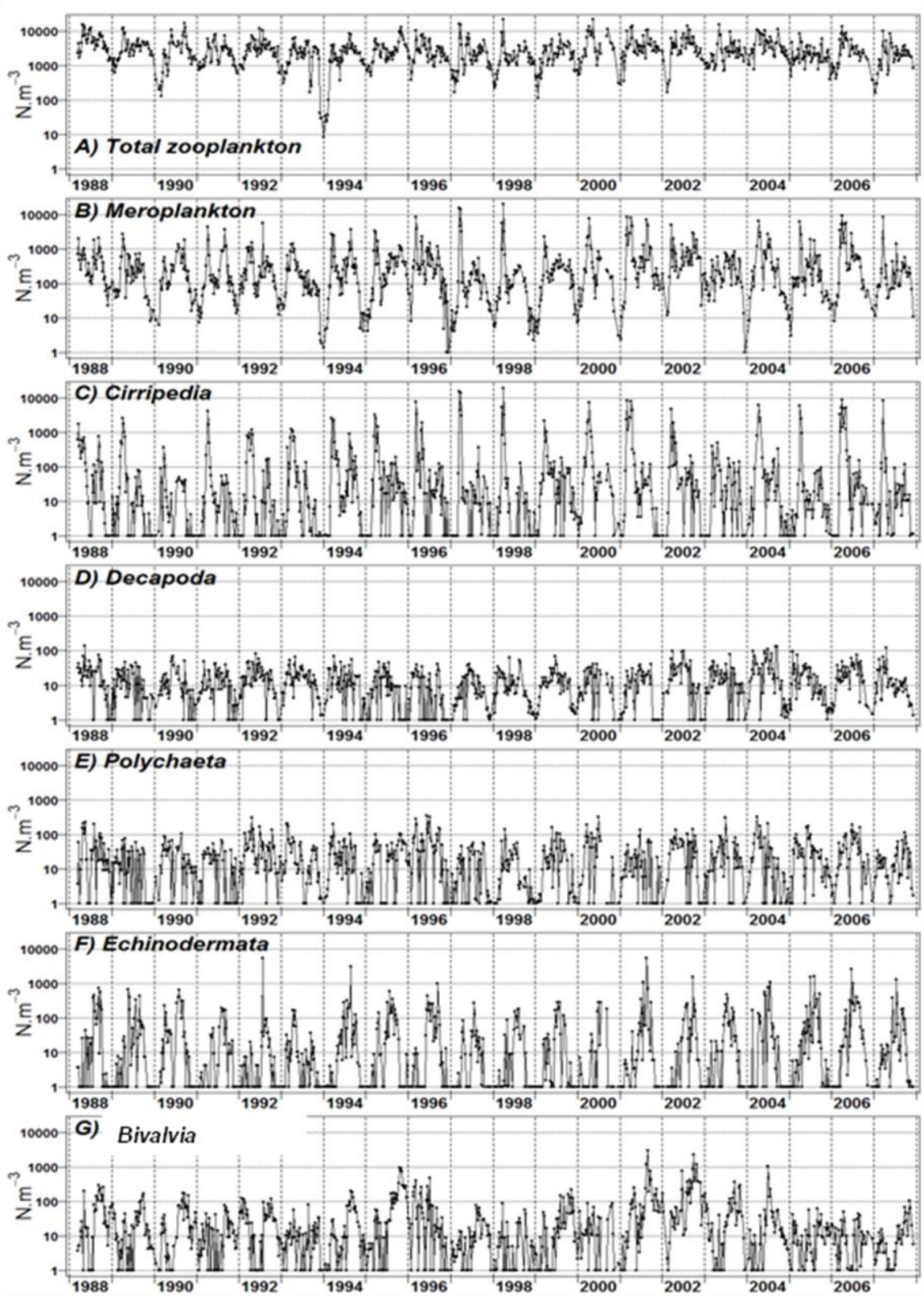

Figure. 3.3. Plot of raw data showing the changes in abundance over the time-series for (A) total zooplankton, (B) total meroplankton, (C) Cirripedia, (D) Decapoda, (E) Polychaeta, (F) Echinodermata and (G) Bivalvia at Station L4.

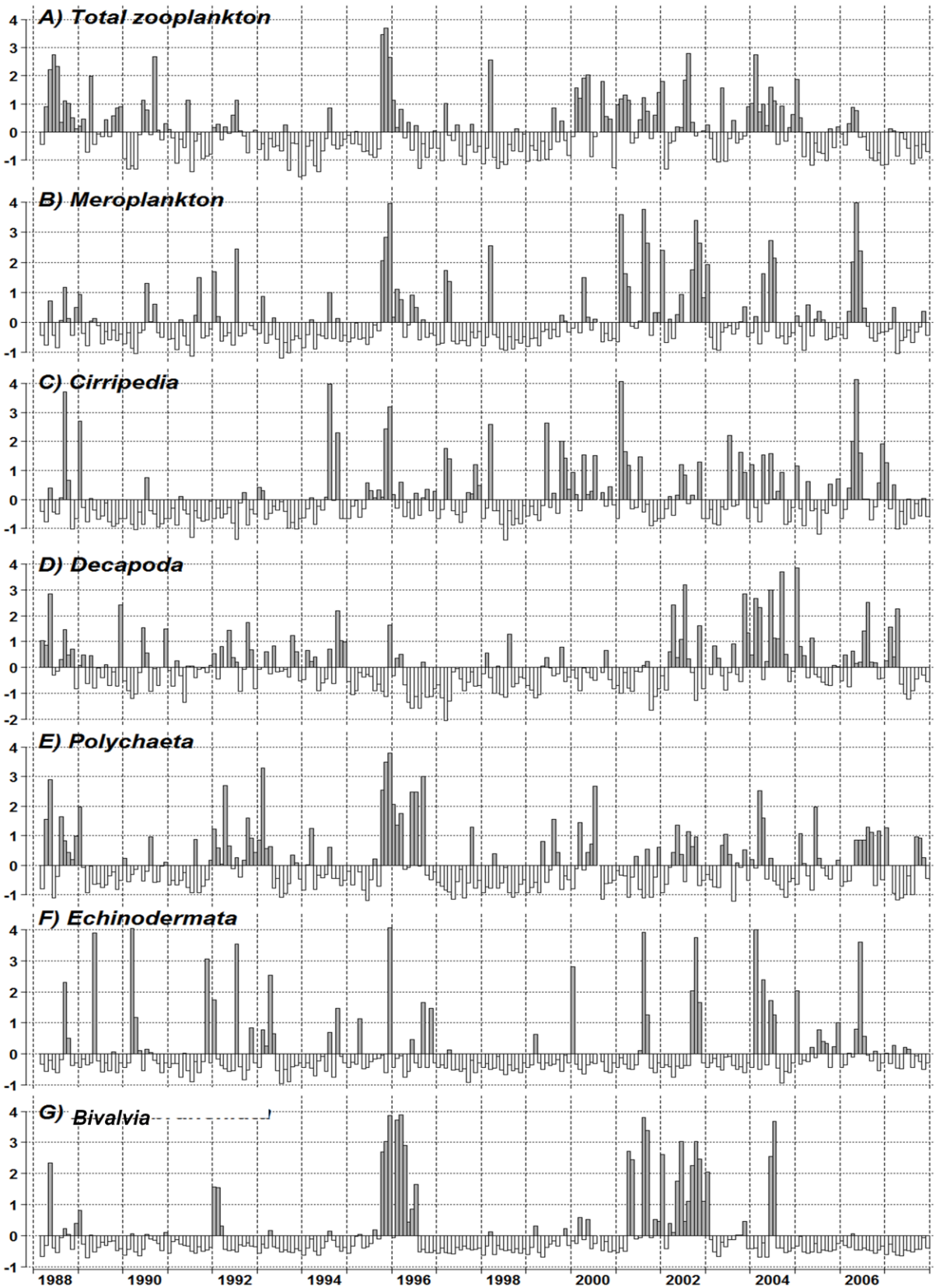

Figure. 3.4. The monthly anomalies in the data for (A) total zooplankton, (B) total meroplankton (C) Cirripedia, (D) Decapoda, (E) Polychaeta, (F) Echinodermata and (G) Bivalvia at Station L4.

3.3.4. Seasonal cycles.

3.3.4.1. Total zooplankton & total meroplankton.

The average seasonal cycles of total zooplankton (Fig. 3.5A) and total meroplankton (Fig. 3.5B) at Station L4 are both characterised by large increases in abundance during March and April, which are driven by the large increases seen in ciirpede larval numbers at this time. These increases coincide with the onset of sea temperature increase. This is then followed by a steady decrease in total zooplankton for the rest of the year. For total meroplankton, there is a decrease in May followed by a slight increase during August and September before falling again during the autumn and winter months. The average seasonal cycle for sea surface temperature was overlaid on each seasonal cycle figure so that increases in abundance can be visualised with sea surface temperature.

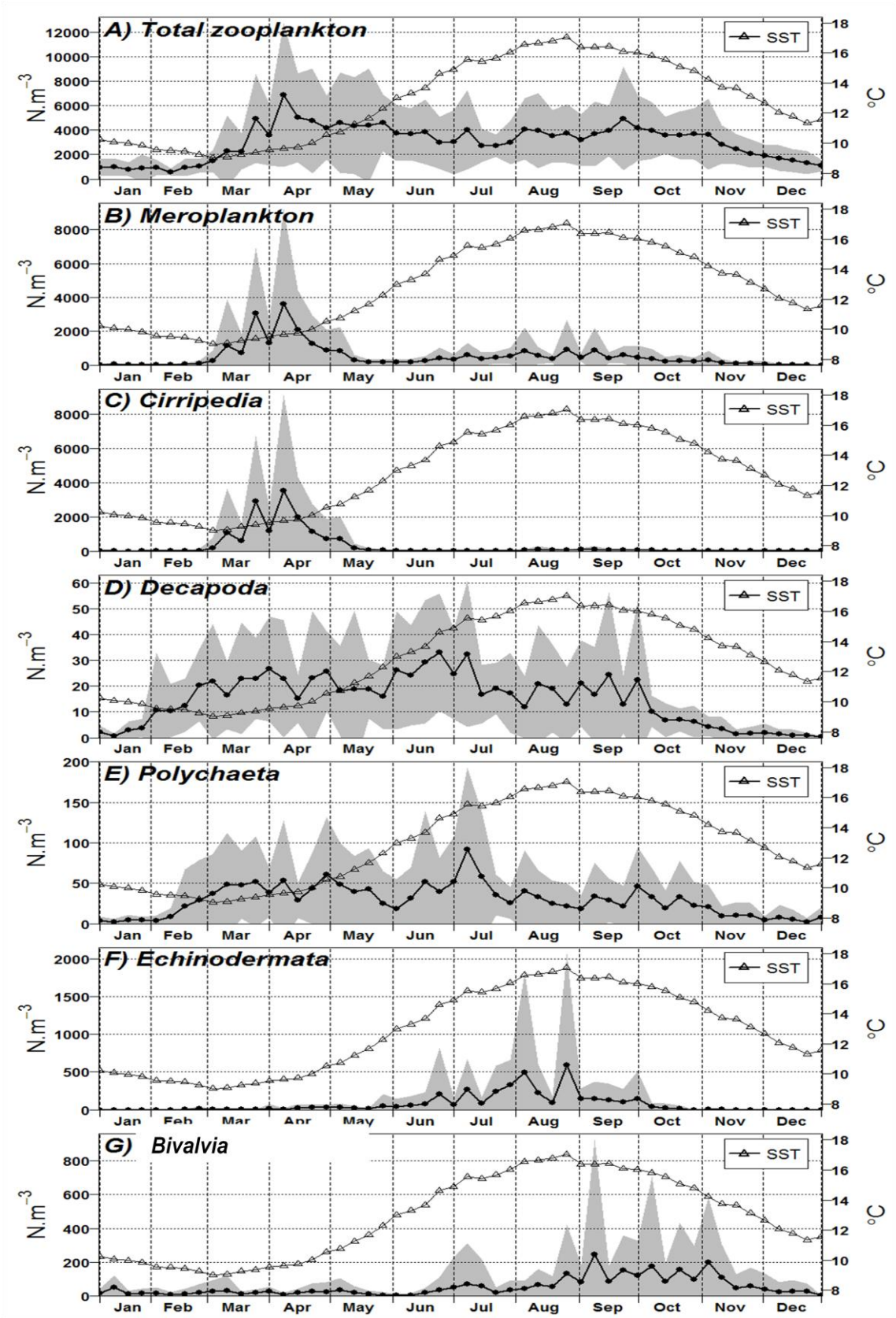

Figure. 3.5. Changes in the weekly average abundance N m^{-3} for (A) total zooplankton, (B) total meroplankton, (C) Cirripedia, (D) Decapoda, (E) Polychaeta, (F) Echinodermata and (G) Bivalvia at Station L4, calculated from 20 years data. Solid line indicates the mean; grey band indicates ±1 SD. Mean weekly sea surface temperature (°C) is also shown.

Anomaly analysis (Fig. 3.4A, 3.6A) shows no significant variation (p = 0.542 Table 3.I) in total zooplankton abundance over the time-series. However, a significant (p = 0.032) overall increase in abundance was seen in total meroplankton (Fig. 3.4A, 3.6A, Table 3.I) over this period.

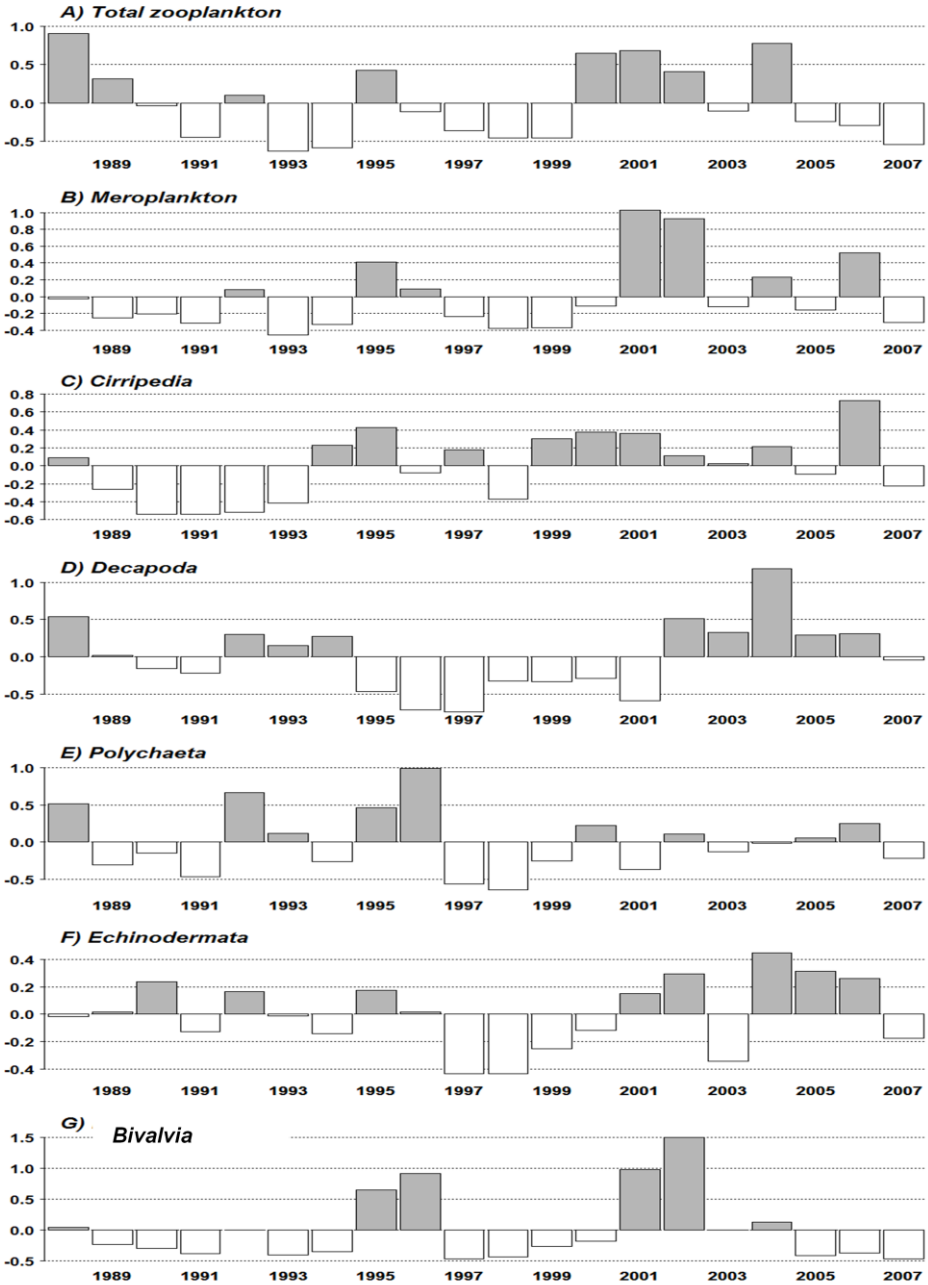

Figure. 3.6. Annual anomalies in the data for (A) total zooplankton (B) total meroplankton, (C) Cirripedia, (D) Decapoda, (E) Polychaeta, (F) Echinodermata and (G) Bivalvia at Station L4 as calculated from the monthly anomalies having removed the average abundances.

3.3.4.2. Cirripedia.

Cirripedia larvae occur in relatively low numbers in the water column for most of the year. There is a massive increase in abundance in March and April (Fig.3.5C) coinciding with the spring phytoplankton bloom (Figure 3.1), that can exceed 15,000 $N.m^{-3}$ (15251 $N.m^{-3}$ were recorded on 17/03/1997). This is the largest increase in abundance exhibited by any of the five groups examined in this study. The abundance of cirripede larvae then falls by the end of May and remains relatively low (<100 $N.m^{-3}$) for the rest of the year except for a slight increase in numbers during August and September (e.g. 777 $N.m^{-3}$ on 05/09/1988). Annual anomaly analysis (Fig. 3.6C, Table 3.I) shows a significant increase in abundance ($p<0.007$) over the 20 years of the time series.

3.3.4.3. Decapoda.

Decapod larvae are present in the plankton in every month (Fig. 3.5D). Numbers are generally low, ranging from 0 to 136 $N.m^{-3}$. Few larvae are found from November to January before abundance increases during spring. A peak tends to occur at the end of March before abundances fall in April and become variable. The highest average abundances occur in June and at the start of July, before abundances fall quite dramatically into August. Minor peaks occur over the autumn months before abundances fall to low winter levels. The abundances of decapod larvae begin to increase before SST reaches its lowest value at around 9°C but only reach their highest numbers once the temperature has reached 13-14°C. The analysis of annual anomalies (Fig. 3.6D) shows no significant trend (Table 3.I).

3.3.4.4. Polychaeta.

Polychaete larvae are found in the plankton in low numbers in January and February (Fig. 3.5E) before increasing in numbers and reaching a peak in late spring. Highest abundances occur in June and July (e.g. 172 $N.m^{-3}$ on 10/06/1996). The seasonal cycle of polychaete larvae at L4 is then characterised by a steady decrease towards the winter months reaching the lowest average abundance in December and January (Fig.3.5E). Abundance of polychaete larvae appears to increase before SST reaches its lowest value at around 9°C but their highest levels occur once the temperature has reached 13-14°C. The overall trend in polychaete abundance over the last 20 years (Fig. 3.6E) is not significant (Table 3.I).

3.3.4.5. Echinodermata.

Echinoderm larvae, including ophiopluteii, echinopluteii, and auricularia, are found in low numbers during the winter and spring months before increasing as summer begins (Fig. 3.5F). The larvae are present in their highest numbers (as high as 5546 $N.m^{-3}$) in August and September before decreasing again through the autumn and winter months (Fig.3.5F). The abundances of Echinodermata appear to increase when sea surface temperature is approaching its highest level at around 17°C. The annual anomaly analysis (Fig. 3.6F) shows no significant trend (Table 3.I) over the 20 years of sampling.

3.3.4.6. Bivalvia.

Bivalve larvae are present in the meroplankton throughout the year at L4 (Fig.3.5G). They are found in low numbers from January to June before increasing during the summer months. During September and October there is a very large peak in the numbers of larvae (up to 2940 $N.m^{-3}$), before numbers fall again during the winter. This large increase in the abundance of Bivalvia appears to occur once sea surface temperature has reached its highest level at around 17°C. Changes in annual anomalies (Fig. 3.6G) show no significant trend (Table 3.I).

3.3.5. Statistical analysis.

The composition of the meroplankton community at Station L4, as visualised in MDS, changes throughout the year (Figures 3.7 and 3.8). Intra-annual changes in community composition are larger than inter-annual changes, as symbols representing the same month in different years tend to be grouped together in the ordination (Fig. 3.7). Three seasonal groupings may be seen. The autumn and winter months (October to February) are characterised by low overall abundances, leading to relatively high variability in inter-sample resemblances (Clarke *et al.*, 2006). The assemblage in early spring (March and April) is dominated by Cirripedia (Fig. 3.8) but also contains relatively high numbers of polychaete and decapod larvae. Following the spring outburst the community in June has sometimes almost returned to winter conditions owing to generally low abundances. The summer (May to September) shows an increasing trend in bivalve and echinoderm larvae and high numbers of Polychaeta and Decapoda (Fig. 3.8). The reversion back to winter conditions tends to be abrupt, occurring between September and October.

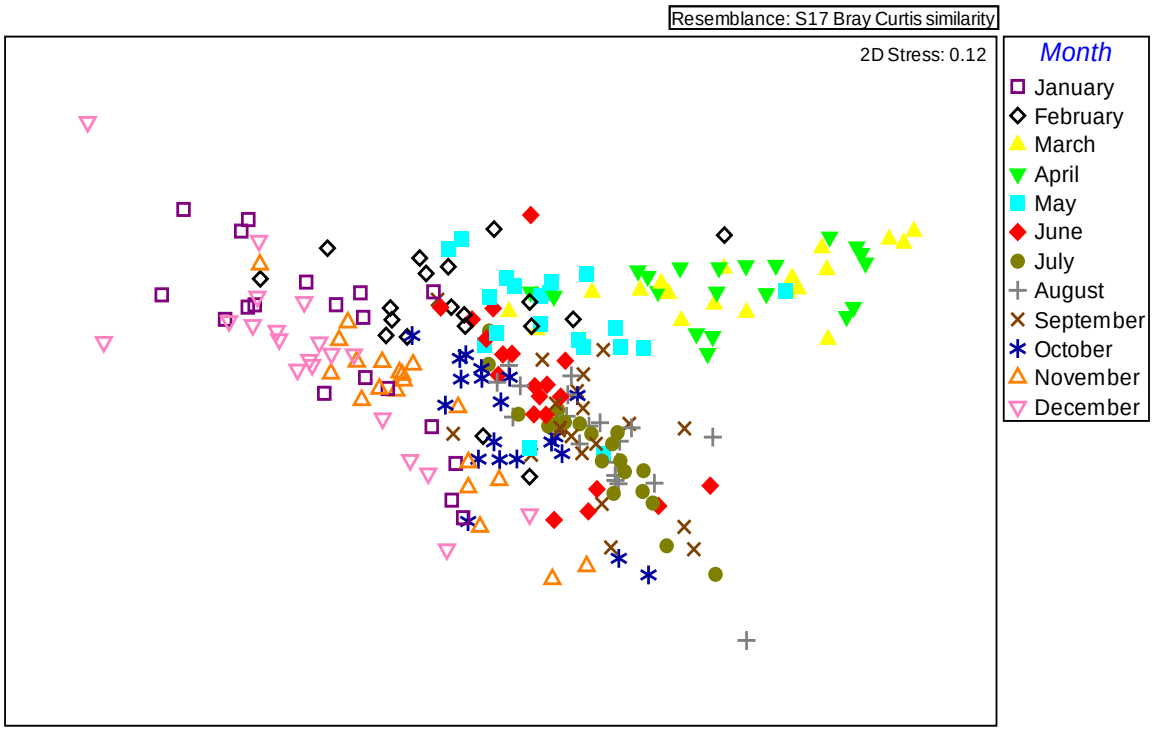

Figure. 3.7. Non-metric multi-dimensional scaling (MDS) ordination of Bray–Curtis similarities among monthly within-year average abundances of the five major meroplankton groups in the study.

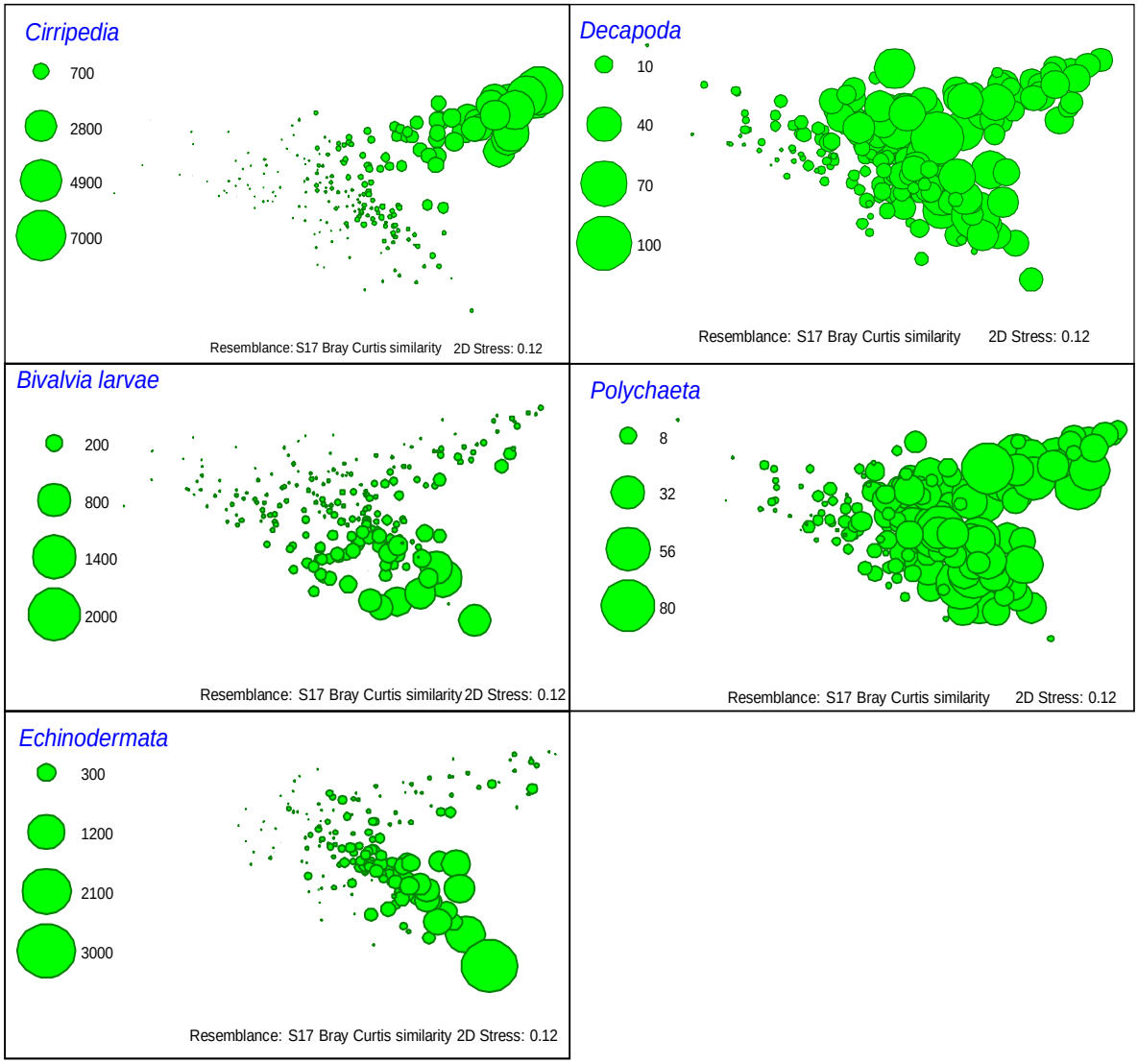

Figure. 3.8. MDS ordinations of Bray–Curtis similarities among monthly within-year average abundances of the five major meroplankton groups (as in Fig. 3.7) overlaid with circles proportional in size to abundances of individual major meroplankton groups.

The two-way ANOSIM test conducted on the differences between year groups across all month groups produced a sample statistic (Global R) of 0.033 with a significance level of 2.2% (Figure. 3.9). This shows that the null hypothesis of there being no significant inter-annual variation between samples can be rejected as p = 0.022. The two-way ANOSIM test conducted on the differences between month groups across all of the year groups produced a sample statistic (Global R) of 0.444 with a significance level of 0.1% (Figure. 3.10). This shows that the null hypothesis that there are no significant

differences between month groups can be rejected (p= 0.001) therefore proving the presence of significant seasonal variation.

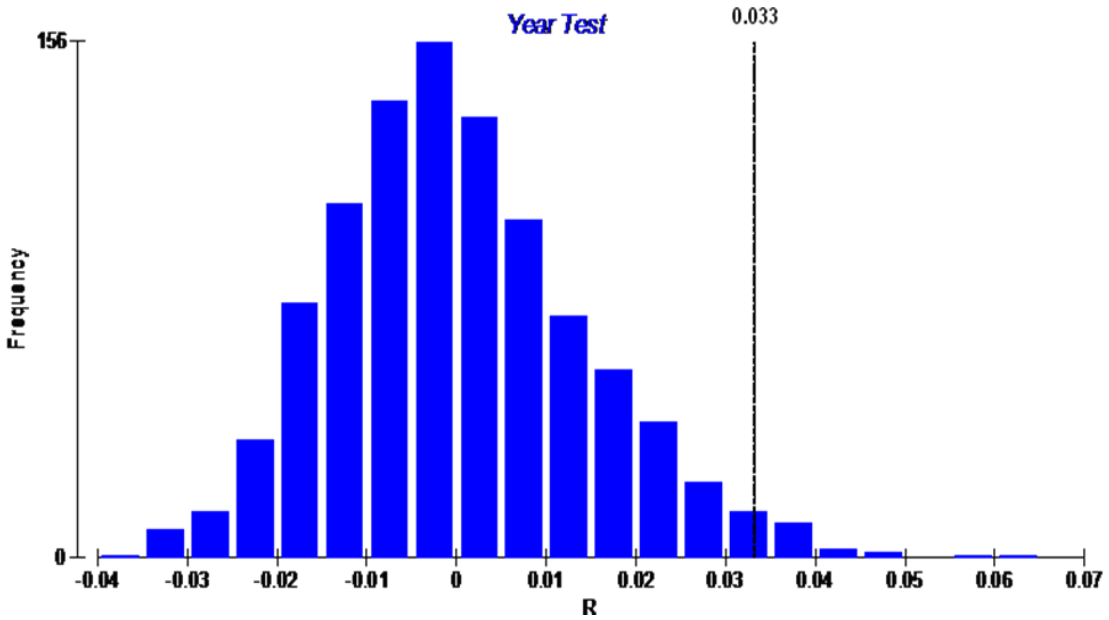

Figure. 3.9. Two-way ANOSIM test conducted on the 1988-2007 Station L4 time-series data examining the differences between year groups across all month groups. The Global R value is 0.033 and the sample statistic significance level is 2.2%.

As the p-value for monthly variation is lower than that of inter-annual variation between the samples, this shows that seasonal changes make a more important contribution towards structuring community composition in the meroplankton community at Station L4 with there being less inter-annual change over the time-series. This is shown by the R-values that support the conclusion that monthly variation has a greater effect than inter-annual variation, suggesting that the seasonal cycles of abundance observed in the data occurred every year over the time-series.

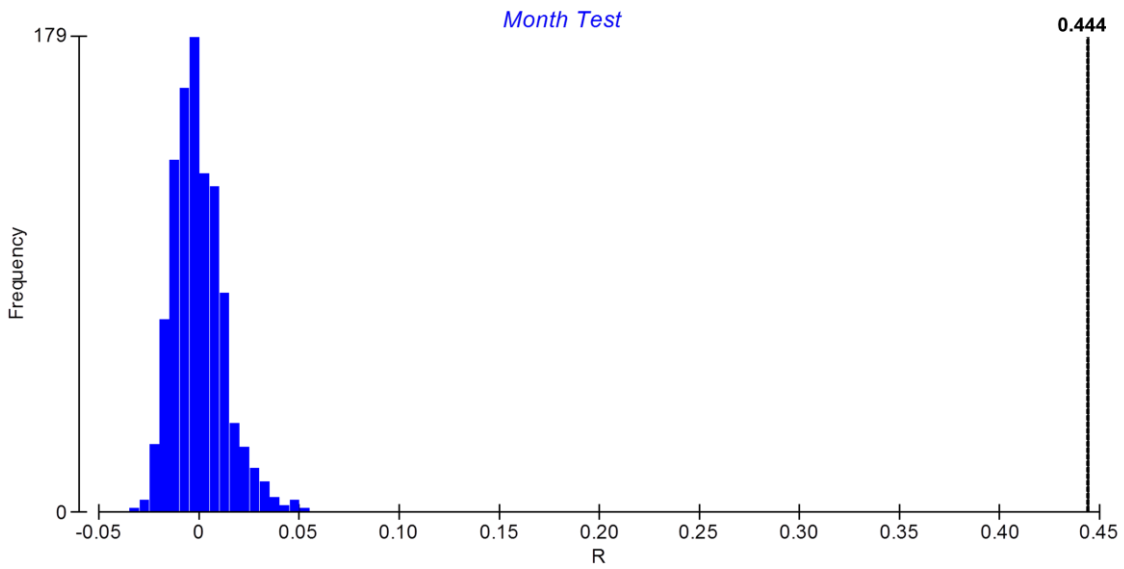

Figure. 3.10. Two-way ANOSIM test conducted on the 1988-2007 Station L4 time-series data examining the differences between month groups across all year groups. The Global R value is 0.444 and the sample statistic significance level is 0.1%.

3.4. DISCUSSION.

The relationship between the spring phytoplankton bloom and the release of meroplanktonic larvae has been studied extensively and examples of both direct and indirect induction of spawning in benthic species as a consequence of either blooms or those environmental conditions that trigger blooms have been reported. For example, certain species of barnacle and spider crab spawn upon direct contact with phytoplankton cells (Starr *et al.*, 1991; 1993; Starr *et al.*, 1994; Andreu & Duarte, 1996) whilst the Green Sea Urchin, *Stronglycentrotus droebachiensis* (Müller) and the Blue Mussel, *Mytilus edulis* (Linnaeus), both undergo spawning upon detection of a heat-stable metabolite released by certain phytoplankton species (Starr *et al.*, 1990; 1992).

The groups examined in this analysis occur as adults in the benthic and intertidal communities in the Plymouth Sound area (Allen, 1899; Marine Biological Association

of the United Kingdom, 1957; Holme, 1961) and so these meroplankton groups can be said to be representative of the existing adult populations in the local ecosystem. Historical data (Lebour, 1947) show that the larvae of the cirripede *Semibalanus balanoides* (Linnaeus), in particular, undergo their main settlement during March and April (Marine Biological Association of the United Kingdom, 1957). *S. balanoides* is one of those species that initiates spawning upon direct contact with phytoplankton cells (Starr *et al.*, 1991). It is known to store embryos in its mantle cavity until the presence of a food source is detected such as the diatom *Skeletonema costatum* (Gaston *et al.*, 1997) which contributes heavily to the spring bloom at Station L4 (Barnes, 1962). Thus the timing of the massive release of cirripede larvae during this time period is probably determined by the spring phytoplankton bloom that occurs every year in the waters in and around Plymouth Sound (Boalch, 1987; Aiken *et al.*, 2004; Southward *et al.*, 2004).

It would be expected that most of the cirripede larvae sampled at Station L4 are released by adults occupying the hard substrate habitats in the Plymouth Sound area and adjacent coastline. The small but significant increase in cirripede abundance over the past 20 years is responsible for the significant increase seen in meroplankton abundance over the same time period ($p= 0.032$) given that cirripede larvae occur in greater numbers than any other group. Adult cirripedes are known to inhabit the rocky intertidal habitats around the Plymouth Sound area in large numbers (Southward, 1991) with the common species being: *S. Balanoides*, *Chthamalus stellatus* (Poli), *Chthamalus montagui* (Southward), and *Elminius modestus* (Darwin). It is known that the abundance of these species is linked to environmental temperature (Southward, 1991) and therefore, the small increase in sea surface temperature observed over the time-series may have contributed to the small increase seen in cirripede larvae. Other factors such as a

decrease in predation on adult cirripedes or an increase in suitable intertidal habitats for colonisation can affect adult populations and the numbers of larvae produced.

Decapod larvae are not as abundant in the plankton community as cirripede larvae. Cirripede larvae are much smaller than decapod larvae and so it is likely that their superior numbers reflect the lower relative per-individual energetic costs of producing smaller larvae (Thorson, 1946). Adult cirripedes exist in much larger numbers than decapod species in the Plymouth area (Southward, 1991; Marine Biological Association of the United Kingdom, 1957) and this will also account for the larger numbers of cirripede larvae recorded in this study. Historical records from the early 20th century show that, while some decapod species breed throughout the year, many reproduce during the spring and summer (Lebour, 1928a; Marine Biological Association of the United Kingdom, 1957) and so larvae are more common in the plankton in these months Although not discriminated to species, the pattern observed at Station L4 over the last 20 years in this study suggests that the overall seasonal cycle has remained the same, and there is no evidence of a trend in changing abundance over this period.

Historical records of the plankton in and around Plymouth Sound (Lebour, 1947; Marine Biological Association of the United Kingdom, 1957), show that polychaete larvae were found in the water column in their highest abundances in the spring and summer months. Timing varied between different polychaete species but all tended to decrease during the autumn and winter period. The patterns observed in this study are similar. There are a wide range of life history strategies among the Polychaeta, and among their larvae, with some polychaete larvae feeding upon other planktonic larvae (Thorson, 1946). Therefore, some polychaete species release their larvae in response to

the available food source provided by other planktonic larvae that in turn are produced as a result of the spring bloom.

During the summer period, there are a number of peaks in echinoderm larval abundance, characterised by rapid increases and decreases. This suggests that different species are spawning, and their larvae do not have a long residence time in the plankton. The historical data show that echinoderm larvae, particularly the ophiopluteii of *Ophiothrix fragilis* (Abildgaard), were common in the plankton in and around Plymouth Sound from July to September (Lebour, 1947). Increases in abundance of echinoderm larvae correlated to increasing sea surface temperatures have been reported from the North Sea (Kirby *et al.*, 2007; Kirby *et al.*, 2008), but although there has been an increase of 0.6°C per decade over the last 20 years at the study site (Smyth *et al.*, 2010) there was no significant increasing trend in echinoderm larval abundances over the same period at Station L4.

The large increase in bivalve larval numbers in September and October is related to the autumn phytoplankton bloom which provides a readily available food source for the larvae. Water temperature is known to be a principal factor in affecting gonadal development in marine bivalves (Loosanoff & Davis, 1963; Chávez-Villalba *et al*, 2002) suggesting that this release occurs in the autumn rather than spring as a result of higher water temperatures than those corresponding with the spring bloom. This also allows the adults of the species to assimilate energy by feeding on the phytoplankton in the blooms during the summer in order to produce gametes. This large increase in the late summer is known from historical records (Lebour, 1938) that show, for example, the larvae of *Lima hians* (Gmelin) displaying the most pronounced change in abundance. *L. hians*, however, has disappeared from the meroplankton and benthic

communities during the intervening years but the peak in bivalve larval abundance has occurred annually during the time-series at Station L4 and analyses of anomalies show no significant trend of change over the same period.

Variation in community composition throughout the year is a result of the different spawning triggers for the species that comprise the five groups under study. The species in these groups often possess differing ecologies and vary in the time scales in which they appear in the water column. This determines the observed changes in the composition of the meroplankton community throughout the year. The seasonal cycles of the five groups examined at L4 exhibit low numbers followed by rapid increases during the spring and, in some cases, autumn months before declining towards the winter period. There is significant seasonal variation in the composition of meroplankton community at Station L4 over the year in terms of the five groups studied, with Cirripedia being the dominant member of the community in March and April, and Bivalvia being the dominant member in terms of abundance in September and October.

Multivariate analysis shows that seasonal variation accounts for the majority of variability in community composition at the study site (Figures 3.8 & 3.9) rather than inter-annual variability, as the same months from each of the 20 years tend to be clustered together on the MDS plot. Two-way crossed ANOSIM tests support this conclusion with significant intra-annual variability occurring and little inter-annual variation being shown. Similar patterns of long-term variability have been shown in other studies where seasonal variation was more pronounced than inter-annual variation which, in terms of species richness and diversity, remained relatively constant despite a few species showing overall changes in abundance (Pitois *et al.*, 2009).

Cirripedia abundance corresponded with the spring bloom and thus the associated increase in sea surface temperature recorded over the time series, while bivalve and echinoderm larvae exhibited their highest numbers during the autumn bloom. This bloom is associated with nutrient release caused by increased vertical mixing and the breakdown of stratification in the water column as a result of the onset of cooling and increased wind action (Findlay *et al.*, 2006). Light levels remain high enough not to inhibit photosynthesis and phytoplankton grazer populations are diluted by the vertical mixing, thus promoting the onset of the autumn bloom. Decapod and polychaete larvae appear to have less correlation with sea surface temperature as their numbers start to increase as the temperature is still falling in February. However, the highest abundances in both of these groups occur when sea surface temperature is approaching its maximum. Previous studies have highlighted the correlation between timing of peak abundance and sea surface temperature for each of the groups in this study (Edwards & Richardson, 2004).

Photoperiod duration plays an important role in the timing of larval release (Macho *et al*, 2005) as cirripedes are known to release larvae during daytime hours in large numbers to induce larval aggregation which, through swamp effect over predators, increases larval survival. Therefore the increasing day-length during spring combined with an ample food source provided by phytoplankton blooms enhances the chances of larval survival (Macho *et al*, 2005). Lunar cues trigger spawning events in marine organisms. The cirripede, *Chthamalus,* is known to release larvae during spring tides which occur in conjunction of full and new moons, and certain decapod larvae that are spawned during the largest amplitude lunar tides are transported by ebb currents to deeper waters where predation is reduced thus increasing chances of survival (Christy, 1986).

The coupling between phytoplankton abundance and the release of meroplankton larvae is a driving force in the seasonal cycles of these organisms and, in turn, is linked to a number of factors including sea surface temperature, nutrient cycling, physical processes such as wind, currents, and stratification of the water column (Findlay *et al.*, 2006; Kirby *et al.*, 2008). Various biological factors such as the trophic interactions between different species, predation, competition, larval mortality and dispersal also play a major role in structuring meroplankton communities (Eckman, 1996; Todd, 1998; Bhaud, 2000).

The availability of long-term data-sets such as that from Station L4 allows us to study the temporal variability in the taxa recorded and provides a valuable insight into the long-term cycles in their life-histories. Such long-term time series data can provide insights into short- and long-term patterns of abundance and community change in a given area, and so can be used to study long-term zooplankton dynamics (Pitois *et al.*, 2009; Batten & Burkill, 2010) and can be used to detect evidence of possible shifts in the timing of larval release by marine benthic organisms. There was no evidence in this study, however, of such a shift in any of the groups studied. Long-term data-sets also facilitate phenological studies such as those in the North Sea (Greve *et al.*, 2005) and of the Continuous Plankton Recorder (Edwards & Richardson, 2004) in the North-West Atlantic. It is important to continue this time-series in order to further understand changes that occur over a longer time period than 20 years and to allow us to monitor the effects of possible climate change, northward expansion of southern species' distribution, and the contraction of northern/ arctic species' distribution as is being observed elsewhere today (Greve *et al.*, 2005).

CHAPTER 4. SEASONAL DYNAMICS OF THE MEROPLANKTON ASSEMBLAGE AT STATION L4 2008-2010.

4.1. INTRODUCTION.

It has been previously mentioned that little thought has been given to the study of meroplanktonic larvae and their seasonal life cycles until recently, where analyses of meroplankton time-series have started to show evidence of coupling between meroplanktonic larvae and the biological and physical factors affecting them (Fernandez *et al*, 2012). These studies and those detailed in Chapter 3 reveal the potential for understanding patterns of meroplankton abundance by examining long-term datasets.

Records from the environs of Plymouth Sound and surrounding areas were absent for a number of years since the studies of Lebour described data derived from qualitative plankton samples collected from within the Plymouth Sound breakwater during the years 1940-45 (Lebour, 1947). This was the first time that such detailed notes on the occurrences of meroplanktonic larvae were published. These qualitative data were reported in the form of presence/absence in conjunction with anecdotal observations as to the occurrences and abundance of the larvae based on some earlier studies (Lebour, 1927; 1928a; b; 1930; 1931; 1936; 1937a; b; 1938; 1940; 1943; 1944). The data from these earlier studies comprise both larvae sampled with tow-nets, and those hatched from egg-carrying adults collected from the surrounding area and grown under laboratory conditions. Chapter 1 describes these observations and the full dataset is displayed in Appendix 1. They show an indication of the different breeding seasons of the various meroplanktonic organisms present. Although most of the data are derived from the plankton samples from within the Plymouth Sound breakwater, anecdotal evidence of larval occurrences from outside the breakwater are also detailed. These data are described in those records as either inshore or offshore.

The Plymouth Marine Fauna (Marine Biological Association of the United Kingdom, 1957) was a detailed report of the distribution and comparative abundance of the marine species inhabiting Plymouth Sound and the surrounding areas. It updated the previous versions published in 1904 and 1931 and contained many of the records detailed in the studies of Lebour. These resources provide an indication of the meroplankton community at the time and represent the last occasion that such detailed reports were made.

Chapter 3 detailed the analysis of meroplankton data derived from samples from the Western Channel Observatory during the years 1988-2007 and revealed the patterns of seasonal abundance that typified the meroplanktonic organisms in the study area. This study highlighted the need for long-term meroplankton time-series in order to understand the life-cycles of several meroplankton groups that contained both commercially important species and those that play a vital role in marine ecosystems and food webs. This analysis was limited to five groups defined at a broad taxonomic level (Bivalvia, Cirripedia, Decapoda, Echinodermata, and Polychaeta) and revealed considerable seasonal variation in the abundance of these groups. This level of taxonomic resolution limited the patterns of seasonal and annual variation that could be revealed by this analysis. Other studies have used this level of resolution and showed similar results (Fernandez *et al*, 2012).

The object of the study described in this chapter was to gain further insight into the composition of the meroplankton community of the Plymouth area today by increasing the level of taxonomic resolution of the larvae sampled. This allowed for the patterns of seasonal abundance and species composition of the meroplankton community at Station L4 to be further identified. A higher taxonomic resolution allowed for groups not recorded in the 1988-2007 time-series to be examined and this demonstrates its potential for revealing further patterns of interannual variation in

meroplanktonic communities around the world. This supplements the picture developed in Chapter 3, and, by comparison with the data described by Lebour in 1947, provides an indication of any change in the meroplankton community over longer periods of time that was not evident in the 1988-2007 Station L4 time-series data which showed little inter-annual variability.

4.2. MATERIALS AND METHODS.

4.2.1. Field sampling.

Prior to the main sampling effort, it was necessary to determine the most suitable mesh size for the survey. Therefore, samples were taken at Station L4 on the research vessel *Plymouth Quest* using three nets with different mesh sizes: a "coarse" net (710µm mesh) taken on 28th January 2008, a "medium" net (335µm mesh) taken on 13th February 2008, and a "fine" net (142µm mesh) taken on 28th January 2008. These nets were each towed at a fixed depth of 10m for 10 minutes at 1.5 knots. The samples were washed into cool boxes with fresh seawater in order to keep the organisms alive until they could be returned to the laboratory. The samples were then sieved through a 100µm mesh sieve and fixed in 4% formaldehyde solution overnight. They were then washed and sieved to remove any residual formalin and transferred to 70% ethanol. All meroplanktonic organisms were picked from the samples and then identified to the lowest possible taxonomic level. They were then counted and recorded using a stainless steel tally counter (Table 4.I).

From 19th May 2008, monthly zooplankton samples were collected at Station L4, weather permitting, from the research vessel, *Plymouth Quest* with a plankton tow net (335µm mesh,

440mm diameter) using the same sampling, preservation, and processing protocols described previously. These samples were collected on a monthly basis with some samples collected on a more regular basis such as wekly, fortnightly, and every three weeks owing to weather complications. The meroplanktonic larvae from each sample were stored in 70% ethanol in separate labelled scintillation vials. This sampling effort was continued until 21st May 2009.

Further sampling was conducted whilst collecting bivalve larvae for use in molecular experimentation from 26th October 2009 to 23rd March 2010. Sampling was conducted during these months as they represent the time-period with the highest bivalve larval numbers recorded in the earlier part of the survey despite these months having the lowest overall numbers of meroplanktonic larvae. 96 bivalve larvae were removed from these trawls before the remainder of the sample was picked for meroplanktonic larvae. These numbers were recorded and, in the case of bivalve larvae, were added to the numbers that were removed for molecular analysis. This generated a further 5 months of larval counts that have been included in the analysis for the main time-series data in order to understand monthly and seasonal patterns of meroplanktonic larval abundance. A 10 minute tow with a 440 mm diameter net at 1.5 knots (0.77 m.s^{-1}) sampled 69.6 m^{3} of water, so species counts were divided by this factor to convert them to densities.

4.2.2. Data analysis.

For each of the groups under study, graphs showing the seasonal densities in N m^{-3} were created. The historical data derived from the records of Lebour (1947) were converted to a numerical abundance scale: 0 = absent; 1 = rare; 2 = common; 3 = abundant. This represented the descriptive observations made. This method was limited but was felt to be the most suitable manner of

interpreting the original observations in a fashion that could be compared to the more recent numerical data. In order to compare the time-series data with the historical data, total counts were converted to a numerical abundance scale: absent = 0; 1-10 individuals per sample = 1 (rare); 11-100 individuals per sample = 2 (common); 101+ individuals per sample = 3 (abundant). Due to the larger number of species and genera observed by Lebour, only those directly comparable to the time-series data were used in the analysis with several species and genera being amalgamated into the groups detailed in the 2008-2010 data-set.

4.2.3. Statistical analysis.

The PRIMER software package was used for statistical analysis. Nonparametric multivariate methods were used to analyse density data (Clarke, 1993; Clarke & Warwick, 2001; Clarke & Gorley, 2006). Prior to calculating resemblances among samples using the Bray-Curtis coefficient, the contributions of dominant species were weighted using a range of transformations (Clarke *et al.*, 2006). Nonmetric multidimensional scaling (MDS) was used to visualise the relationships among samples in 2 dimensions. An indication of the success of the algorithm in reproducing the rank order of resemblances in the full multidimensional matrix in 2 dimensions was given by a stress value, calculated using Kruskal's stress formula 1. All plots in this study have stress values <0.2. Ordination plots with stress < 0.1 correspond to plots giving an excellent to good representation, while plots with stress values >0.1 but <0.2 give a useful picture, though small details should not be over interpreted (Clarke, 1993). The significance of differences among groups of samples was tested using analysis of similarities (ANOSIM). The non-parametric mantel test RELATE was used to test the significance of correlations between resemblance matrices and the contributions of taxa

to resemblances among samples were examined using 1- and 2-way similarity percentages breakdown (SIMPER).

4.3. RESULTS.

4.3.1. Determination of optimum mesh size.

The medium-mesh net was determined to be the most suitable for use in the survey as the processing time to sample yield ratio was considered to be the most efficient. A smaller number of larvae were sampled than with the fine mesh (Table 4.I), but the richness of the fine-mesh sample resulted in a considerably longer processing time. This led to a considerably lower larval yield to processing time ratio. The coarse-mesh net had the lowest processing time but did not sample sufficient larvae and missed some groups entirely. The medium mesh was considered to adequately sample meroplanktonic larvae in the water column while producing a practical sample processing time. Despite the higher larval numbers and species diversity yielded by the fine mesh, which would have generated a more complete picture of the meroplankton assmeblage, the duration of the sample processing time was considered too long for its use for the time constraints of this survey. All subsequent results are derived from sampling with a medium tow net. As these samples were taken in January and February, there is likely to have been a seasonal effect on the data produced as meroplankton numbers are at their lowest during this time period. This further precluded the use of the fine mesh as sample processing times would have been considerably longer in the months with a higher abundance of meroplankton in the water column. It was considered that the modest number of bivalve larvae recorded from the medium mesh net was due in part to the time of year that

sampling was conducted. It was fully expected that bivalve larvae would be sufficiently sampled at different times of the year.

Table 4.I. The total numbers of meroplanktonic larvae recorded from the three different mesh sizes tested.

	28/01/2008 Fine mesh (142 µm)	**13/02/2008 Medium mesh (335 µm)**	**28/01/2008 Coarse mesh (710 µm)**
Decapoda (Brachyura) Zoea	716	497	238
***Ebalia* zoea**	0	7	0
Caridea	41	47	36
Galathea	66	11	48
Upogebia	1	0	0
Gastropoda larvae	73	12	0
Bivalvia larvae	1	0	0
Cnidaria	0	5	0
Total	898	579	322

4.3.2. Time series raw data.

The data were converted to density in the form of N m^{-3} (Table. 4II). The original 2008-2010 time-series data were recorded in the form of total number of larvae per sample and can be found in Appendix 3.

Table 4.II. Densities (N. m^{-3}) of each of the meroplankton groups recorded during the 2008-2010 Station L4 Time-series using a 335 µm mesh net.

Species	19/05/2008	23/06/2008	28/07/2008	20/08/2008	22/09/2008	29/09/2008	29/10/2008	26/11/2008	13/01/2009	11/02/2009	02/03/2009	23/03/2009	27/04/2009	21/05/2009	26/10/2009	01/12/2009	15/12/2009	04/01/2010	01/02/2010	01/03/2010	23/03/2010
Decapoda (Brachyura) zoea	1.466	34.66	0.39	2.23	0.561	4.47	0.776	0.072	0.489	10.795	29.711	11.629	9.501	29.438	4.086	0.086	0.172	1.121	7.661	1.581	1.121
Decapoda (Brachyura) megalopa	0.029	0.216	0	0	0	0	0.115	0	0.014	0.029	0	0	0.23	0.848	0.086	0	0	0	0	0	0
Necora zoea	0	0	0	0	0	0	0	0	0	0	0	0.719	0.043	3.205	0.014	0	0	0	0.201	0.014	0
Paguridae zoea	0.014	0.101	0	0	0.172	0	0	0	0.331	2.271	2.041	1.049	0.115	0.129	0.172	0.158	0	0.546	0.345	0	0.071
Ebalia zoea	0	0	0	0.014	0.057	0	0.158	0	0.201	0.359	0.072	0.129	0.043	0	0.029	0.014	0	0.101	0.273	0	0
Porcellanid zoea	0.043	6.827	0	0.014	0.086	0.517	0.029	0.014	0	0	0.014	0.201	0.834	0.029	0.014	0	0	0	0	0	0
Caridea	0.144	0.517	0.22	0.862	0.546	2.214	2.932	0.331	0.331	0.891	0.747	0.043	0.69	0.201	6.310	0.129	0.029	0.345	0.172	0.029	0.029
Galathea	0.172	0.719	0	0	0.287	0.029	0.086	0	0.101	0.906	1.351	1.179	1.768	0.402	0.331	0	0.029	0	0.244	0.057	0.086
Upogebia	0.561	1.294	0.6	2.228	1.308	1.739	0.934	0.043	0.014	6	0.115	0.014	1.351	2.458	0.920	0	0.043	0	0	0	0.029
Callianassa	0	0.029	0.014	0	0	0.043	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cirripedia nauplii	0	0.029	0.086	11.341	0.834	0.043	4.37	0.014	0.029	0	0	0.043	0	0	5.247	0.014	0	0	0	0	20.770
Cirripedia cyprid	0	0	0.043	0	0.086	0	0.057	0	0	0	0	0	0	0	0.086	0	0	0	0	0	0
Gastropoda larvae	0.216	0.057	0.604	0.575	0.977	10.997	0.302	0.187	0.187	0.072	0.144	0.201	0.503	0.029	0.604	0	0.014	0.029	0.144	0.029	1.049
Bivalva larvae	0	0	0.129	0.776	9.084	37.301	31.364	0.043	0.014	0	0.014	0	0	0	0.057	0.057	0.043	0.201	0.043	0	0
Mussel larvae	0	0	0	0	0	0	0	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0
Cnidaria	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0	0	0	0	0	0
Polychaete larvae	0.101	0	0.014	0	0	0	0.014	0.043	0	0	0.029	0.144	1.107	0.057	0	0	0	0.014	0.345	0	0.086
Lagis koreni larvae	0	0.014	0	0	0	0	0	0	0	0	0	0	0.043	0	0	0	0	0	0	0	0
Obelia medusa	0	0	0	5.62	0	0.014	0	0.201	0	0	0	0	0	0	0	0	0	0	0	0	0
Echinoderm ophioplutei	0	0.014	0.014	0.043	0.115	0.043	0.115	0	0	0	0	0.086	0.014	0	0	0	0	0	0	0.014	0.273
Echinoderm auricularia	0	0	0	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Echinoidea larvae	0	0	0	0	0.029	0	0	0	0	0	0	0	0.057	0	0	0	0	0	0	0	0
Asteroidea brachiolaria	0	0	0	0	0.043	0	0	0	0	0	0.014	0	0	0	0.762	0	0	0	0	0	0
Asteroidea	0	0	0	0	0.029	0	0.014	0	0	0	0	0	0.014	0	0	0	0	0	0	0	0
Total Meroplankton	2.746	44.477	2.114	23.703	14.228	57.410	41.266	0.948	1.725	21.323	34.252	15.437	16.313	36.810	18.718	0.458	0.330	2.357	9.428	1.724	23.514

4.3.2. Seasonal cycles of meroplankton groups.

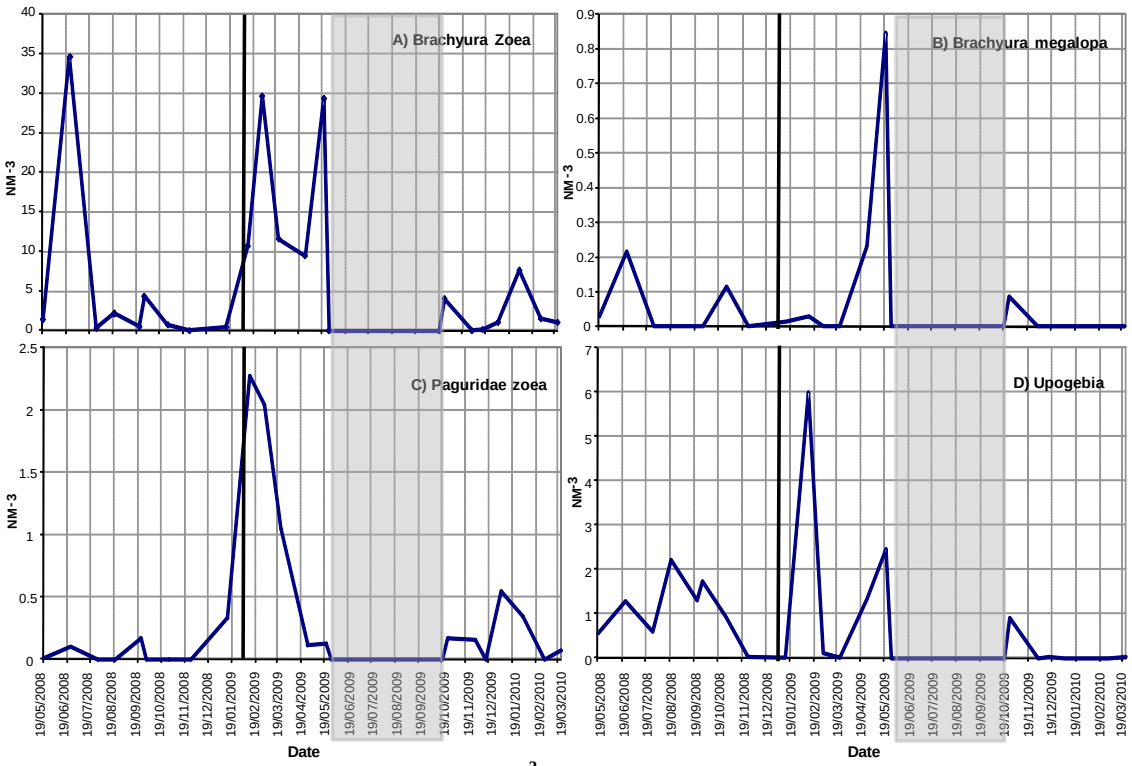

Figure. 4.1. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) Brachyura zoea, B) Brachyura megalopa, C) Paguridae zoea, and D) *Upogebia* at Station L4 from May 2008 – March 2010. Black line indicates year change in the time series. Shaded area indicates no data collected.

The seasonal cycle of Brachyura zoea (Fig. 4.1A) was characterised by large densities (34.66 individuals m^{-3}) during the spring months followed by a sharp decline over summer in 2008 until low densities were reached during the winter months before numbers increased again in spring. Brachyura megalopae (Fig. 4.1B) were observed in low numbers throughout the time series, with a peak in density of 0.848 individuals m^{-3} being recorded in May 2009. Paguridae zoea (Fig. 4.1C) occurred in low numbers throughout the year with the late winter months seeing the largest densities (2.271 individuals m^{-3} in February 2009) before they exhibited a sharp decline in spring where the usual low numbers resumed. The larvae of *Upogebia* were present in the meroplankton in every month of the year. Their seasonal cycle (Fig. 4.1D) showed large

densities in the early spring months (6 individuals m^{-3} in February 2009), followed by smaller increases over the summer months before they decreased over autumn and reached their lowest densities during winter.

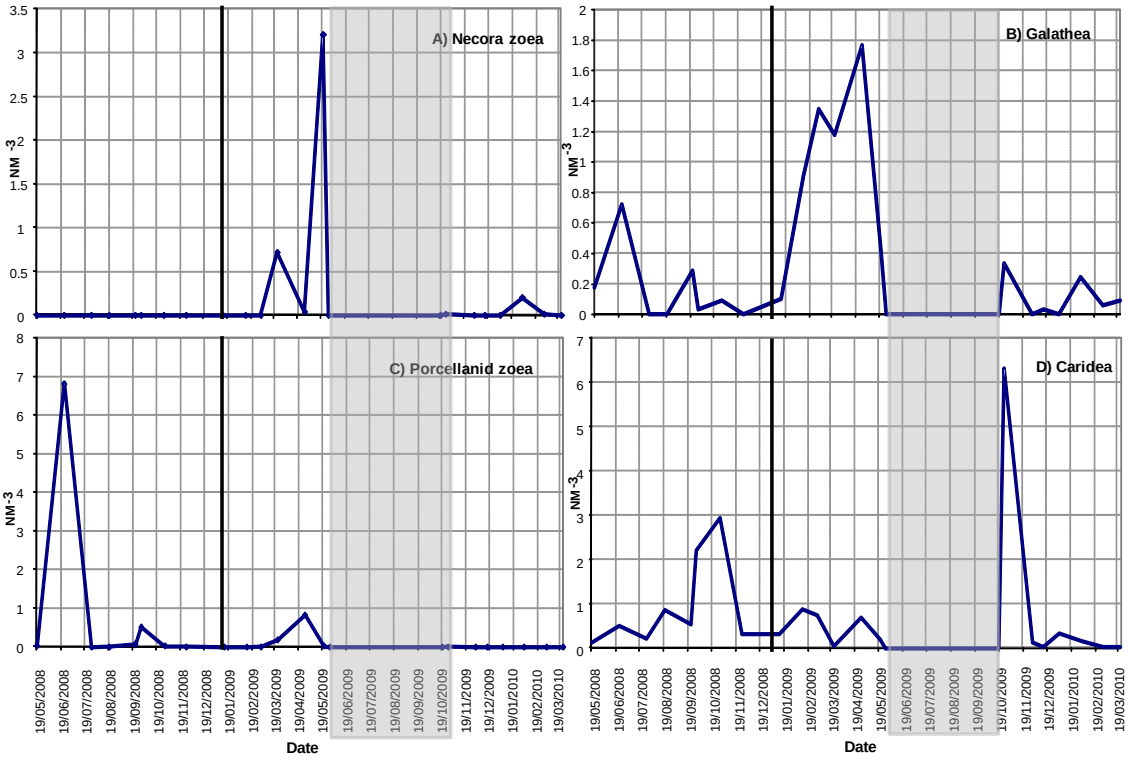

Figure. 4.2. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) *Necora* zoea, B) *Galathea*, C) Porcellanid zoea, and D) Caridea at Station L4 from May 2008 – March 2010. Black line indicates year change in time-series. Shaded area indicates no data collected.

The seasonal cycle of *Necora* zoeae (Fig. 4.2A) was characterised by low densities for most of the year until the spring months when densities increased, reaching 3.205 individuals m^{-3} during May 2009. This peak was the highest level that the numbers of zoeae reached during the survey. The seasonal cycle of the larvae of *Galathea* (Fig. 4.2B) was similar to that of *Upogebia* larvae with the largest densities being recorded during spring (1.768 individuals m^{-3} in April 2009). They occurred throughout the summer months in lower numbers before they decreased in winter. Porcellanid zoeae (Fig. 4.2C) occurred in their highest densities during the spring months (6.827

individuals m^{-3}) before they sharply decreased over summer and into the winter months. The larvae of the infraorder Caridea (Fig. 4.2D) were observed in every month throughout the time series and the seasonal cycle was characterised by a low density during winter followed by increases through the spring and summer months before their highest numbers were reached in late summer and early autumn (6.31 individuals m^{-3} in October 2009), before they fell again during winter.

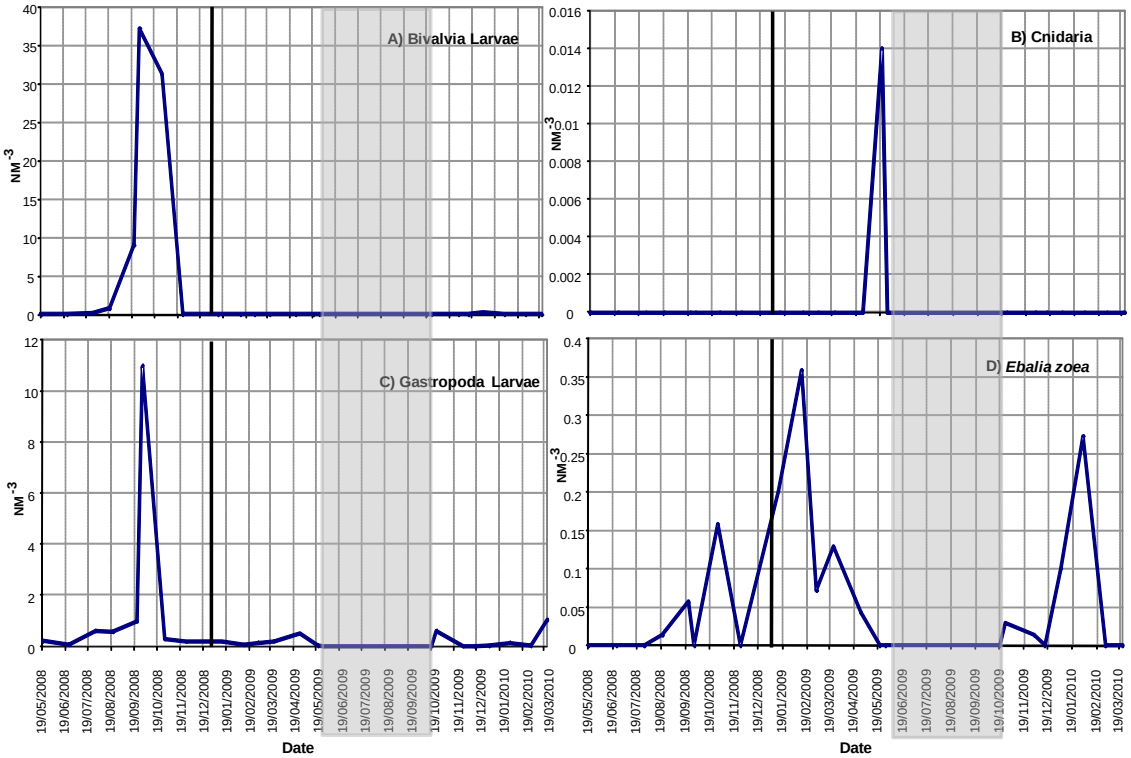

Figure. 4.3. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) Bivalvia larvae, B) Cnidaria medusae, C) Gastropoda larvae, and D) *Ebalia* zoea at Station L4 from May 2008 – March 2010. Black line indicates year change in the time series. Shaded area indicates no data collected.

Bivalve larvae (Figure 4.3A) appeared in the meroplankton in July, where they were recorded in low numbers that gradually rose until a large increase was observed at the end of September where a density of 37.3 individuals m^{-3} was recorded. This peak in density continued throughout October when numbers started to decrease before the

larvae disappeared in February and remained absent during the spring months. An unidentified hydroid medusa (Fig. 4.3B) was only recorded once during the time series, in May 2009. The seasonal cycle of Gastropoda larvae (Fig. 4.3C) was similar to that of Bivalvia with low numbers observed for most of the year before a large increase occurred in September when density reached 10.997 individuals m^{-3} before the numbers of larvae decreased during the winter months. The zoeae of the genus *Ebalia* (Fig. 4.3D) occurred in low numbers throughout the time series and appeared in the meroplankton from late summer to early spring. The highest densities of *Ebalia* zoea were recorded in February 2009 (0.359 individuals m^{-3}).

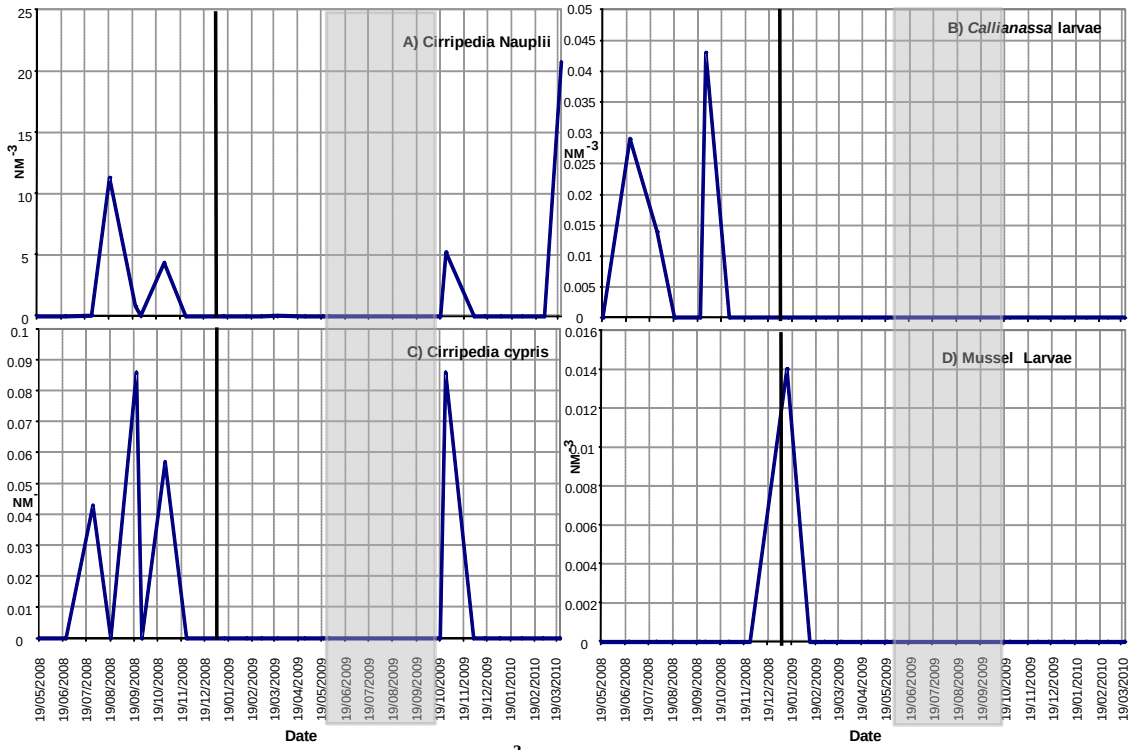

Figure. 4.4. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) Cirripedia nauplii, B) *Callianassa* larvae, C) Cirripedia cypris larvae, and D) Mussel larvae at Station L4 from May 2008 – March 2010. Black line indicates year change in the time series. Shaded area indicates no data collected.

Cirripedia nauplii (Fig. 4.4A) were recorded in much larger densities than Cirripedia cyprid larvae (Fig. 4.4C). Cirripedia nauplii occurred in their largest numbers in the

spring months where they became the dominant member of the meroplankton community at Station L4 with the highest density of 20.77 individuals m^{-3} recorded in March 2010. They were observed in lower numbers during the summer, before they decreased during the autumn months until they were absent from the meroplankton during winter. *Callianassa* larvae (Fig. 4.4B) were only observed in very low numbers in the summer months of 2008. Cirripedia cyprid larvae (Fig. 4.4C) were only recorded during the summer and autumn months in low numbers before disappearing during the winter. Mussel larvae (Fig. 4.4D) were recorded once during the time-series in January 2009.

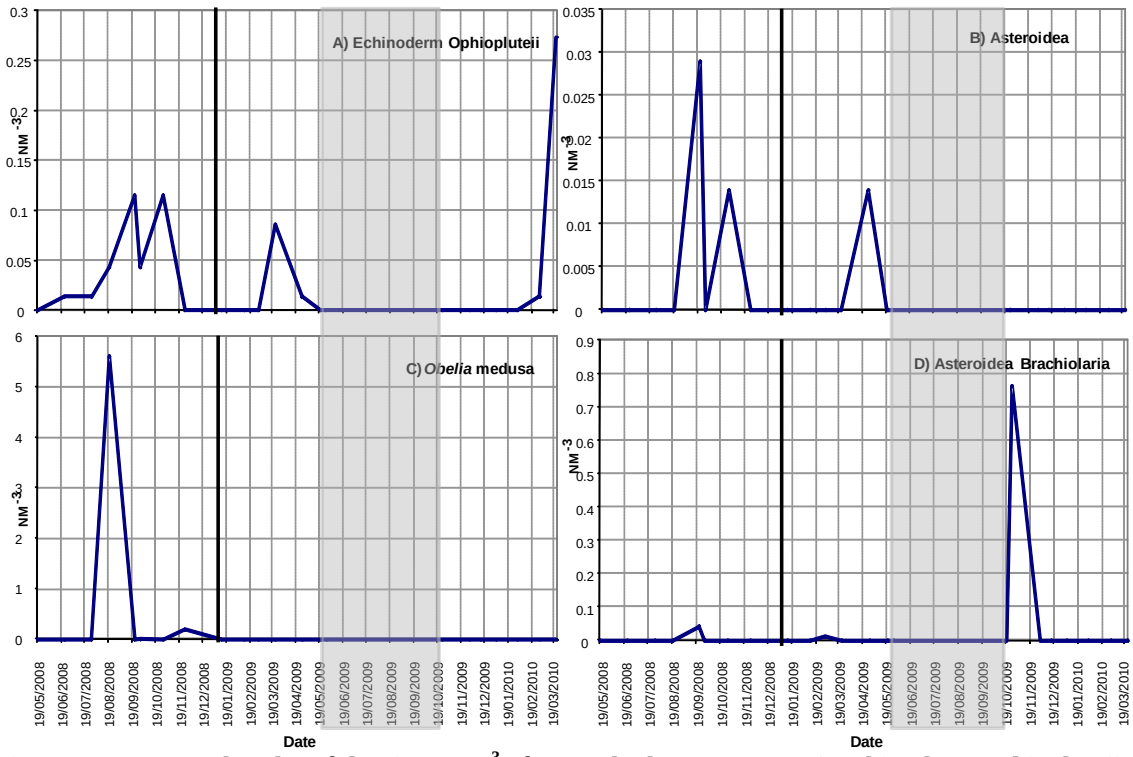

Figure. 4.5. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) Echinoderm ophiopluteii, B) Asteroidea, C) *Obelia* medusa, and D) Asteroidea brachiolaria at Station L4 from May 2008 – March 2010. Black line indicates year change in time-series. Shaded area indicates no data collected.

Echinoderm ophiopluteii (Fig. 4.5A) were recorded during the spring, summer and early autumn months of the time-series in low numbers with the highest density of 0.273

individuals m^{-3} in recorded in March 2010. Asteroidea larvae (Fig. 4.5B) occurred in low numbers in the spring and autumn months. Medusae of the genus *Obelia* (Fig. 4.5C) were observed in late summer and autumn with the highest density of 5.62 individuals m^{-3} recorded in August 2008. Asteroidea brachiolaria (Fig. 4.5D) were recorded in low densities in spring and autumn.

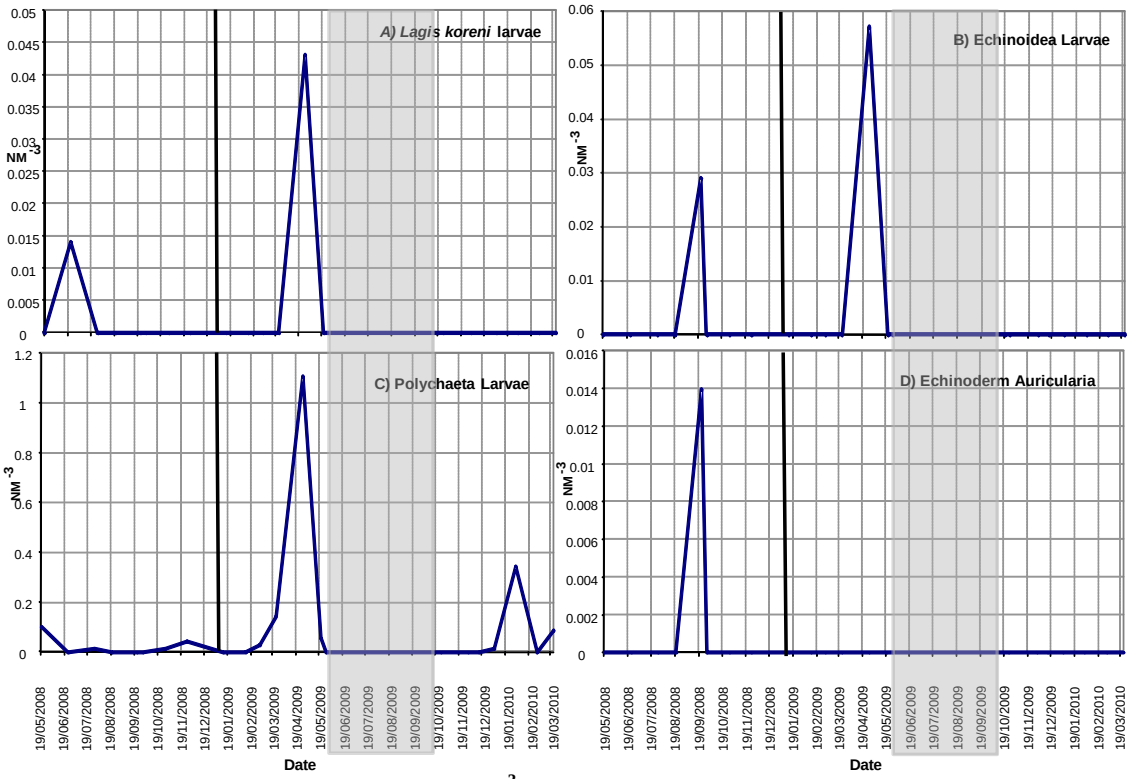

Figure. 4.6. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) *Lagis koreni* larvae, B) Echinoidea larvae, C) Polychaeta larvae, and D) Echinoderm auricularia at Station L4 from May 2008 – March 2010. Black line indicates year change in time-series. Shaded area indicates no data collected.

The larvae of the polychaete *Lagis koreni* (Fig. 4.6A) were recorded on two occasions in the spring and early summer. Echinoidea larvae (Fig. 4.6B) were only recorded in low numbers in September 2008 and April 2009. Unidentified polychaete larvae (Fig. 4.6C) were recorded in most months of the survey with the exceptions of August, September, and December. They occurred in low numbers during the months that they

were observed, with February 2010 seeing the highest density of 0.345 individuals m^{-3}. Due to the number of different species liable to comprise the polychaete larvae sampled, it is not possible to determine a seasonal cycle in this case. Finally, echinoderm auricularia larvae (Fig. 4.6D) were only observed once, in September 2008.

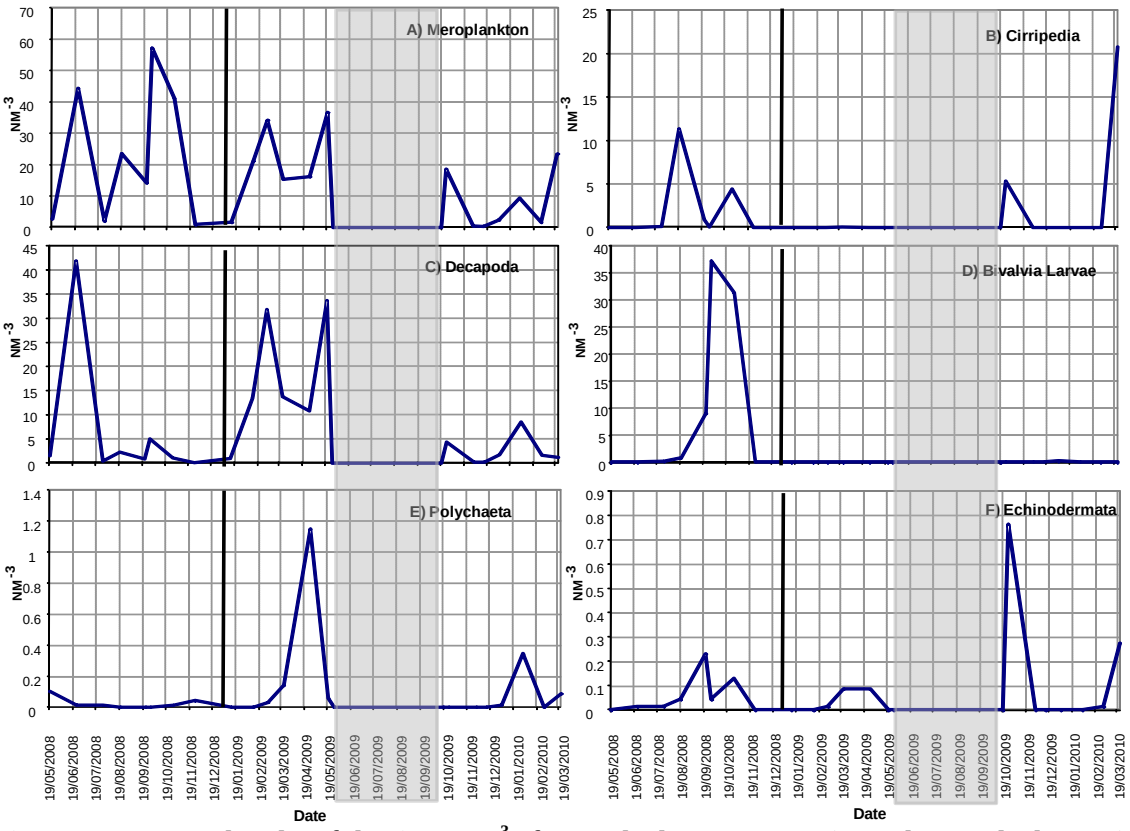

Figure. 4.7. Seasonal cycles of density N. m^{-3} of meroplankton groups: A) Total Meroplankton, B) Total Cirripedia, C) Total Decapoda, D) Total Bivalvia, E) Total Polychaeta, and F) Total Echinodermata. Black line indicates year change in time-series. Shaded area indicates no data collected.

Seasonal cycles of abundance of total meroplankton, Cirripedia, Decapoda, Bivalvia, Polychaeta, and Echinodermata (Fig. 4.7A-F) were constructed in order to see if the patterns observed in the 20 year time-series at Station L4 in the broad taxonomic groups discussed in Chapter 3 were applicable in the following years in this time-series.

The seasonal cycle for total meroplankton (Fig. 4.7A) was characterised by large densities in the spring months which corresponded with the associated spring phytoplankton bloom and is likely to have been determined by the concurrent abundance of decapod larvae (Fig. 4.7C) at this time. A large increase followed during summer, and the largest observed density was in September 2008 when 57.41 individuals m^{-3} were recorded. This was likely to be a result of the spikes in the densities of both Bivalvia (Fig. 4.7D) and Gastropoda larvae (Fig. 4.3C) that have been shown to occur every year around this time at Station L4.

Total Cirripedia (Fig. 4.7B) exhibited the same pattern as described earlier for Cirripedia nauplii (Fig. 4.4A) due to the large numbers of nauplii recorded driving this cycle in comparison to the relatively low occurrences of cyprid larvae. The seasonal cycle for total decapod larvae (Fig. 4.7C) was typified by large densities during the spring and summer months followed by a sharp decline towards winter where recorded numbers were low. The seasonal cycle for Bivalvia (Fig. 4.7D) has already been discussed with the massive densities seen during the late summer and autumn months being the dominant feature of the seasonal cycle. The seasonal cycle for total polychaete larvae (Fig. 4.7E) was dominated by a massive increase in abundance during the spring months of 2009 and a smaller increase during the spring of 2010. Polychaete larvae occurred in low numbers for the rest of the time-series. Total Echinodermata larvae (Fig. 4.7F) were most common during the summer and autumn months with the largest density observed in October 2009. This was followed by a steep decrease during winter that led onto an increase in abundance in spring.

4.3.3. Statistical analysis.

4.3.3.1. Statistical analysis of the 2008-2010 data.

The density data was subjected to a fourth-root transformation to reduce the weighting of the more abundant meroplankton groups in the study, particularly the brachyuran larvae, and plotted in an MDS ordination (Fig. 4.8) using the Bray-Curtis similarity matrix. A degree of seasonality can be seen within the data with the same months being grouped together although this would be evident if a similar pattern was seen in 2011. There are differences between the same months in consecutive years but this is to be expected due to the annual variability in the abundances exhibited by the meroplankton groups in this study. An alternative explanation for this would be the presence of auto-correlation, with the same meroplankton population potentially being sampled in consecutive sampling trips.

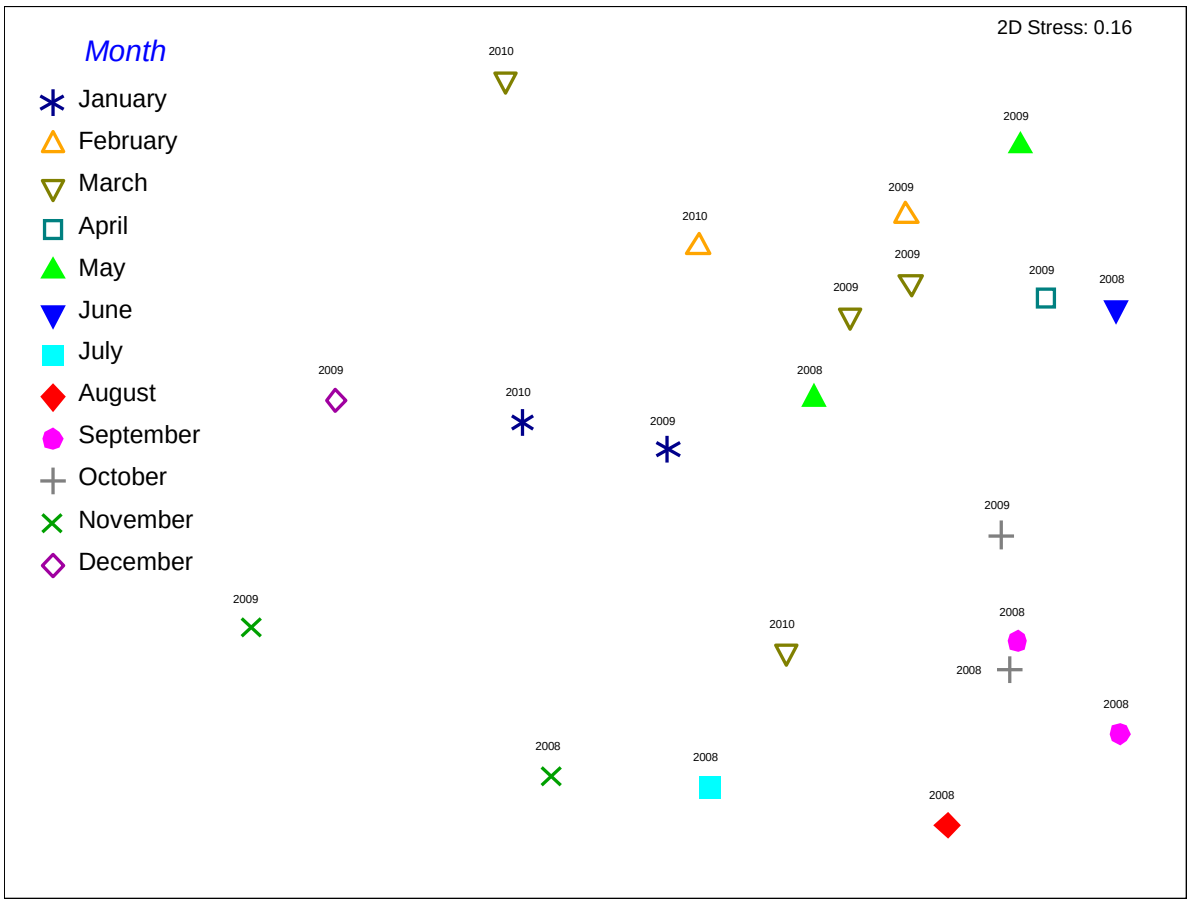

Figure 4.8. MDS ordination based on Bray-Curtis similarities calculated using fourth-root transformed densities from the 2008-2010 meroplankton Station L4 data.

Larval densities were at their highest during the spring, summer and autumn months (Fig. 4.9). There appear to be two distinct major groups in the data: a spring group and a late summer/ autumn group. The MDS ordination displaying taxon number (Fig. 4.10) shows that the highest numbers of taxa were recorded in September, with the highest number of taxa observed being recorded during the same months as the highest larval densities. Simprof and cluster analysis (Fig. 4.11 & 4.12) reveal the presence of four groups of samples within the data and confirm that the spring and late summer/ autumn groups are the largest and most closely related. Other groupings are present but are not statistically different from one another. The two groups (C & D) contain the months that represent the two largest periods of larval density for meroplanktonic organisms (Fig. 4.12).

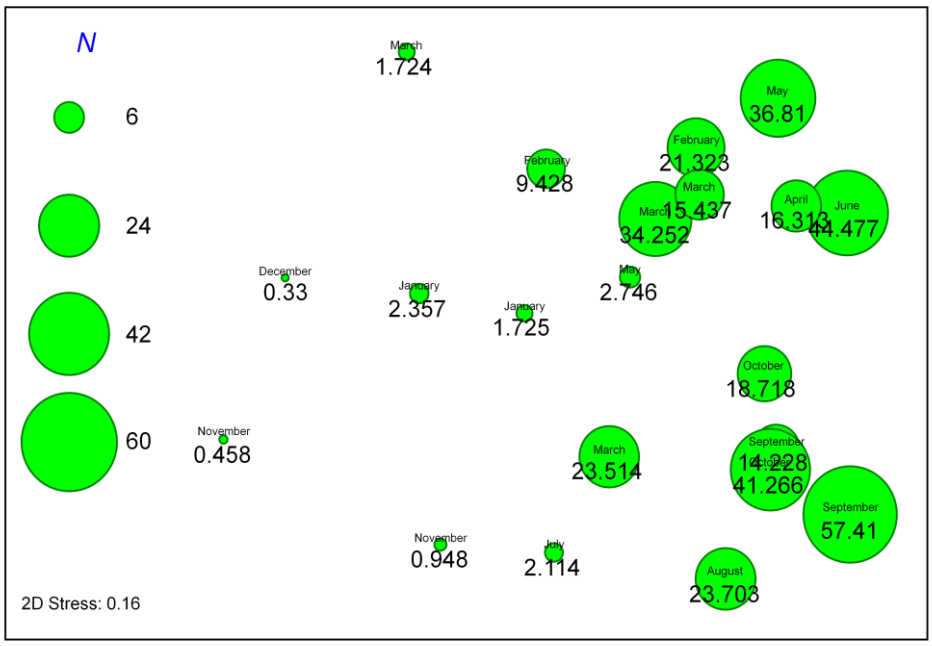

Figure 4.9. MDS ordination based on Bray-Curtis similarities calculated using fourth-root transformed densities from the 2008-2010 meroplankton Station L4 data, with larval densities (N) overlaid.

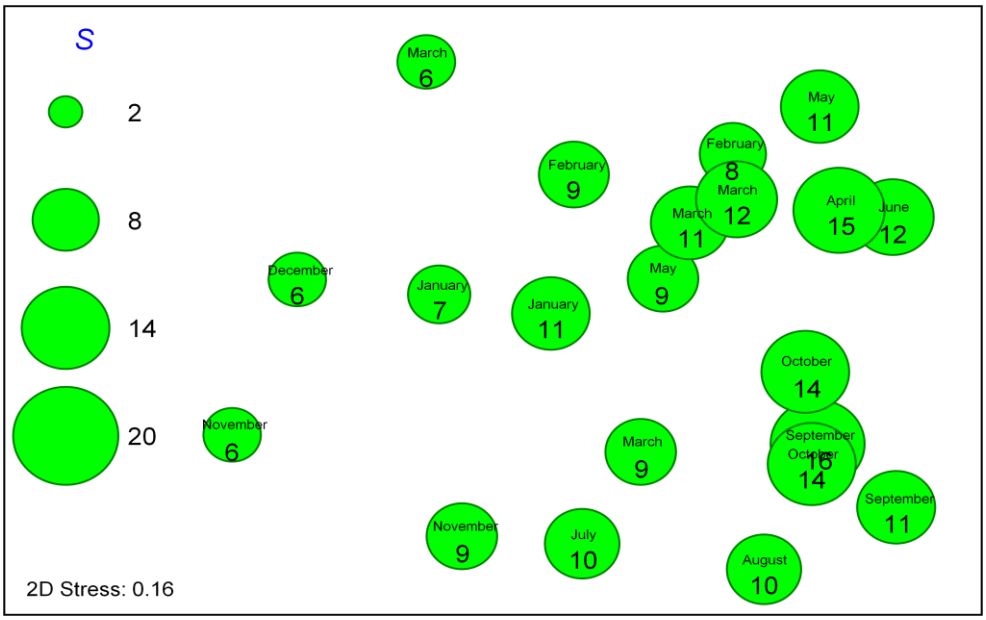

Figure 4.10. MDS ordination based on Bray-Curtis similarities calculated using fourth-root transformed densities from the 2008-2010 meroplankton Station L4 data, with taxa numbers (S) overlaid.

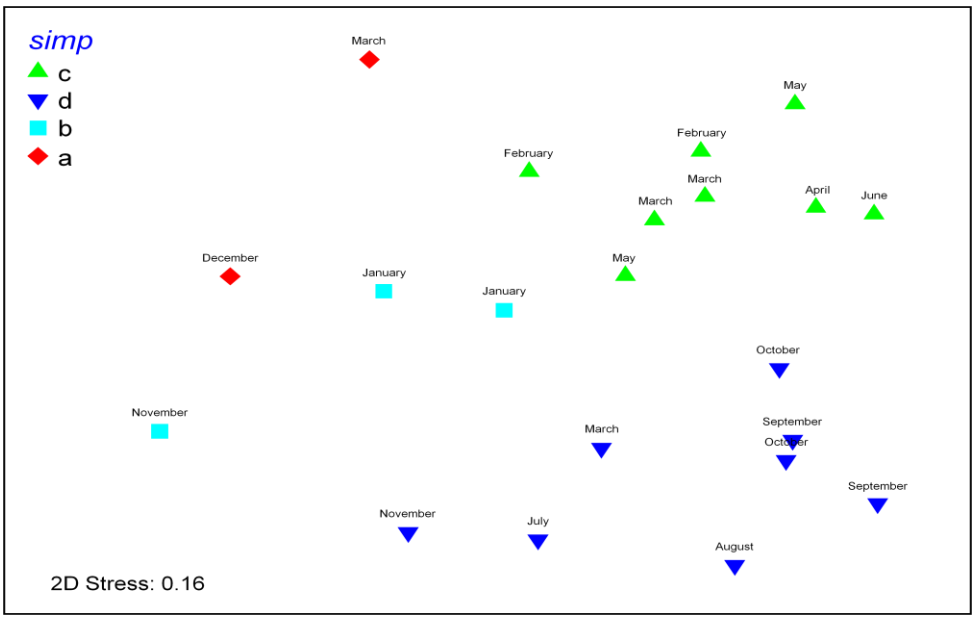

Figure. 4.11. MDS ordination displaying the results of SIMPROF analysis showing the presence of four groups within the 2088-2010 Station L4 density data.

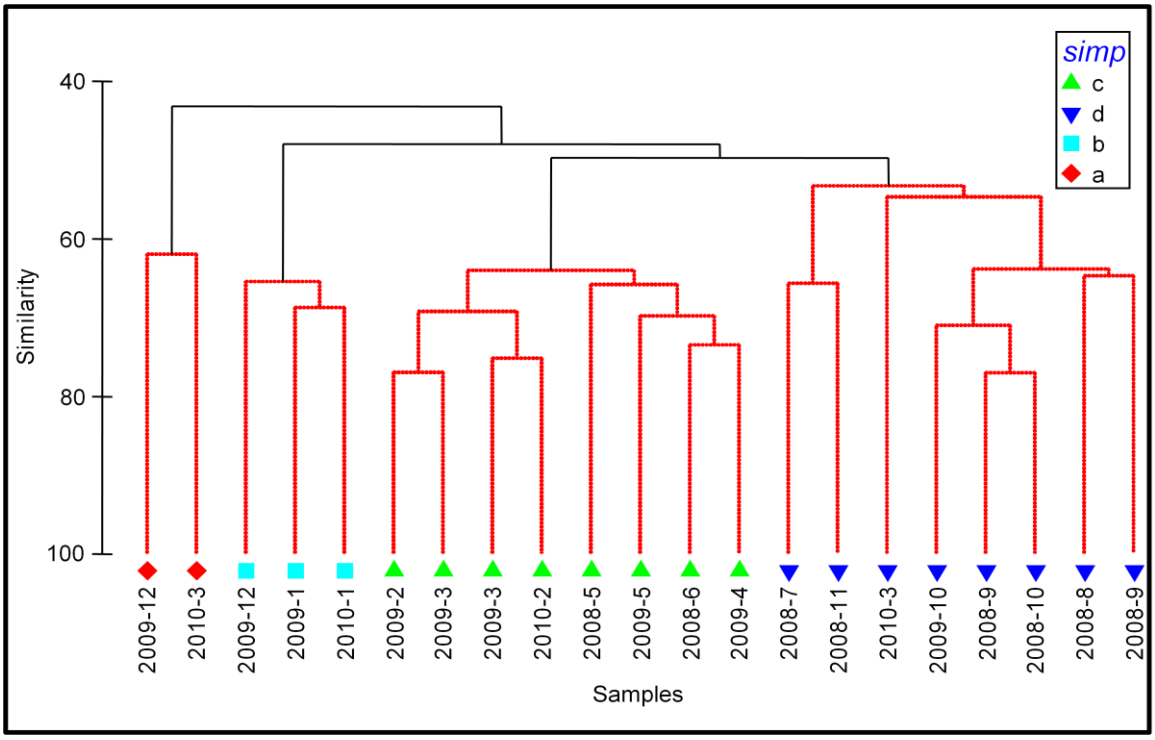

Figure. 4.12. Dendrogram showing the results of cluster analysis. 4 statistically significant groups can be seen with a similarity of 50%.

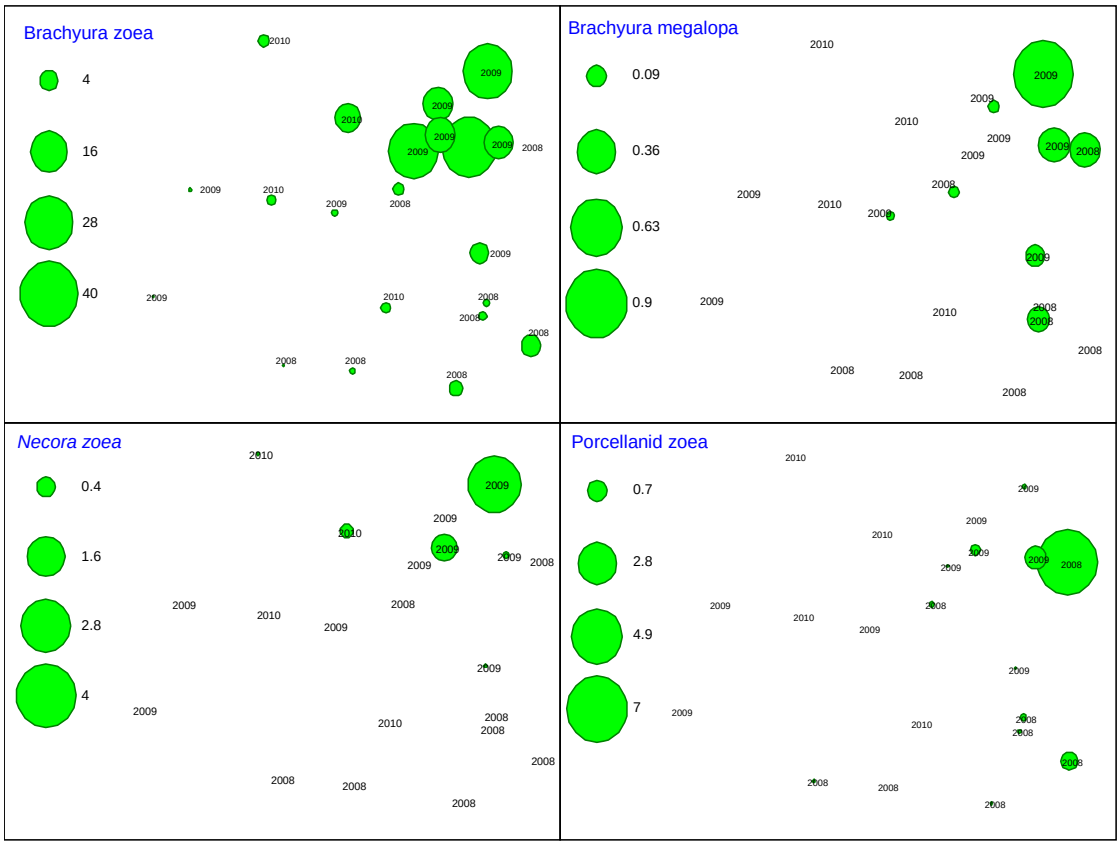

Figure 4.13. MDS ordinations based on the Bray-Curtis similarities calculated using the fourth-root transformed 2008-2010 data, with the individual densities of: Brachyura zoea, Brachyura megalopa, *Necora* zoea, and Porcellanidae zoea displayed as proportional circles (2D stress= 0.16).

Displaying the individual densities of each of the meroplankton groups recorded in the form of bubble plots (Figs. 4.13-4.17) shows that most of the occurrences of these larvae are either within the spring or late summer/ autumn groups.

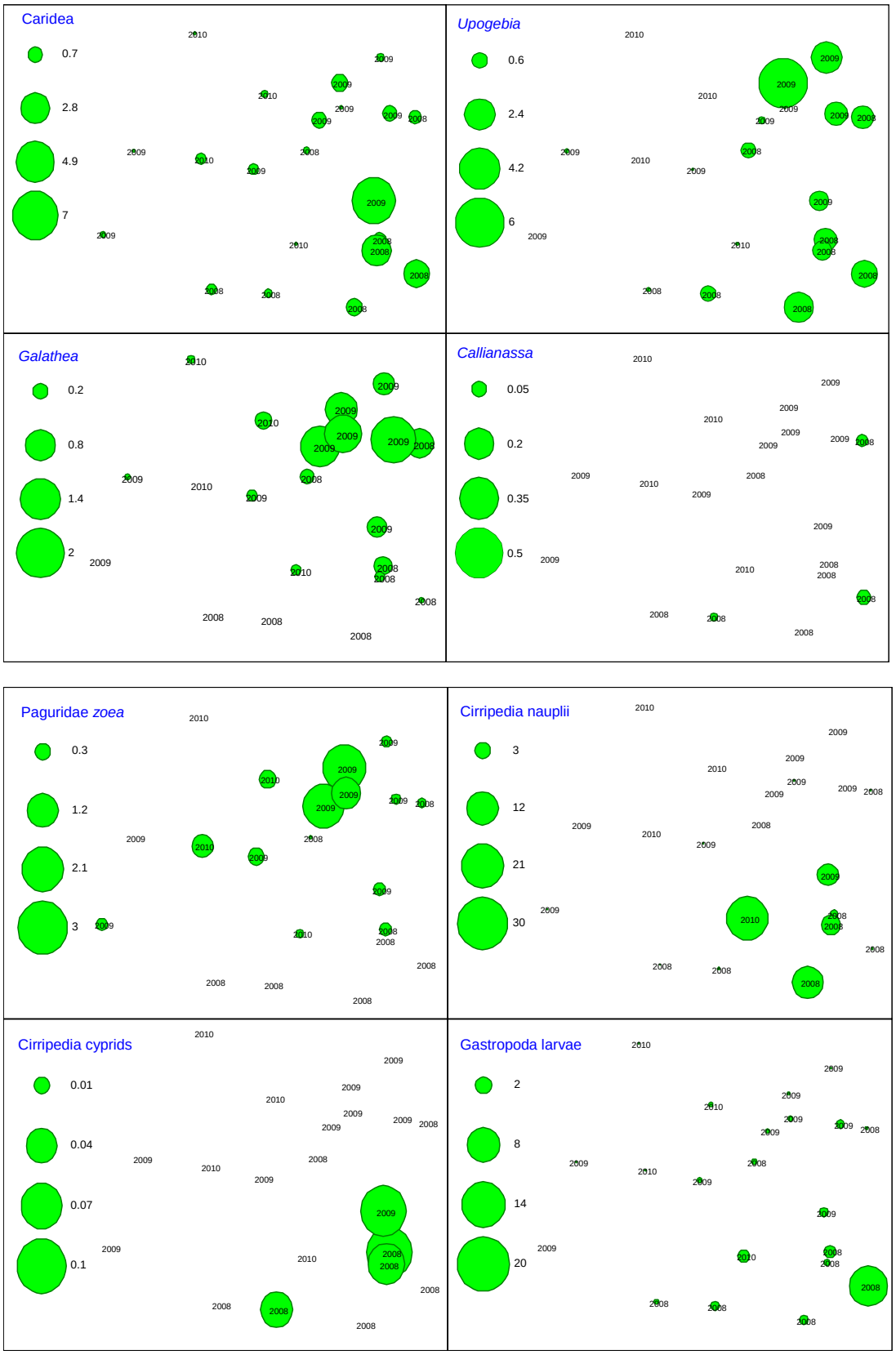

Figure 4.14. MDS ordinations based on the Bray-Curtis similarities calculated using the fourth-root transformed 2008-2010 data, showing the individual densities of: Caridea larvae, *Upogebia* larvae, *Galathea* larvae, *Callianassa* larvae, Paguridae zoea, Cirripedia nauplii, Cirripedia cyprid larvae, and Gastropoda larvae displayed as proportional circles (2D stress=0.16).

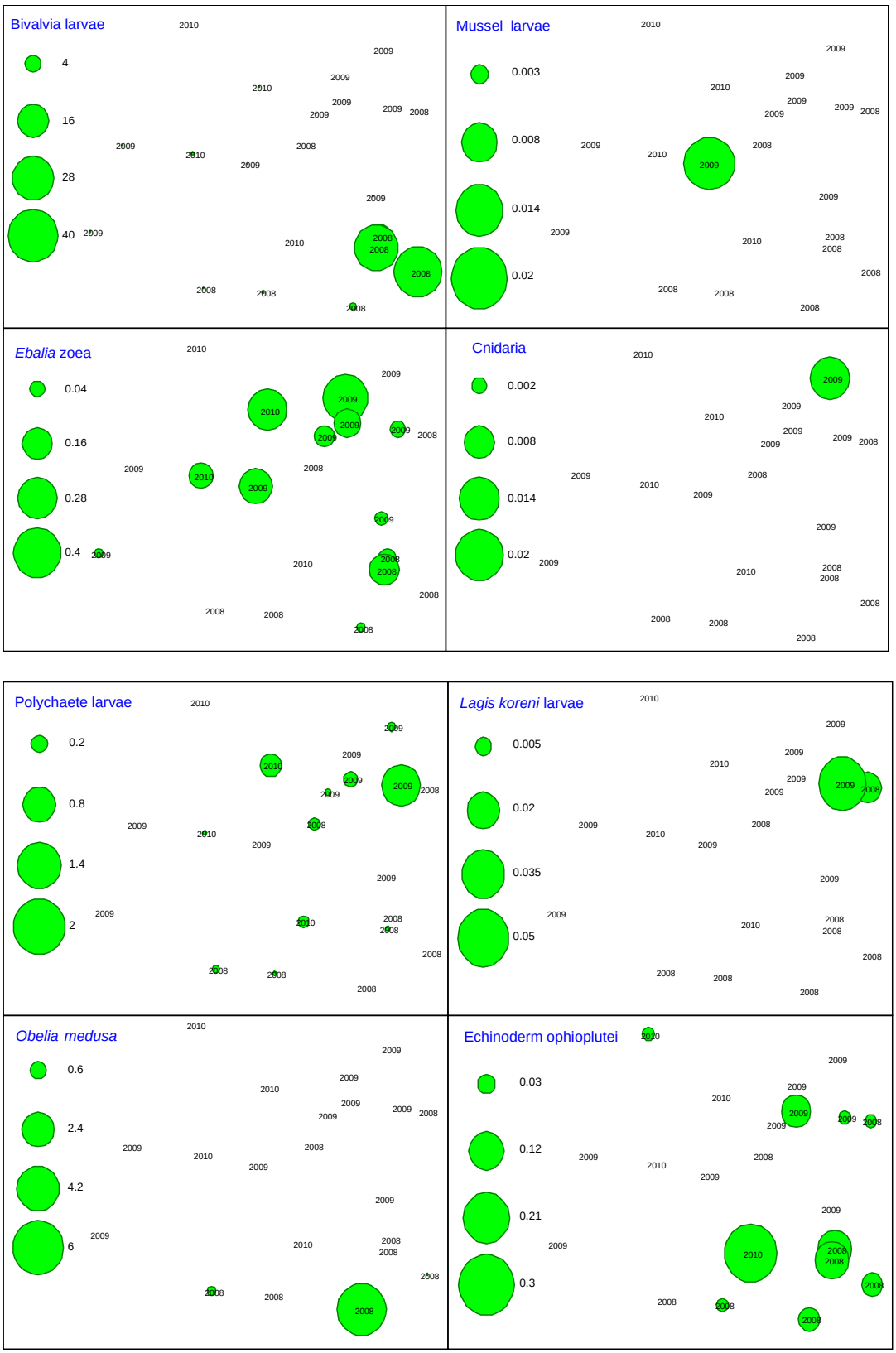

Figure 4.15. MDS ordinations based on the Bray-Curtis similarities calculated using the fourth-root transformed 2008-2010 data, showing the individual densities of: Bivalvia larvae, Mussel larvae, *Ebalia* zoea, Cnidaria, Polychaeta larvae, *Lagis koreni* larvae, *Obelia* medusae, and Echinoderm ophiopluteii displayed as proportional circles (2D stress=0.16).

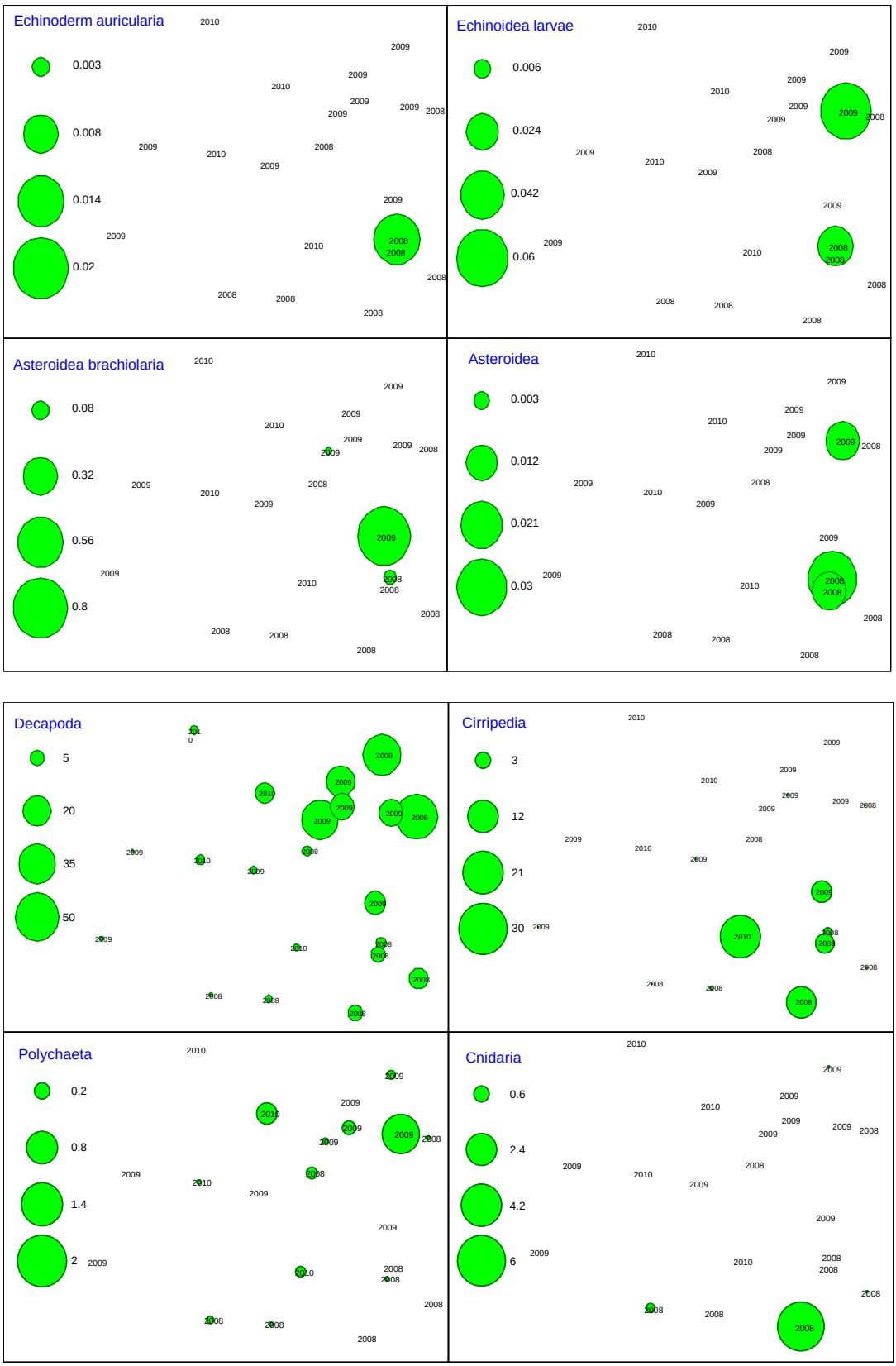

Figure 4.16. MDS ordinations based on the Bray-Curtis similarities calculated using the fourth-root transformed 2008-2010 data, showing the individual densities of: Echinoderm auricularia, Echinoidea larvae, Asteroidea brachiolaria, Asteroidea larvae, Total Decapoda, Total Cirripedia, Total Polychaeta, and Total Cnidaria displayed as proportional circles (2D stress=0.16).

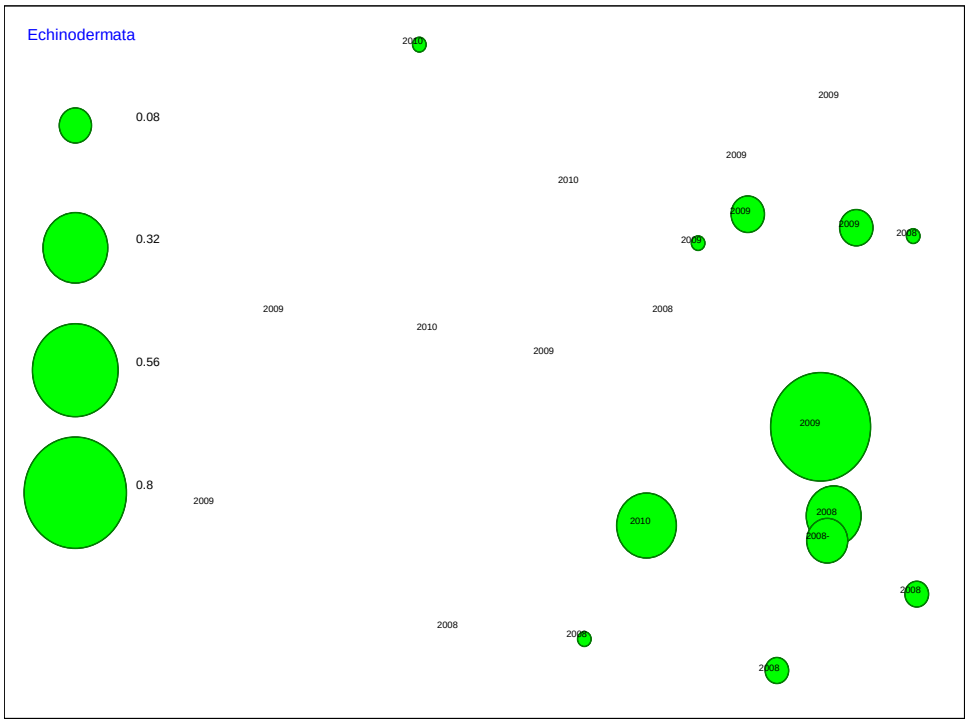

Figure 4.17. MDS ordination based on the Bray-Curtis similarities calculated using the fourth-root transformed 2008-2010 data, showing the individual densities of Total Echinodermata displayed as proportional circles (2D stress=0.16).

Three clear states exist in the 2008-2010 Station L4 time-series data: a winter state with some transitional samples (groups A & B), where few larvae and meroplanktonic species are observed, a spring state (Group C) and a late summer/autumn state (group D), where most of the meroplanktonic species and larval numbers occur.

4.3.3.2. Statistical analysis of historical data.

MDS of the untransformed data from Lebour (1947) (Appendix 1) indicates a clear seasonal cycle in meroplankton composition (Fig. 4.18). This high degree of cyclicity suggests the presence of different spawning periods amongst the benthic organisms in the survey area. This cyclicity is confirmed by a RELATE test (Fig. 4.19) with a model matrix representing samples arranged in a circle (cyclicity) in which the null hypothesis, of no relationship is rejected (Rho= 0.684, $p<0.0001$).

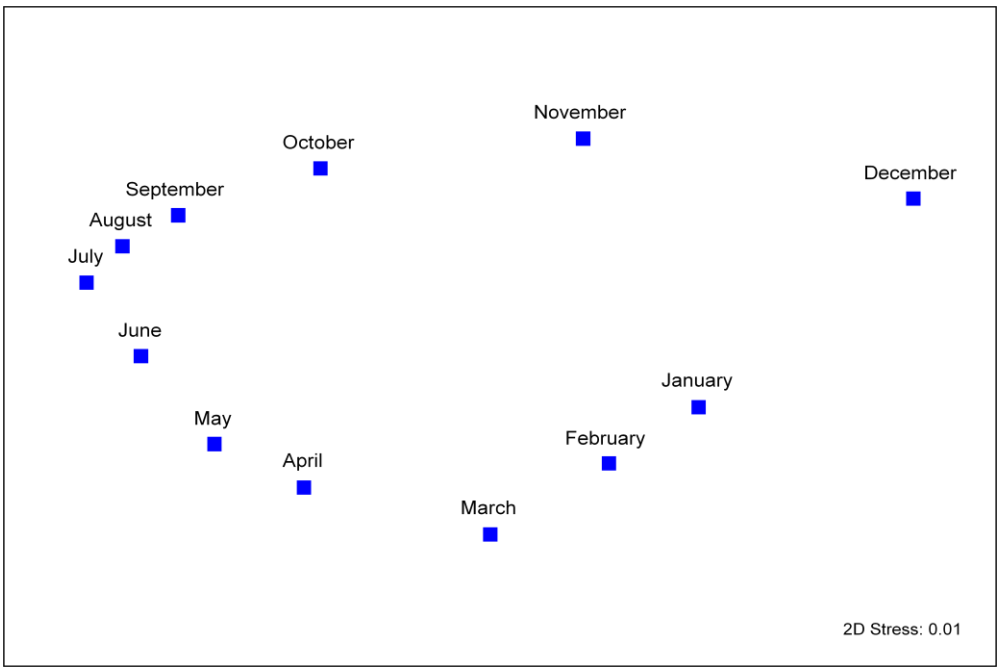

Figure. 4.18. MDS ordination based on the Bray-Curtis similarities calculated by month on the untransformed historical abundance data derived from Lebour (1947).

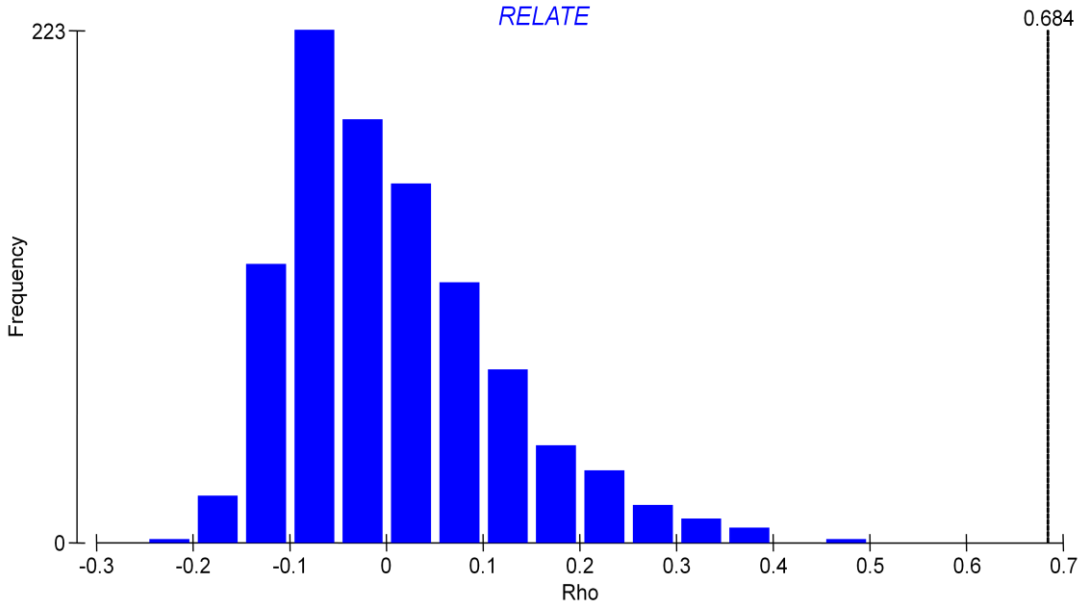

Figure. 4.19. Graph showing the results of the RELATE test examining the degree of cyclicity present in the historical data (Lebour, 1947). Rho= 0.684.

Separate MDS ordinations of the untransformed inshore and offshore data (Figs. 4.20. & 4.21) show that both data-sets exhibit clear seasonal progression in terms of species composition and a degree of cyclicity. It is, however, evident that the months in the

inshore data are considerably more closely related to one another than those in the offshore data.

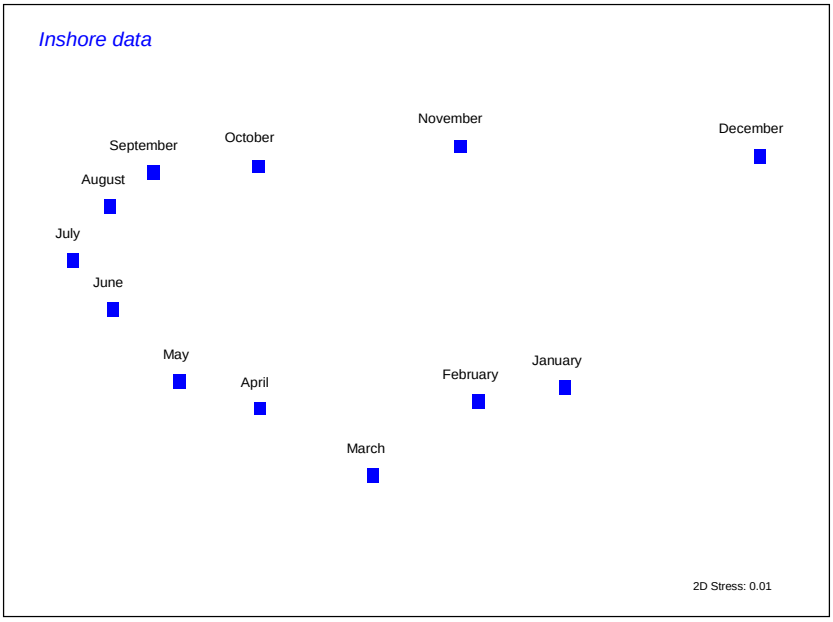

Figure. 4.20. MDS ordination based on the Bray-Curtis similarities calculated by month on the untransformed historical inshore abundance data derived from Lebour (1947).

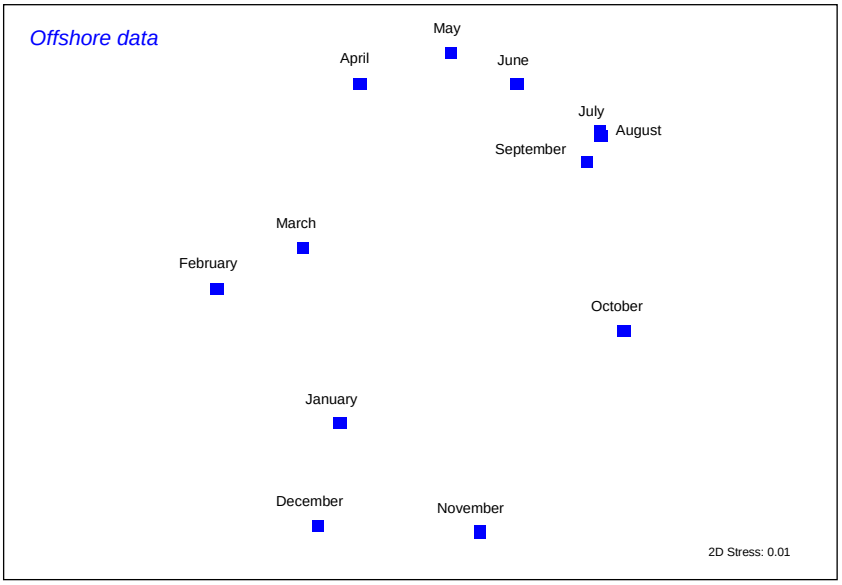

Figure. 4.21. MDS ordination based on the Bray-Curtis similarities calculated by month on the untransformed historical offshore abundance data derived from Lebour (1947).

The seasonal progression and cyclicity shown in the inshore and offshore data is confirmed by RELATE test with a null hypothesis of no cyclicity (Figs. 4.22. & 4.23). Sample statistics (Rho) were 0.666 for the inshore data and 0.757 for the offshore data. The significance level of the sample statistic in both instances was 0.1% and these allow for the null hypothesis to be decisively rejected.. Although MDS suggests that cyclicity is stronger inshore, RELATE shows that it is stronger offshore due to the higher sample statistic (0.757) observed.

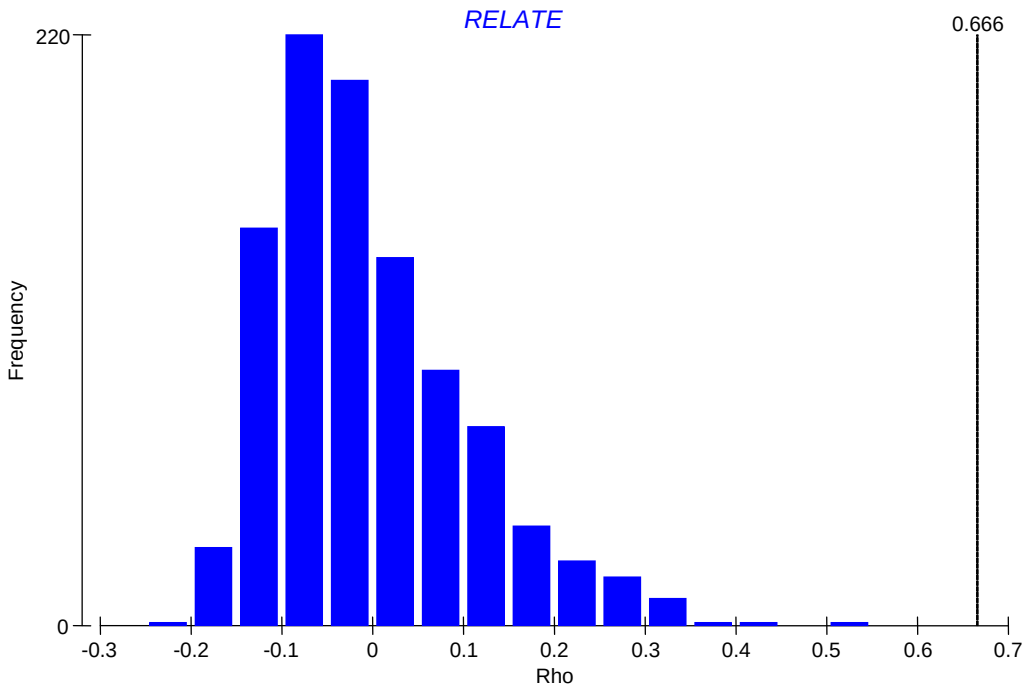

Figure. 4.22. Graph showing the results of the RELATE test conducted on the inshore historical data to examine the degree of cyclicity present in the data (Rho=0.666).

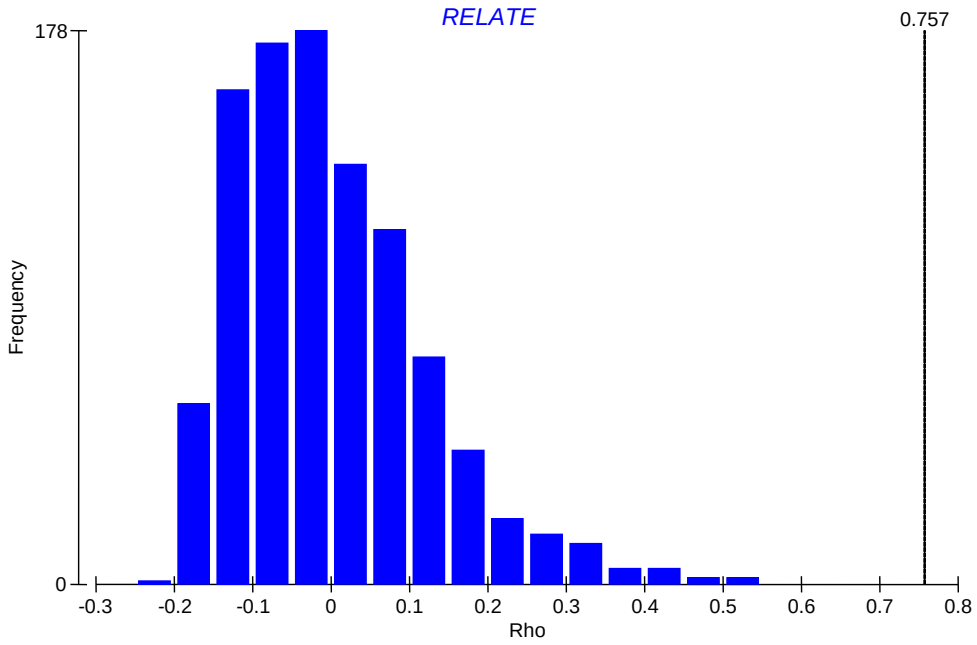

Figure. 4.23. Graph showing the results of the RELATE test conducted on the offshore historical data to examine the degree of cyclicity present in the data (Rho=0.757).

The historical data derived from Lebour (1947) shows clear patterns of succession and cyclicity. This is due to the historical data being produced from a number of years of observations and sampling (1940-45), resulting in an average abundance per month for each species observed.

4.3.3.3. Statistical comparisons between the 2008-2010 data and the historical data from Lebour (1947).

In order to compare the historical and 2008-2010 data, it was necessary to remove those species not recorded in both datasets and convert the data into a comparable abundance scale with the historical inshore and offshore data being combined (Tables 4.III and 4.IV).

Table 4.III. Table showing the combined inshore/ offshore abundance data derived from the observations of Lebour (1947). Only those comparable organsisms between the historical and 2008-2010 data are displayed.

Species	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Decapoda	2	2	3	3	3	3	3	3	3	3	2	2
Necora	1	1	3	3	3	3	3	2	2	1	1	0
Porcellanidae	1	1	2	2	2	3	3	3	2	1	1	0
Caridea	2	2	2	3	3	3	3	3	3	3	2	2
Galathea	2	2	2	3	3	2	2	2	2	1	0	0
Upogebia	0	0	0	2	2	2	3	3	3	2	2	0
Callianassa	0	0	0	0	0	3	3	3	3	3	0	0
Paguridae zoea	2	2	2	2	2	2	2	2	2	2	2	0
Gastropoda larvae	2	2	3	3	3	3	3	3	3	3	3	3
Bivalvia larvae	2	1	2	1	1	1	1	2	2	3	3	1
Mussel larvae	1	1	0	1	2	1	1	1	0	1	1	0
Ebalia zoea	0	1	1	1	1	1	2	2	2	1	1	0
Polychaete larvae	1	1	2	2	2	2	2	2	2	2	2	1
Echinoderm ophioplutei	0	0	0	0	0	0	1	1	1	1	0	0
Echinoderm auricularia	0	0	1	1	1	1	1	0	0	0	0	0
Echinoidea larvae	0	0	0	0	1	1	2	2	1	1	1	0
Asteroidea	0	0	0	0	0	1	1	0	0	0	0	0

Table 4.IV. Table showing the converted abundance data from the 2008-2010 meroplankton time-series. Only those comparable organisms between the historical and 2008-2010 data are displayed.

Species	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Decapoda	2	3	3	3	3	3	2	3	3	3	1	2
Necora	0	2	2	1	3	0	0	0	0	0	0	0
Porcellanidae	0	0	2	2	1	3	0	1	2	1	1	0
Caridea	2	2	2	2	2	2	2	1	3	3	2	1
Galathea	1	2	2	3	2	2	0	0	2	2	0	1
Upogebia	1	1	1	2	3	2	3	2	3	2	1	1
Callianassa	0	0	0	0	0	1	1	0	1	0	0	0
Paguridae zoea	2	3	3	1	1	1	0	0	2	2	0	2
Gastropoda larvae	2	1	2	2	2	1	2	2	3	2	2	1
Bivalvia larvae	2	1	1	0	0	0	1	2	3	3	1	1
Mussel larvae	1	0	0	0	0	0	0	0	0	0	0	0
Ebalia zoea	2	2	1	1	0	0	0	1	1	2	0	1
Polychaete larvae	1	2	1	2	1	1	1	0	0	1	1	0
Echinoderm ophioplutei	0	0	3	1	0	1	1	1	1	1	0	0
Echinoderm auricularia	0	0	0	0	0	0	0	0	1	0	0	0
Echinoidea larvae	0	0	0	0	0	0	0	0	1	0	0	0
Asteroidea	0	0	1	1	0	0	0	0	1	2	0	0

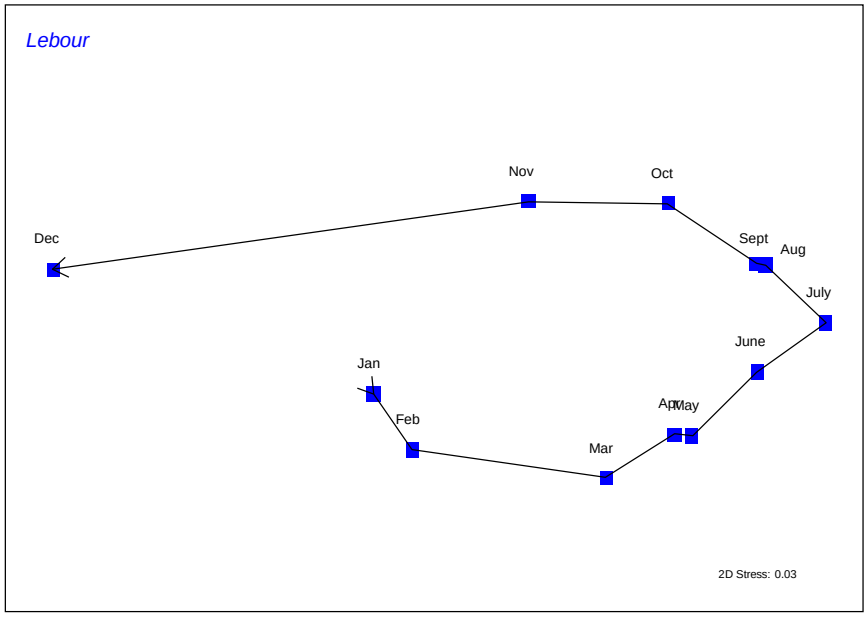

Figure. 4.24. MDS ordination based on the Bray-Curtis similarities calculated by month from the untransformed historical combined inshore and offshore abundance data derived from Lebour (1947). (2D stress=0.03).

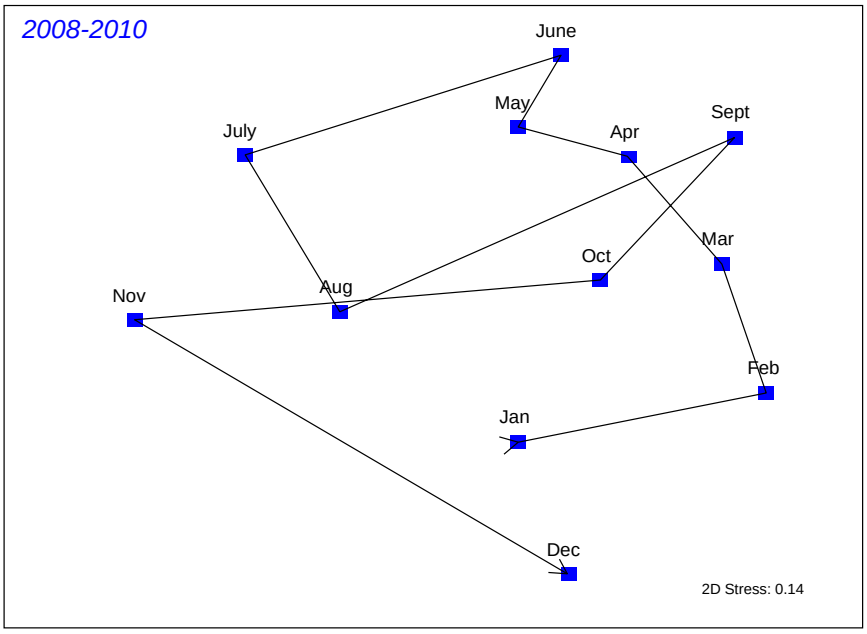

Figure. 4.25. MDS ordination based on the Bray-Curtis similarities calculated by month from the untransformed 2008-2010 abundance data (2D stress=0.14).

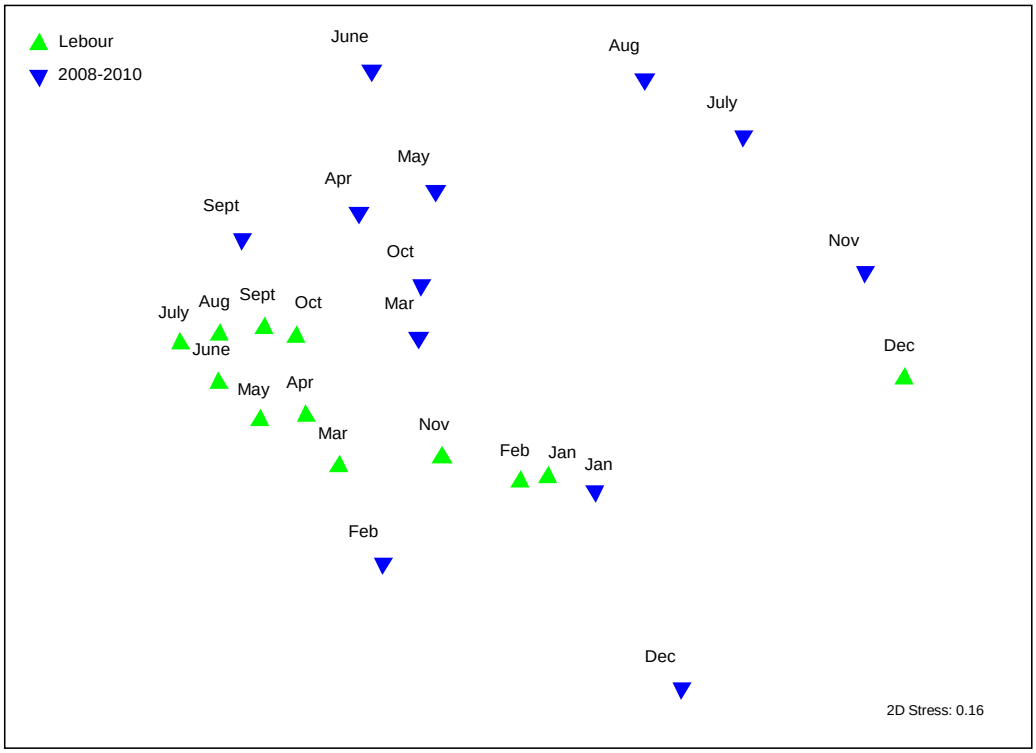

Figure. 4.26. MDS ordination based on Bray-Curtis similarities calculated by month using the untransformed merged 2008-2010 abundance data and historical data derived from Lebour (1947). (2D stress=0.16).

MDS ordinations of the combined historical data and the 2008-2010 Station L4 time-series data (Figs. 4.24 & 4.25) show a considerably higher degree of cyclicity present in the historical data than in the time-series data. Trajectories have been overlaid to give visual representation of the seasonal succession and degree of cyclicity present in the data. The time-series data does exhibit seasonal progression but the inter-annual and intra-annual variation present in the data prevents a clearer picture from being formed. MDS ordination of the two merged data-sets (Fig.4.26) shows the degree of which the historical data are more closely related to each other than the data from the 2008-2010 time-series, which shows considerable variation.

The differing degrees of cyclicity within the two data-sets were confirmed by RELATE tests (Figs. 4.27 & 4.28.) with the null hypotheses of no cyclicity or succession being rejected. The stronger degree of cyclicity in the historical data is shown by the higher Rho value of 0.532 compared with 0.387 for the 2008-2010 data. This reveals a weaker correlation supporting the lower degree of cyclicity observed. The sample statistic significance level for both tests was 0.1%. The lower degree of cyclicity in the 2008-2010 data was to be expected as the historical data is an average of several years sampling effort.

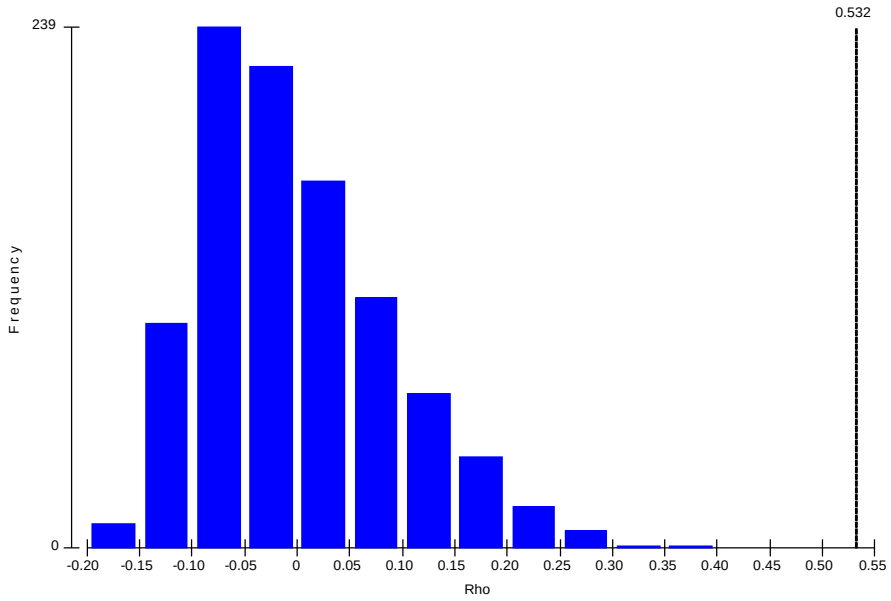

Figure. 4.27. Graph showing the results of the RELATE test conducted on the historical data to examine the degree of cyclicity present in the data. (Rho=0.532).

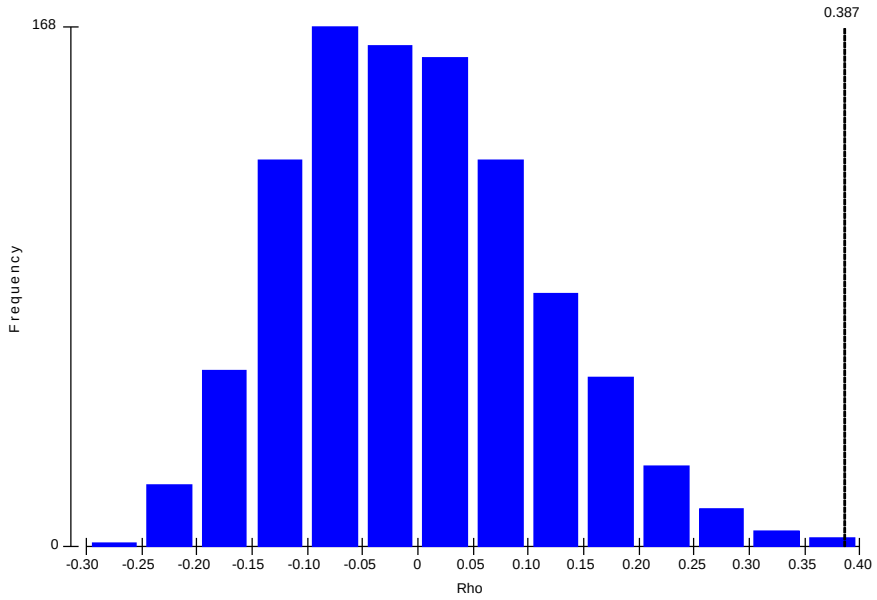

Figure. 4.28. Graph showing the results of the RELATE test conducted on the 2008-2010 data to examine the degree of cyclicity present in the data. (Rho=0.387).

Concordance in pattern between the datasets was confirmed by a RELATE test, in which the null hypothesis, of no correlation between corresponding elements in the underlying resemblance matrices, was rejected (Fig. 4.29). A sample statistic (Rho) of 1 and a sample statistic significance level of 0.3% shows that there is correlation present.

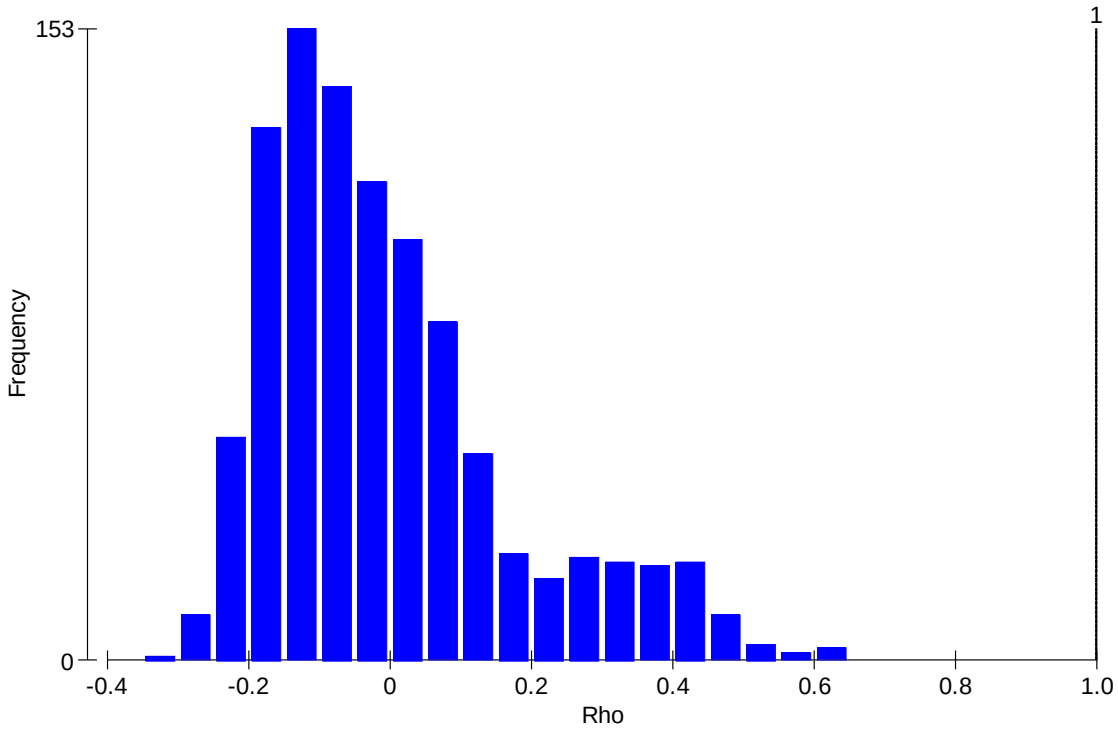

Figure. 4.29. Graph showing the results of the RELATE test conducted on the 2008-2010 data and historical data derived from Lebour (1947) to examine the level of similarity between the two datasets (Rho=1).

A resemblance matrix using Bray-Curtis similarity was contructed to compare the level of similarity between each month of the two data-sets in terms of taxa abundance and composition so that ANOSIM could be conducted. One-way ANOSIM (Global R=0.221, significance level=0.3%) allows for the null hypothesis of 'no difference between datasets' to be rejected although R is low so the separation is not large (Fig. 4.30).

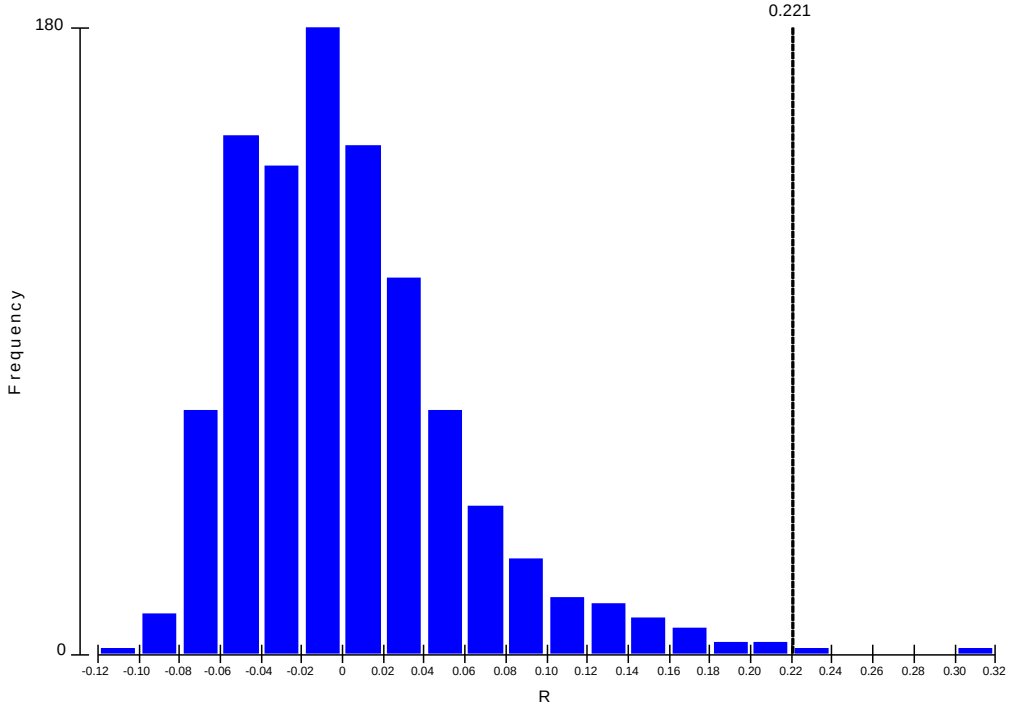

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Figure. 4.30. One-way ANOSIM test conducted on the merged resemblance data from the historical and 2008-2010 datasets (Global R value=0.221, sample statistic significance level= 0.3%).

Two-way SIMPER analysis conducted on the resemblance data from the two datasets showed an average dissimilarity of 30.86 with the occurrences of *Necora* zoea in the two datasets providing the largest percentage of this dissimilarity at 10.12%. MDS of the presence/ absence-transformed merged data shows that there is still considerable variation between the two datasets, and between the samples of the 2008-2010 data at this level (Fig. 4.31).

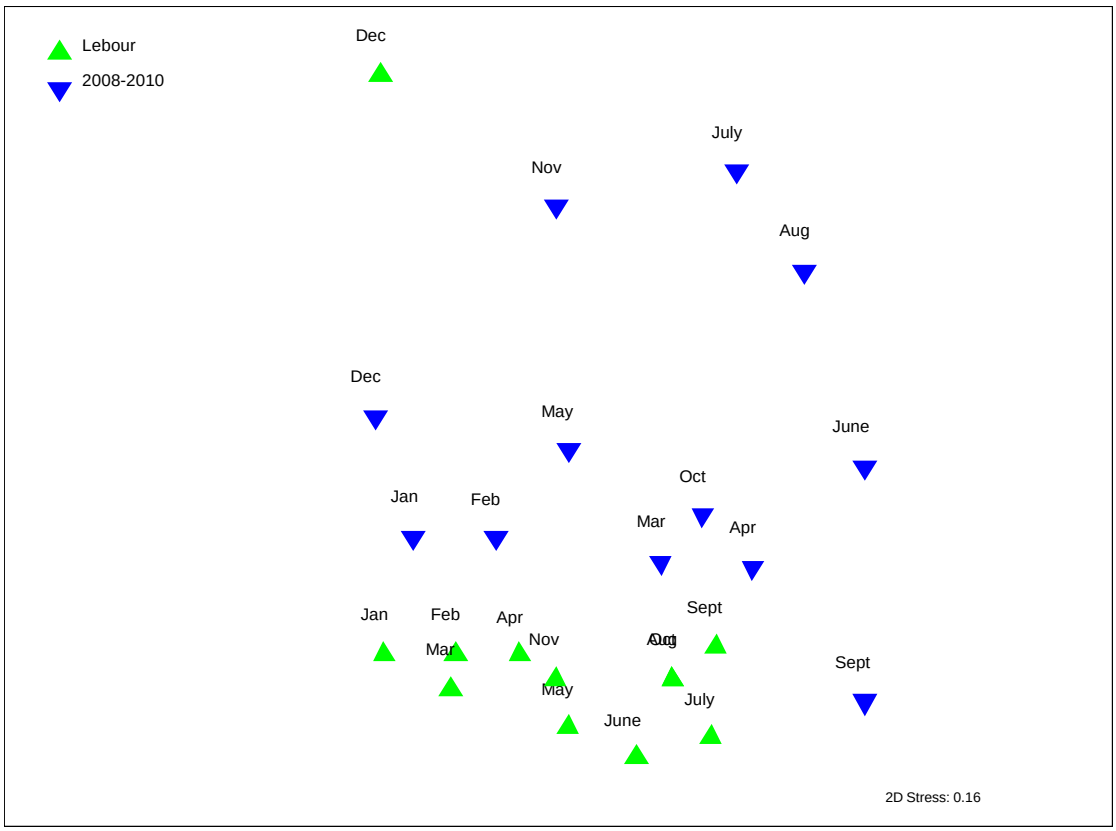

Figure. 4.31. MDS ordination based on the Bray-Curtis similarities calculated by month from the presence/absence transformed merged 2008-2010 abundance data and historical data derived from Lebour (1947).

The DIVERSE univariate diversity index conducted on the presence/absence data produces an indication of species richness by producing a taxon count. It shows that there were more taxa recorded in the historical data than in the 2008-2010 data. This was to be expected as the sampling period for the historical dataset covers a much longer time period than that of the 2008-2010 dataset and, therefore, is more likely to have sampled a greater number of taxa. A further two-way SIMPER test conducted on this presence/absence merged data produced an average dissimilarity of 23.96 with the occurrences of Mussel larvae within the two datasets accounting for the largest percentage of the dissimilarity at 11.92%.

4.4. DISCUSSION.

4.4.1. Seasonal patterns in species composition and abundance in 2008-2010 data.

As discussed in Chapter 3, species with a meroplanktonic phase have complex life cycles where dispersal and subsequent recruitment are vital in structuring populations (Pan *et al.*, 2011) and link primary production with higher trophic levels by acting as a major trophic link themselves (John *et al.*, 2001). Analysis of the 20 year time-series data from Station L4 showed that seasonal variation was the driving factor in the variability in community composition at the study site rather than inter-annual variation. This conclusion was drawn from the analysis of broad taxonomic groups. The 2008-2010 time-series data was subject to increased taxonomic resolution that allowed for the patterns in annual abundance of different taxa to be examined.

The seasonal cycle of density displayed by decapod species in the 2008-2010 time-series followed the same pattern as that was shown in the 1988-2007 Station L4 time-series (Fig. 3.5D), with low numbers during the winter months followed by large densities in spring and occasional subsequent peaks in density in the summer months that are likely to represent different spawning events. By splitting Decapoda into several constituent genera and species, it was possible to determine the individual spawning events of those separate groups. *Ebalia* zoeae were seen to be present in the meroplankton community during the winter months and early spring whereas the zoeae of various Porcellanidae species were most common in the late spring and early summer months.

Brachyura zoeae were the most common meroplanktonic organism (Table 4.II) over the entire time-series, with cirripede nauplii and bivalve larvae being the second and third most abundant. Most of their abundance however, occurred in sudden bursts over short periods of time: Cirripedia nauplii in the spring months, and Bivalvia in late summer and early autumn. Brachyura zoeae tended to rapidly increase but continued to be recorded in higher numbers over a longer time period. Brachyuran zoeae were considerably more abundant than brachyuran megalopae and this seasonal variation in the occurrences of distinct decapod life stages may be a result of the natural mortality between developmental stages. Dispersive migration may occur from the study site as individuals develop (Lindley, 1987) and increase their dispersal potential as stronger swimming abilities and functional morphological features appear (Lee *et al.*, 2004). This would lead to less megalopae being sampled. The peak shown in early spring was in all likelihood an effect of the spring phytoplankton bloom providing a ready food source for the larvae. Subsequent peaks in summer corresponded to subsequent phytoplankton blooms.

Cirripedia nauplii accounted for the majority of the cirripede individuals recorded in this survey, appearing in spring, with cyprid larvae appearing in the plankton in much lower numbers in the summer and autumn following metamorphosis from nauplii. This is to be expected due to the naturally high mortality rate experienced from one life stage to another (Eckman, 1996) and dispersal from the study site (Lindley, 1987). In theory, advection of nauplii into the study site would counteract some of this loss of larvae (Eiane *et al*, 1998). The effects of temporal autocorrelation cannot be discounted as it is possible that the same larval cohort was sampled in consecutive sampling trips, which would lead to apparent seasonal patterns of abundance appearing more pronounced than is actually the case (Beaugrand & Reid, 2003).

Statistical analysis reveals the presence of four distinct groups within the samples of the 2008-2010 data. The two most closely related groups (spring and late summer/ autumn) contained the majority of samples and therefore larval individuals and species recorded, and this is confirmed by Simprof and cluster analysis. These results are to be expected given that they represent the times of the year where food supply is most plentiful and most larval spawning occurs. The other two groups occur in winter and early spring where fewer larvae are found in the plankton community at the study site. This represents a degree of seasonality present in the data with different species comprising the two dominant groups observed (Fig. 4.14 – 4.17).

Differences between the 1988-2007 and 2008-2010 data-sets could be due to several reasons. Due to the mesh size of the plankton tow net (335 µm), it is possible that many smaller meroplanktonic organisms were not sampled as a result. The WP2 haul net used for the 1988-2007 time-series has a finer mesh size of 200 µm (John *et al.*, 2001) and is liable to have sampled a greater number of smaller meroplanktonic organisms, such as bivalve larvae, than the net used in this study. As a result of their small size, it is likely that more lamellibranch larvae were present in the water column but only those large enough to be retained by the plankton tow net were sampled. Previous studies have examined the differences in individual species abundance between two different mesh sizes used in both the Continuous Plankton Recorder survey and the Dove sampling station time-series in Northumberland (Clark *et al.*, 2001; John *et al.*, 2001; Pitois *et al.*, 2009). It was found that while they had an effect upon the size and number of species sampled, differing mesh size was not wholly responsible for these dissimilarities. Active avoidance of sampling gear and passive avoidance due to hydrodynamic factors are two of the possible explanations for this (Clark *et al*, 2001).

The samples from the 1988-2007 time-series were collected using a vertical haul whereas the 2008-2010 survey used a horizontal tow at 10m depth for 10 minutes. The vertical haul sampled every depth and was more likely to have encountered those larval species that inhabit lower depths especially those that do not travel far from the seabed or those that exhibit diel migration (Colebrook *et al.*, 1961; John *et al.*, 2001). A vertical haul is also more likely to encounter newly spawned larvae earlier as they are released and later developmental stages as they begin to settle on the benthos due its sampling of lower depths than the horizontal tow, possibly revealing slightly different peaks in density than are evident in the tow data (Pan *et al.*, 2011). This is likely to provide a different cadre of species than those encountered by the horizontal tow. However, the horizontal tow covers more distance than the vertical haul (463m as opposed to ~51m depth for the vertical haul). As a result of covering a greater distance and thus sampling a greater volume of the water column, the number of larval individuals sampled is theoretically greater.

The patchiness of meroplankton distribution in the water column (Wiebe & Holland, 1968; Clark *et al.*, 2001) means that it is likely that a horizontal tow will encounter a greater number of meroplanktonic organisms than a vertical haul. Another reason for the disparity in the results in the two surveys is the size of sampling gear resulting in certain planktonic species avoiding being sampled. This is likened to predator avoidance in that some organisms are liable to be able to detect the oncoming "bow-wave" of a sampling device and therefore avoid it. However, this depends upon the speed and size of the sampling apparatus (Clark *et al.*, 2001).

The samples in both datasets were collected at roughly the same time of day thus minimising the effects upon species diversity and abundance caused by diel migration

by meroplanktonic organisms (Pitois *et al.*, 2009). The 1988-2007 time-series samples were collected on a weekly basis (weather permitting) whereas the samples from the 2008-2010 data were collected on a much more varied time-scale: ranging from weekly, fortnightly, and monthly depending on the weather. This is likely to have had a considerable effect on the number and abundance of the species encountered, with the sampling effort in the 1988-2007 time-series more likely to detect any increases in abundance due to spawning events. By the time a monthly sampling effort occurs, it is possible that those larvae produced by a sudden onset of spawning may have dispersed from the study site. This may be due to larval stage duration, mortality, behaviour, and settlement (Eckman, 1996).

The data from the 2008-2010 survey generally exhibit the same general patterns of abundance/density and species composition identified in the 1988-2007 time-series data at Station L4 with seasonal variation in community composition accounting for the majority of the changes seen throughout the study. Compared to the 1988-2007 time-series, it is evident that this cycle repeats itself on an annual basis, with the 2008-2010 data displaying little dissimilarity with the earlier dataset. This supports the conclusion drawn in Chapter 3 that there have been no major changes at Station L4 since 1988. While the methods of data collection differ and may have resulted in slightly different patterns in the two datasets, these remain broadly similar. Seasonal variation remains the driving factor in determining species composition over the course of the year with the onset of the spring phytoplankton bloom triggering the release of meroplanktonic larvae from several groups of benthic organisms. This is followed by subsequent spawning events throughout the summer and autumn months.

It is important to consider the possible and future effects of trophic mismatch on the meroplankton dynamics at Station L4. It has been shown that the spring diatom bloom has remained relatively constant and is presumably related to day-length or light intensity (Edwards & Richardson, 2004) and so it is likely that the link between the spring bloom at Station L4 and the spawning of meroplanktonic larvae will continue. Other organisms that rely on temperature to stimulate physiological development or release larvae, however, have been shown to considerably advance their seasonal cycles in response to temperature increase. The level of this response varies considerably in different organisms and has led to a mismatch between subsequent trophic levels in terms of the timing of primary, secondary, and tertiary production (Edwards & Richardson, 2004). Warmer waters have been associated with the earlier appearance of the planktonic larvae of several fish species in the North Sea (Greve *et al*., 2005). This could result in later larval spawning events being affected by changes in the phytoplankton dynamics at Station L4 in response to temperature increase through climate change.

4.4.2. Comparison with historical data.

In order to detect any patterns of long-term variation in the meroplankton community, it was necessary to examine data prior to the commencement of the 1988-2007 time-series at Station L4. Data derived from the observations of Lebour (1947) showed a considerable degree of cyclicity and seasonal progression in species composition over the course of the year. MDS ordination suggested that there is greater similarity amongst the species in the inshore samples than within the offshore data (Figs. 4.20 - 4.21). This suggests that while a greater diversity of species was recorded offshore, both the inshore and offshore data show a level of cyclicity and seasonal progression in

species composition. RELATE tests (Figs. 4.22 & 4.23) confirm this and show the higher degree of cyclicity in the offshore data. This is likely to be due to the higher level of variation both in the number and types of species recorded in the offshore data.

There is a considerably smaller level of cyclicity present in the 2008-2010 data although seasonal succession in species composition is still evident (Fig. 4.25). The patterns identified in the historical data are liable to be clearer and well-defined as the data were produced from a number of years of observations and sampling (1940-45), resulting in an average abundance per month for each species observed thus allowing the effects of inherent annual and seasonal variation over short time-periods to be minimised. The 2008-2010 time-series data covers two years with some months only being sampled once so there is are limitations on the seasonal patterns that can be discerned. It is likely that the higher number of species and individuals recorded in the historical data is due to the longer sampling time period than the 2008-2010 survey. Therefore, patterns of species composition and abundance will be less defined and harder to identify in the latter owing to the natural variation in larval recruitment and mortality present over such a small time-scale. Greater variation is present in the samples of the 2008-2010 study than in those of the averaged historical data (Fig. 4.26), and RELATE tests (Figs. 4.27. & 4.28) confirm that cyclicity is more prevalent in the historical data than in the 2008-2010 data.

These two datasets are broadly comparable but the differences in the length of time over which the records were collected and the qualitative nature of the historical observations mean that, while there is no evidence of major change at the simple abundance scale used, there may well be differences in both species composition and abundance that have been missed. Due to the level of taxonomic discrimination applied to the 2008-

2010 time-series being of a lower resolution than that applied to the historical observations, the disappearance of certain species from the study site over the years is likely to have been missed. The level of taxonomic resolution was lower in the 2008-2010 due to the expertise of the researcher and so larvae were identified to as low a taxonomic level as possible. Observed increases in the abundance of certain species such as *Necora*, since the historical studies, may be due to the normal seasonal and annual fluctuations in adult fecundity, larval survival, and dispersal as a result of oceanographic processes, winds, tides, or currents (Eckman, 1996; Queiroga *et al.*, 2006) rather than being an indicator of any long-term change. Generally, there has been little overall variation in the species composition of the meroplankton community in the Plymouth Sound area since the historical data were collected.

The observed increase in *Necora* larval numbers may also be as a result of an increase in sea surface temperature such as the increase of 0.6°C per decade observed during the 1988-2007 time-series (Smyth *et al.*, 2010), but without definite empirical data showing such an increase in temperature since the 1940s, this conclusion cannot be drawn. However, due to the qualitative nature of the historical data, it is possible that the differences in the abundances of the various groups in question are an artefact of differing abundance interpretations between the two data sets. The numerical definition of 'common' in the 2008-2010 data may be considerably different from that of Lebour (1947) and so on.

Comparison with historical data shows some dissimilarity between the two datasets, but there is no evidence of definite overall change in either species abundance or composition. The levels of seasonality and cyclicity are considerably higher in the historical data than in the 2008-2010 study, but this is likely to be a result of the greater

number of samples taken in the earlier studies. Therefore, it is evident that long-term datasets need to continue sampling an area in order to truly understand long-term patterns of community composition and variation, both in the benthic and pelagic environments, which 2 year or 20 year surveys cannot identify. The comparison of current and historical data is limited by variations in sampling methodology and the level of taxonomic resolution applied. Using the same methodology over a longer period of time would allow further comparison, and would possibly reveal patterns of long-term temporal variation.

4.4.3. Evidence of Benthic-pelagic coupling and links with benthic survey.

The study site has been subject to a number of benthic surveys and these have reported the loss or reduction in the abundance of certain species since the records of Lebour (1947), e.g. *Lima hians* (Gmellin). These are most likely due to the effects of disturbance by beam trawling and other demersal fishing activities as the area in question is an important location for beam trawling and scallop dredging (Capasso *et al.*, 2010). This loss or reduction is likely to have had serious implications for the composition of the meroplanktonic community at Station L4 as has been demonstrated in the North Sea (Kirby et al, 2007, 2008), and on the plankton community as a whole (Lindley & Batten, 2002). This will continue until such time as a population is revived by recruitment from a viable population outside of the immediate area. This, however, is dependent on a number of pre- and post-settlement factors such as predation, suitable available substrata, oceanographic processes and so on (Todd, 1998, Pechenik, 1999), and the assumption of the presence of a suitable viable population. This may be the reason that such species have failed to recolonise the benthos and contribute larvae to the meroplanktonic community.

CHAPTER 5. SEASONAL DYNAMICS OF BIVALVE LARVAE AT STATION L4 AND THE MOLECULAR METHODS USED TO FURTHER THEIR IDENTIFICATION.

5.1. INTRODUCTION.

Marine bivalve larvae are notoriously difficult to identify using traditional microscopic methods to examine morphological features that are often very similar, if not identical, in the larvae of a number of different species (Garland & Zimmer, 2002; Livi, 2006; Sawada *et al.*, 2008; Wight *et al.*, 2009). The process can also be considerably time-consuming due to the number of individual larvae involved in any study examining the dynamics of bivalve larvae (Garland & Zimmer, 2002). As such, a number of different molecular techniques have been employed in an attempt to enhance the level of taxonomic discrimination currently available in the study of identification (Sawada *et al.*, 2008).

The use of procedures such as quantitative PCR (Wight *et al.*, 2009), multiplex PCR (Hare *et al.*, 2000), PCR-Restriction Fragment Length Polymorphism (RFLP) , PCR-Single Strand Conformation Polymorphism (SSCP) (Livi, 2006), *in situ* hybridisation (Le Goff-Vitry, 2007b), and DNA markers either through the use of probes (Bell & Grassle, 1998) or restriction enzymes (Hare *et al.*, 2000) have all facilitated the identification of bivalve larvae. However, some of these methods, such as the use of specific PCR primers (Morgan & Rogers, 2001), can only be used for qualitative assessment of bivalve larvae and therefore can only give an indication of presence/absence of a particular species within a mixed group rather than one of abundance (Comtet *et al.*, 2000; Livi, 2006).

Other techniques such as PCR-SSCP, PCR-RFLP, and multiplex PCR can be used in studies with a large number of individuals, however, they tend to be designed for a few species within a given genus (Livi, 2006). Studies examining the changes in the species

composition and relative abundances of a community of bivalve larvae over a given time-span at a particular site require a technique that can be used on large numbers of larvae and one that is not limited to a small number of species.

5.2. JUSTIFICATION OF STUDY.

The purpose of this chapter was to examine how the species composition of the bivalve larval community at Station L4, and the relative abundances of the individual species changed over an extended time period. Previous time-series studies at Station L4 have recorded these larvae as merely 'bivalve larvae'. As a consequence it has been impossible to examine whether there are any variations in the species composition of larvae being sampled on an annual or seasonal basis. It is also impossible to determine whether the larvae of certain bivalve species have appeared or disappeared from the study site and its immediate vicinity over the years since studies by Lebour (1947) and the descriptions from the Plymouth Marine Fauna (Marine Biological Association of the United Kingdom, 1957) first revealed the occurrences of the characteristic bivalve species of Plymouth Sound and its surrounding areas.

The investigation detailed in this chapter aimed to identify bivalve larvae sampled at Station L4 on a weekly basis to a species level and thus, determine any patterns of species composition and abundance. In addition, the larval species found were compared with the adult species recorded during the ongoing benthic survey carried out by Plymouth Marine Laboratory at the study site. In order to accomplish this, it was necessary to use a molecular technique that worked on individuals, thus allowing for the identification and resolution of individual bivalve larvae. Such a method allows for a

large number of larvae to be processed in a manner that is both relatively rapid and cost-effective and is suitable for use on numerous bivalve species and not restricted to a single genus or family. The application of 'universal' primers for PCR of DNA from individual bivalve larvae (such as those designed against conserved gene regions) permits amplification from as many different species as possible and facilitates their identification following sequencing. The use of this technique allowed the resolution of seasonal succession in the spawning times of the bivalve species characteristic of the study site and its environs thus revealing patterns of abundance that have previously been unknown.

5.3. MATERIALS AND METHODS.

5.3.1. Sampling.

Sampling for bivalve larvae was conducted at Station L4 on a weekly basis (weather permitting) from 26/10/2009 until the 23/03/2010. A plankton tow net (335µm mesh, 440mm diameter) was used according to the same methods as described in Chapter 4 and the contents were washed into a cool-box and topped up with seawater from the sampling station for transit back to the laboratory.

Where possible, 96 bivalve larvae were picked from each sample using a 200µM mesh sieve and a pipette to transfer the larvae first to a petri-dish containing seawater, before further transfer to a second dish to ensure that the bivalve larvae were completely isolated. The seawater in the dish was then removed using 25mm glass micro-fibre filter discs (Whatman), leaving only dry larvae. These larvae were then transferred using a

needle to a 96-well plate, with each larva occupying an individual well, and subsequently stored in a -20°C freezer until use.

It was not always possible to pick out 96 larvae from each week due to their scarcity in some of the samples, and the following table (Table 5.I) shows the dates of each sample and the number of larvae selected.

Table 5.I. Table showing bivalve sampling dates and the number of individual larvae collected from each sampling effort.

Sample Date	No. of Bivalve larvae collected
26/10/2009	96
08/11/2009	96
09/11/2009	96
01/12/2009	73
15/12/2009	24
04/01/2010	17
18/01/2010	96
26/01/2010	96
01/02/2010	96
08/02/2010	96
15/02/2010	96
23/02/2010	96
01/03/2010	96
23/03/2010	32
Total	1106

5.3.2. PCR protocol for bivalve larvae.

5.3.2.1. PCR protocol development.

Following the protocol described by Webb *et al* (2006) bivalve larvae were each rehydrated in 200 µL of distilled water for 3 hours before being transferred to 20 µL of colourless Promega PCR 5x Buffer solution and spun for 10 minutes at 1811 RCF in a centrifuge. This protocol was developed further by homogenising the larvae using a

hand-held needle homogeniser (Kontes) once they were placed in the buffer solution. This served to mechanically disrupt the larval tissue and improve the success of the PCR reactions. Following this procedure, the larvae were then frozen for 24 hours in a -20°C freezer.

Two genes, the nuclear 18S rRNA gene and the mitochondrial Cytochrome oxidase I (COI) gene, were tested for their suitability for bivalve larvae identification. The 18S forward - $^{5'}$-GCCAGTAGCATATGCTTGTCTC-$^{3'}$ - and reverse primers – $^{5'}$-AGACTTGCCTCCAATGGATCC-$^{3'}$ (Holland, 1991) were used in the PCR reactions utilising the 18S gene, and the universal COI primers LCO1490 - (forward) $^{5'}$-GGTCAACAAATCATAAAGATATTGG-$^{3'}$ - and HCO2198 (reverse) $^{5'}$-TAAACTTCAGGGTGACCAAAAAATCA-$^{3'}$ (Folmer *et al.*, 1994) were used in PCR reactions utilising the COI gene. Three different reactions for a subset of larvae were conducted to determine the optimum volume of larval homogenate to use: 1 µL, 2 µL, 5 µL, and a no template control (NTC). The optimum volume of DNA template was determined to be 2 µL and this volume was used for reactions using both the 18S and COI genes.

5.3.2.2. PCR protocol.

96-well plates were used for each PCR reaction so that the 2 µL of DNA template taken from every larva could be indentified from its original location in the 96-well plate. Details for the different PCR mixes used for reactions with each gene are listed in Tables 5.II and 5.III

Table 5.II. PCR mix details for 18S and COI genes in bivalve larvae.

PCR Mix	
DNA Template	10-120 ng
dNTPs	0.02 mM
Promega Gotaq Flexi Buffer	1x
$MgCl_2$	2.5 mM
18S Forward primer / LCO1490	20 pmol
18S Reverse primer / HCO2198	20 pmol
Gotaq DNA polymerase	1.25 units
Molecular-grade water	To a final reaction volume of 50µL

As the larvae were placed in 5x PCR buffer and 2 µL of this DNA template solution was used in the reaction, the volume of PCR buffer added to the reaction mix was reduced by 2 µL to allow for the extra buffer solution added with the DNA template.

The cycle used for the PCR reaction using the 18S gene (Webb *et al.*, 2006) comprises: an initial denaturing stage of 2.5 minutes at 94°C, followed by 30 cycles of a denaturing stage of 1 minute at 94°C, an annealing stage of 1 minute at 58°C, and an extension stage of 1 minute at 72°C. The larvae were then subjected to an elongation stage of 5 minutes at 72°C before being stored at 4°C until use (Figure. 5.1).

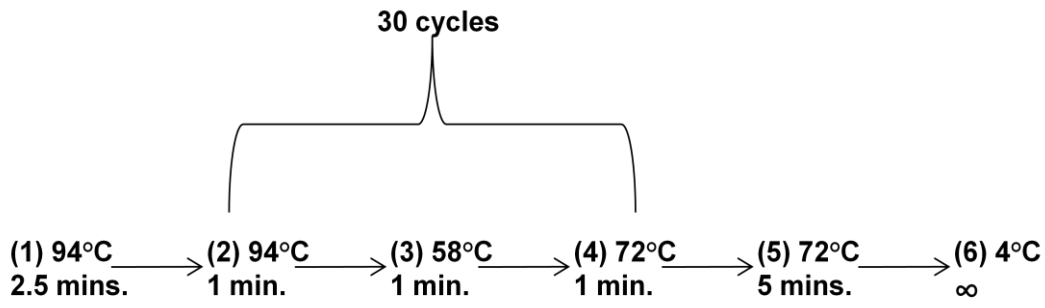

Figure. 5.1. A visual representation of the 18S PCR reaction.

The PCR cycle used for the reactions utilising the COI gene was also taken from Webb *et al* (2006) and comprised an initial denaturing phase of 2.5 minutes at 94°C, 40 cycles consisting of a denaturing phase of 1 minute at 94°C, an annealing phase of 1.5 minutes at 52°C, and an extension phase of 1 minute at 72°C. Once the 40 cycles had finished, an elongation stage of 72°C for 5 minutes followed before the samples were stored at 4°C until use (Figure.5.2).

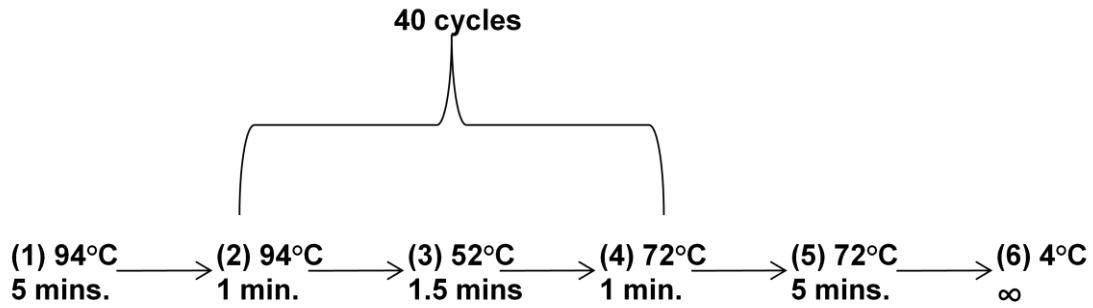

Figure. 5.2. A visual representation of the COI PCR reaction.

The resulting PCR products were subjected to gel electrophoresis on a 1% (w/v) agarose gel in 1 x TAE for 45 minutes at 90 volts. Gels were subsequently viewed on a UV transluminator with successful reactions producing a band with a fragment length of

710 bp for the COI gene, and 550 bp for the 18S gene. The above protocol was repeated for each plate of bivalve larvae collected.

5.3.3. Genetic analysis of larval samples.

For each successful reaction, a 10 µl aliquot was placed into a separate vial and sent to LGC Genomics GmbH. Each reaction was sequenced in a forward direction only using the 18S and COI primers used in the PCR reaction. The sequences were initially examined using the MEGA and BioEdit software packages to discard incomplete sequences and to check ambiguous bases using the associated chromatograms. The remaining sequences were then analysed using the Basic Local Alignment Search Tool (BLAST) function of the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Each sequence was subjected to a BLASTn search and those library sequences with over 97% query coverage and 95% identity similarity were added to the sequence library for this study.

Examples of each of the database sequences of bivalve adults identified in the BLASTn searches and the equivalent larval sequences were then aligned using the BioEdit software package. The sequences were trimmed to remove all of the primer sequences from the larval sequence and to ensure every sequence was of a uniform length to allow for better comparison. A distance-based phylogenetic tree was created using the PHYLIP ver. 3.68 software package (DNAdist and Neighbour) to examine and test the degree of similarity between the sequences derived from the NCBI/ Genbank database and larval sequences generated in this study. In order to test the robustness of the phylogenetic tree produced, bootstrapping was conducted using the PHYLIP package (Seqboot) using 1000 replicates. A consensus tree was constructed using PHYLIP

(Consense) that displayed the reliability of each grouping within the phylogenetic tree as a percentage. Percentages over 70% were considered to represent sufficient reliability in confirming the groups of different sequences. The distance tree and consensus bootstrap trees were combined to form a single tree.

5.3.4. Statistical analysis.

The PRIMER statistics package was used to analyse the data using nonparametric multivariate methods (Clarke, 1993; Clarke & Warwick, 2001; Clarke & Gorley, 2006). Uncontrolled sampling effort was accounted for by standardisation which converted the data to a percentage of sample totals. The data was untransformed and resemblances among samples were calculated using the Bray-Curtis coefficient (Clarke *et al.*, 2006), and relationships among samples were visualised using MDS in 2 dimensions. Stress values were calculated using Kruskal's stress formula to give an indication of the success of the algorithm in reproducing the rank order of resemblances in 2 dimensions. All plots in this chapter have stress values of <0.1 which corresponds to excellent to good representations (Clarke, 1993). Hierarchical cluster analysis by variable was conducted to test for multivariate structure within the standardised data. A Simprof (similarity profile) test was used to identify statistically significant evidence of genuine clusters within the samples.

5.4. RESULTS.

5.4.1. Bivalve sequences.

During the weekly sampling effort a total of 1106 bivalve larvae were collected (Table 5.I) and subjected to PCR. Following sequencing and subsequent sequence analysis, a total of 406 usable 18S sequences and 66 COI sequences were obtained (Table 5.III). The overall success rates for amplification and sequencing were 37.6% for the 18S gene and 6.0% for the COI gene. The proportion of successful, usable sequences obtained from the original bivalve larvae collected varied considerably ranging from 7.3% to 93.8% success rates in 18S reactions, and 0% to 37.5% in COI reactions per weekly sample (Table 5. III).

Table 5.III. The number of successful 18S and COI sequences obtained from each L4 sampling trip.

Sample Date	No. of 18S sequences	No. of COI sequences
26/10/2009	43	4
08/11/2009	7	36
09/11/2009	26	4
01/12/2009	25	0
15/12/2009	18	9
04/01/2010	11	5
18/01/2010	22	0
26/01/2010	44	4
01/02/2010	22	2
08/02/2010	35	0
15/02/2010	28	1
23/02/2010	90	1
01/03/2010	16	0
23/03/2010	19	0
Total	406	66

It is evident that PCR amplification was considerably more successful on the 18S gene than with COI. The successfully amplified bivalve COI gene sequences did not return

any significant hits using the BLASTn search, therefore, it was evident that there were not sufficient reference sequences on the database. The 18S gene sequences from the bivalve larvae did return successful species hits for every individual from BLASTn searches. As a result of the poor successful amplification rates and lack of sufficient BLASTn hits, the COI gene had little current application for the identification of bivalve larvae to a species level in this study. Therefore, the decision was taken to focus solely on the analysis of the 18S sequences. The full sequences in MEGA format for the 18S and COI genes in each bivalve larva can be found in Appendix 4.

Based on the BLASTn searches of these 18S sequences, a total of 279 were found to be the larvae of *Phaxas pellucidus* (Pennant), 4 were found to be *Ensis* sp., 30 were those of *Hiatella arctica* (Linnaeus), 16 were *Kellia* sp, 25 larvae were *Spisula* sp., 28 were of the genus *Musculus*, 18 were *Barnea* sp., 2 were found to be *Mytilus* sp., 1 larva was *Lyrodus pedicellatus* (Quatrefages), and 3 larvae were those of *Leiosolenus obesus* (Philippi). This information is summarised in Table 5.IV. Very few of the species recorded during this sampling effort are present as adults as recorded during the concurrent benthic survey conducted by Plymouth Marine Laboratory at the Station L4 sampling site. The data from this survey can be found in Appendix 4.

Table. 5.IV. The total numbers and occurrences of BLAST hits from bivalve larvae sampled from Station L4 during this time-series.

	Phaxas pellucidus	*Ensis* sp.	*Hiatella artica*	*Kellia* sp.	Mactridae	*Musculus* sp.	*Barnea parva*	*Mytilus* sp.	*Lyrodus pedicellatus*	*Leiosolenus obesus*	Total
26/10/2009	1	0	20	8	0	6	6	2	0	0	43
08/11/2009	1	0	0	2	0	3	1	0	0	0	7
09/11/2009	10	0	0	3	1	5	7	0	0	0	26
01/12/2009	1	0	7	2	0	10	4	0	1	0	25
15/12/2009	13	0	1	1	1	2	0	0	0	0	18
04/01/2010	10	0	0	0	0	1	0	0	0	0	11
18/01/2010	13	0	0	0	8	0	0	0	0	1	22
26/01/2010	44	0	0	0	0	0	0	0	0	0	44
01/02/2010	22	0	0	0	0	0	0	0	0	0	22
08/02/2010	26	0	1	0	6	0	0	0	2	0	35
15/02/2010	28	0	0	0	0	0	0	0	0	0	28
23/02/2010	84	3	0	0	3	0	0	0	0	0	90
01/03/2010	16	0	0	0	0	0	0	0	0	0	16
23/03/2010	10	1	1	0	6	1	0	0	0	0	19
Total	279	4	30	16	25	28	18	2	3	1	406

5.4.2. Phylogenetic tree analysis.

The phylogenetic tree shows the similarity between the larval sequences generated in this study compared to those available in the database, with shorter branch lengths between sequences corresponding to higher sequence similarity. A distance phylogenetic tree was produced using the PHYLIP software package and the values produced during the boot-strapping procedure were overlaid (Fig. 5.3).

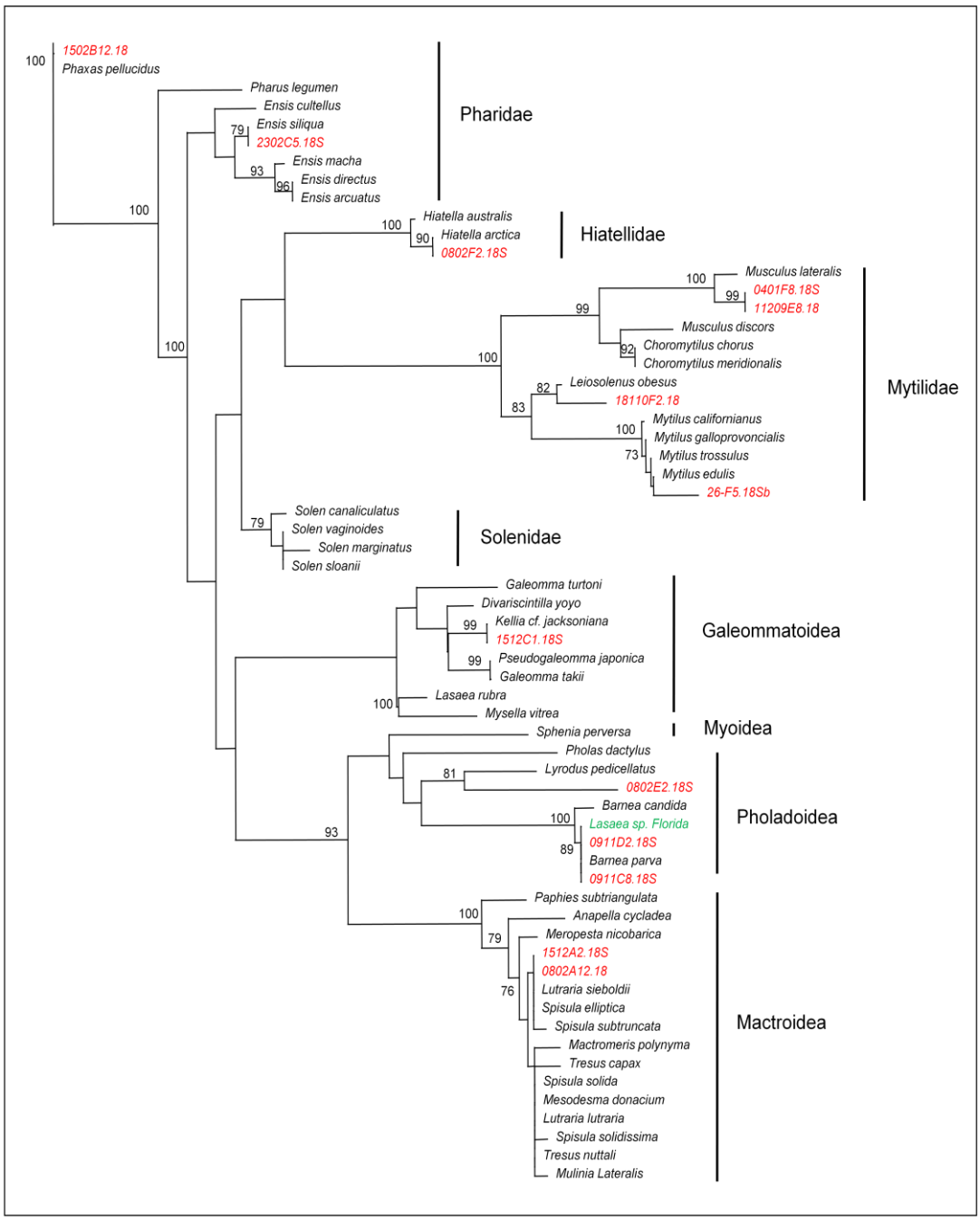

Figure 5.3. Unrooted neighbour-joining tree based on a 550 bp alignment of partial sequences of the 18S gene using a distance algorithm between sequences generated from larvae in this study (highlighted red) and other bivalve species from GenBank (Neighbour, in PHYLIP version 3.68). Bootstrap values were retrieved from 1000 replicates and are indicated at the nodes. The different bivalve families are indicated on the tree. *Lasaea sp. Florida* is highlighted in green as it belongs to the superfamily Galeommatoidea and not the superfamily Pholadoidea.

From this, it is evident those 279 sequences identified as that of *Phaxus pellucidus*, with a high degree of certainty and a 100% score in the bootstrapping test, are indeed the larvae belonging to this species. Those sequences initially identified as *H. arctica* can be confirmed by phylogenetic analysis as the larvae of *Hiatella arctica* with a bootstrap value of 90%. Larval sequences that were originally identified as a species of *Barnea* by BLASTn were shown by phylogenetic analysis to be identical to *Barnea parva* (Pennant) with the grouping supported by a bootstrap value of 89%. Phylogenetic analysis could not separate samples 0911C8.18S and 0911D2.18S from the sequences of *B. parva* and *Lasaea* sp. Florida. *Lasaea* sp. Florida, however, is a species of *Lasaea* recorded from Florida, USA, left at the genus level, therefore it is acceptable to assume that these larvae were indeed produced by *B. parva* as this is a known local species (Marine Biological Association of the United Kingdom, 1957).

Those larvae identified as a species of *Mytilus* were unable to be reliably assigned to an individual species following phylogenetic analysis. However, this is not surprising given the known occurrences of *Mytilus edulis* (Linnaeus) / *Mytilus galloprovincialis* (Lamarck) hybrids in the study area (Hilbish. *et al.*, 2002). Therefore it is likely that the larval sequences from Station L4 are indeed those of a hybrid of the two species. Another mussel genus, *Musculus*, was represented in the larval sequences (0801F8.F8S, 11209E8.18S) by BLASTn but the phylogenetic tree shows that these sequences are unique and group separately from other *Musculus* species. Therefore, while it is probable that these larvae are of the genus *Musculus*, the identity of the particular species remains unknown most likely due to the absence of a suitable reference sequence.

Many of the sequences within the superfamily Mactroidea are highly similar, including *Spisula* sp. and *Lutraria* sp, and cannot be reliably resolved further by the phylogenetic analysis. Therefore, the sequences 1512A2.18S and 0802A12.18S that were initially identified as a species of *Spisula* were unable to be resolved to species level. The bootstrap value and therefore the degree of certainty in grouping these species was lower than 70% and so phylogenetic analysis could only reliably ascertain that the larval sequences belonged to the superfamily Mactroidea.

16 larvae were originally determined to be those of the genus *Kellia* and phylogenetic analysis shows that the representative larval sequence (1512C1.18S) was most similar to that of *Kellia* cf. *jacksoniana* (Smith) with a bootstrap score of 99.2%. However, as this is an Indo-Pacific species, these larvae are most likely to be *Kellia suborbicularis* (Montagu), a species known to inhabit the Plymouth area. As the sequence of *K. orbicularis* is absent from the NCBI database, we can assume that adults of this species have not been sequenced. Therefore, it cannot be reliably proven that the larval sequences match the adults and they must be left resolved at genus level and recorded as *Kellia*.

The sequence 0802E2.18S, is reliably grouped with *Lyrodus pedicellatus* (supported by a bootstrap value of 81%), however, the sequences are different suggesting this sequence represents a different species of this genus particularly as. *L. pedicellatus* is an Indo-Pacific species. The same can be said of the larval sequence (18110F2.18S) identified as that of *Leiosolenus obesus* by BLASTn, another Indo-Pacific species from the family Mytilidae. A bootstrap score of 82% suggests that they are from the same genus, however, the branch length between them indicates the sequences are different and the sequence from this study most likely represents a different species. Four larvae

that matched reference sequences of species from the genus *Ensis* using BLASTn. The phylogenetic tree shows that the sequences are identical to *Ensis siliqua* (Linnaeus) and the grouping is supported by a bootstrap value of 79%.

5.4.3. Statistical analysis.

The untransformed standardised data displayed in MDS ordination shows two distinct groups within the samples (Fig. 5.4). Cluster analysis using a Simprof test confirms their presence (Fig. 5.5). While there are other groupings within the data, it is only the two groups identified at a 20% similarity level that are statistically significant.

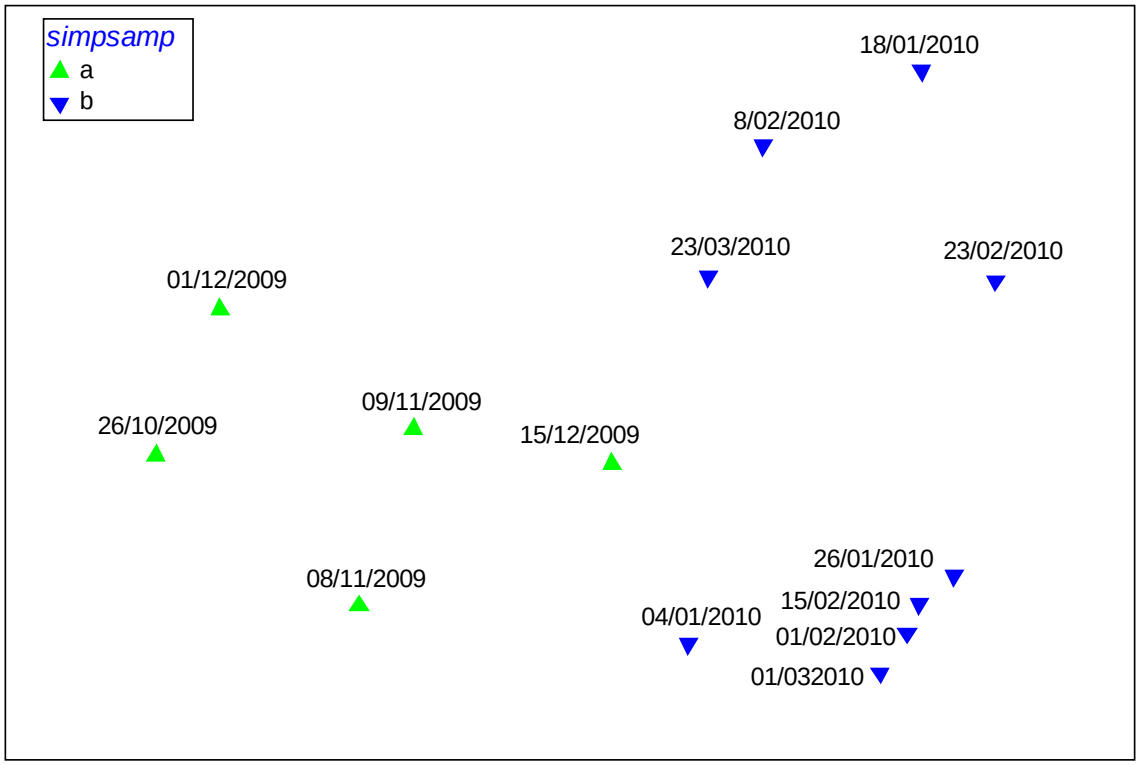

Figure. 5.4. MDS ordination of samples based on Bray-Curtis similarities calculated using the sample data standardised by total (2D stress=0.08). Two groups are shown based on the results of the Simprof test.

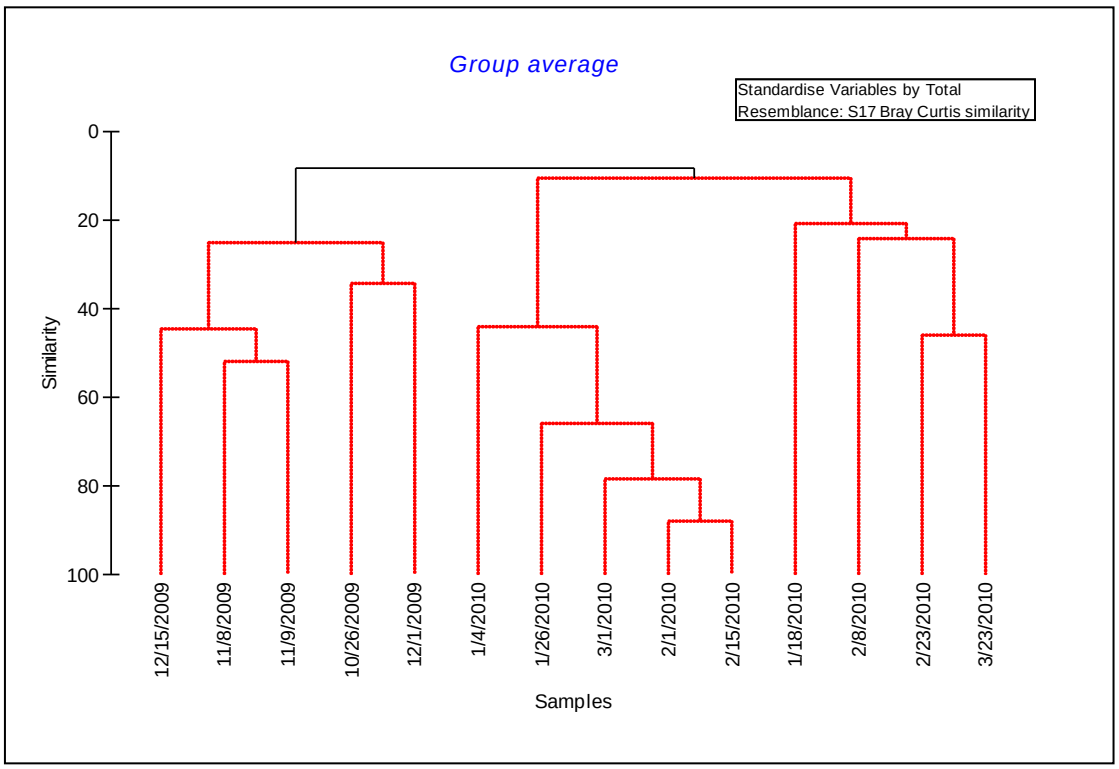

Figure. 5.5. Dendrogram showing the results of cluster analysis and Simprof test on the standardised samples. Branches highlighted in red are not statistically reliable.

Bubble plots were created to display the percentage of each taxon in each sample in order to visualise when each taxon were most abundant in each sample (Figs. 5.6 & 5.7). It is clear that different taxa are more abundant at different times of year.

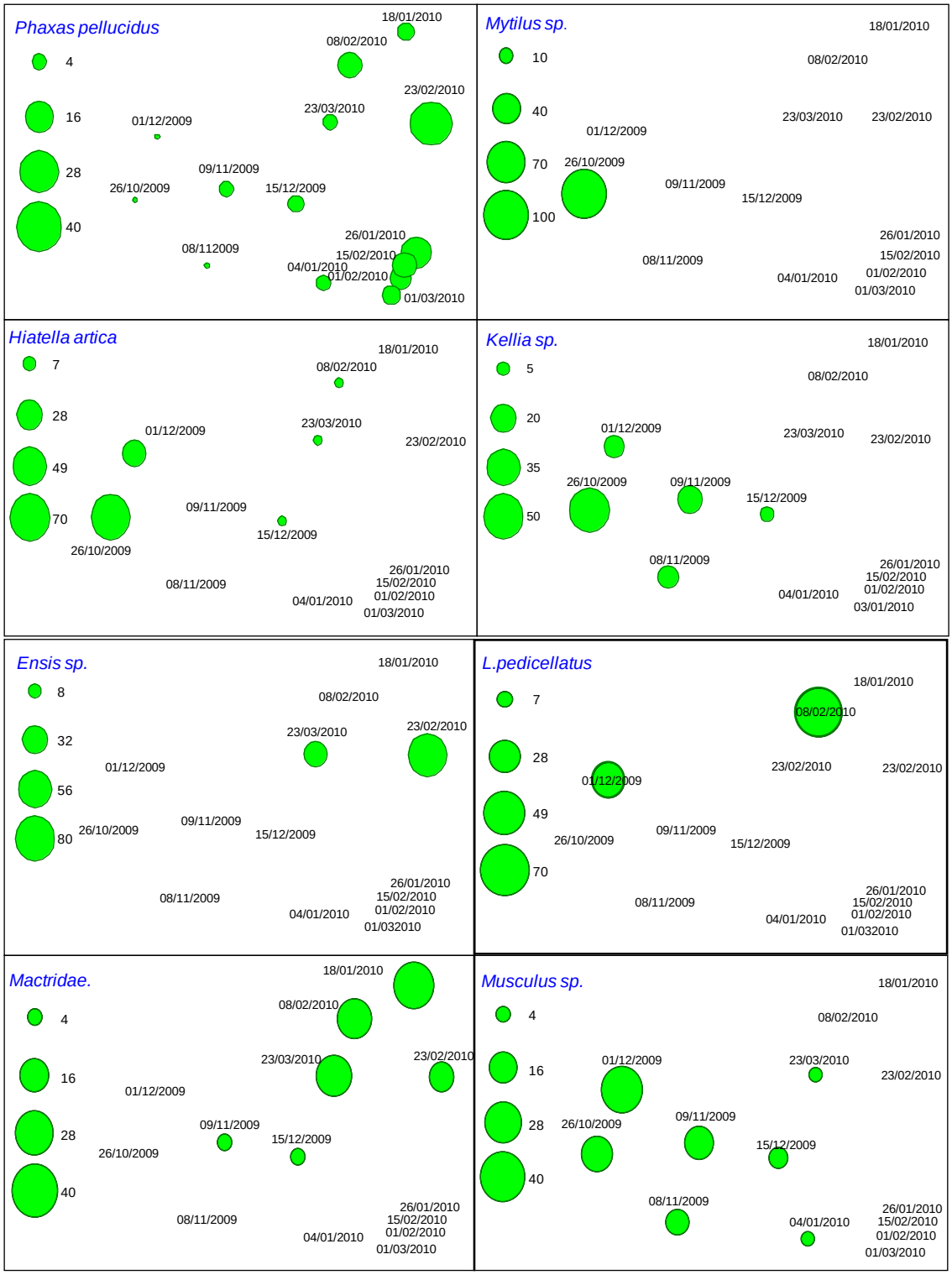

Figure. 5.6. MDS ordinations of samples based on Bray-Curtis similarities calculated using the sample data standardised by total (2D stress=0.08) and displayed as bubble plots showing the percentage of each taxon in each sample for *P. pellucidus, H. arctica, Kellia* sp., *Mytilus* sp., Mactridae, *Musculus* sp., *Ensis* sp., and *L. pedicellatus*.

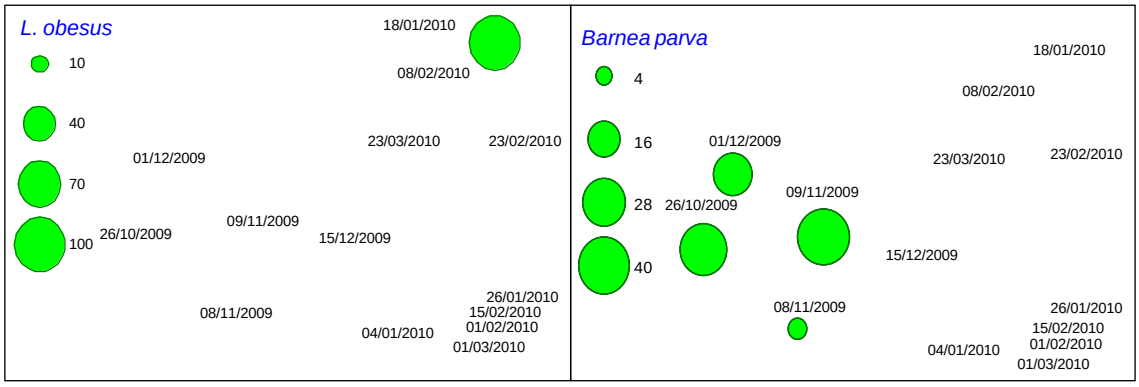

Figure. 5.7. MDS ordinations of samples based on Bray-Curtis similarities calculated using the sample data standardised by total (2D stress=0.08) and displayed as bubble plots showing the percentage of each taxon in each sample for *B. parva*, and *L. obesus.*

MDS of the variables (taxa) standardised by total reveals which taxa have similar patterns of density across the samples, and shows the presence of two groups of taxa (**A** & **B**) (Fig. 5.8). Cluster analysis and Simprof confirm the presence of these two groups that are statistically different from one another at a similarity level of 5% (Fig. 5.9). Further groupings were revealed within group **A** following these analyses. *Lyrodus pedicellatus* was found to be separate from the other taxa in group **A** at a similarity level of 20% and *Kellia* sp, *Barnea parva*, and *Musculus* sp. were found to be different from *Hiatella arctica* and *Mytilus* sp. at a similarity level of 47%. *Kellia* sp. and *B. parva* were found to be different from *Musculus* sp. at a similarity level of 69% and *Kellia* sp. and *B. parva* were separated at a similarity level of 71%. *H. arctica* and *Mytilus* sp. were unable to be reliably separated any further from one another. The similarities between the species in group **B** are unable to be resolved any further with any degree of certainty.

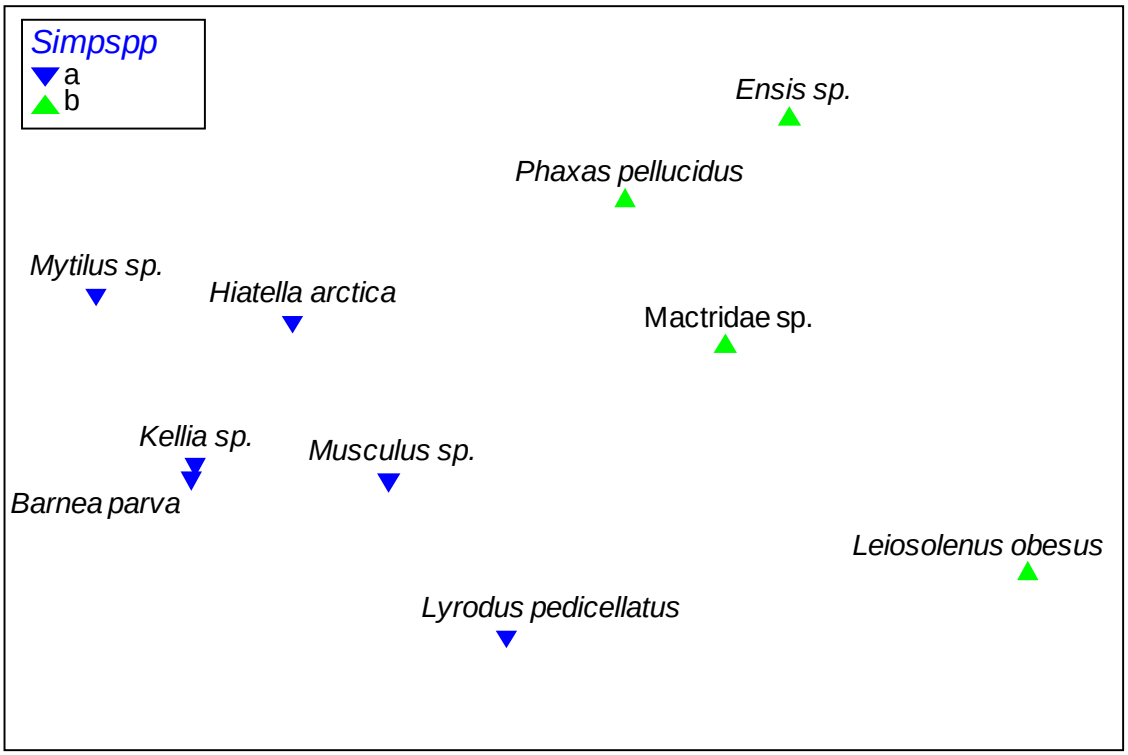

Figure 5.8. MDS ordination based on Bray-Curtis similarities calculated using the variable data standardised by total (2D stress=0.02).

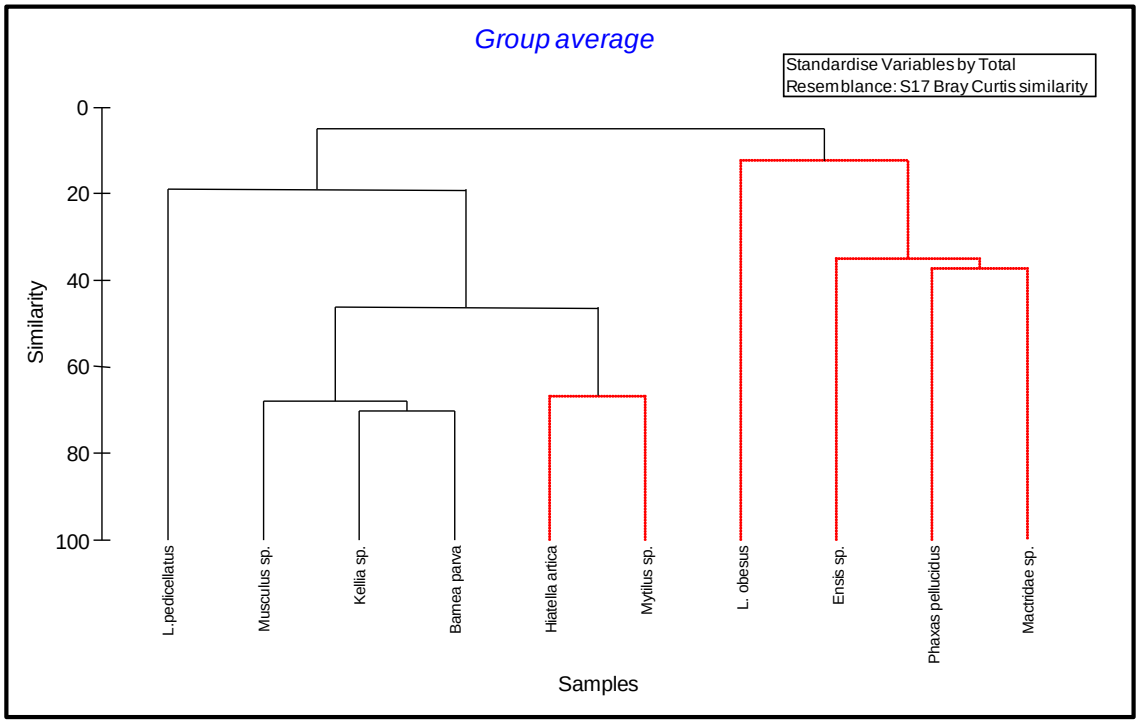

Figure. 5.9. Dendrogram showing the results of cluster analysis and Simprof test on the samples. Clusters formed by branches highlighted in red are not statistically reliable.

5.5. DISCUSSION.

5.5.1. Factors affecting the efficiency of the PCR and sequencing protocols.

The percentage of successful PCR reactions varied considerably across all of the larvae in this study. Only 37.6% of larvae collected during the study were able to successfully produce 18S sequences. While this has provided a large amount of usable data, it is likely that had more sequences been successfully identified then more patterns could have been discerned from the data, a higher number of species may have been observed, and those that were observed might have been able to be resolved to a higher taxonomic level than was possible.

There are likely to be numerous reasons for these failed PCR reactions. During the pre-treatment phase, larvae may not have been adequately broken down using the needle homogeniser as they are very small and it is possible that they remained intact despite homogenisation. This would then inhibit the efficiency of the buffer solution in breaking down cell membranes and allowing access to the DNA. Studies have assessed the success rates of different pre-treatment methods (Sawada *et al.*, 2008) and found that pre-treatment methods are essential in successfully amplifying larval DNA and the choice of method can have major implications on the success of the amplification process. This study used the method described by Webb *et al* (2006) and this type of method was found to have a success rate of around 65% by Sawada (2008).

The thawing process can cause degradation of larval DNA thus hindering PCR (Dessauer, 1996), as can the presence of a shell (Bell & Grassle, 1998) and other unidentified factors (Sawada *et al.*, 2008). It may also be that the presence of certain

PCR inhibiting compounds within the larvae may have caused the failure of a number of the PCR reactions. If these compounds are species-specific then it is possible that the presence of certain species may have been missed. This will have potentially skewed the results as those larval species that are more amenable to PCR will be recorded and others missed. With only 37.6% of larvae sampled able to produce suitable 18S sequences, this is a distinct possibility as the identities of 62.4% of the larvae sampled remain unknown.

The use of the 18S rRNA gene to distinguish between bivalve larval species has been well documented (Bell & Grassle, 1998; Garland & Zimmer, 2002; Giribet & Wheeler, 2002; Webb *et al.*, 2006; Taylor *et al.*, 2007) and has proved adequate in this study. The use of the mitochondrial gene COI has also been highlighted (Wight *et al.*, 2009) but its utilisation in this study provided a lower number of successful PCR reactions and subsequent sequences than the 18S gene. The greater number of successful 18S sequences and greater coverage of sufficient 18S adult bivalve sequences from the NCBI database than those of COI meant the use of the 18S gene was preferred. The COI sequences produced did not match bivalve database sequences and so it would be useful to generate the COI sequences of the adults of bivalve species known to inhabit the Plymouth area to create a reference sequence library with which to compare the larval sequences generated in this study, possibly revealing the presence of species that have not been recorded in this survey so far. To accomplish this, the reasons behind the low PCR success rate need to be investigated.

5.5.2. Abundance and species composition of bivalve larvae at Station L4.

The species composition of the bivalve larval assemblage varies throughout the time series. The larvae of *Phaxus pellucidus* were by far the most abundant sampled and occurred in every sample with the highest numbers being recorded on 23rd February 2010. On this date, 3 larvae were identified as another member of the family Pharidae, E*nsis siliqua*. The larvae of several species that bore into hard substrata were recorded in varying numbers. *Hiatella arctica, Barnea parva,* and larvae similar to L*yrodus pedicellatus* were all recorded from the autumn and winter months. *L. pedicellatus* is an Indo-Pacific species of ship-worm that has been introduced to the Black Sea and eastern Mediterranean (Vishwakiran *et al.*, 2001; Luyten *et al.*, 2006) and bootstrapping shows that the larval and reference sequences are grouped in 82% of the boot-strap replicates. However, it is likely that the actual species identity of the larvae is one whose 18S sequence is not yet on the NCBI database.

A total of 28 larvae from the genus *Musculus* were recorded in October, November and December 2009. Bootstrap analysis shows that the larval sequences were different from that of the library sequences of *Musculus discors* (Linnaeus, 1767), *Musculus lateralis* (Say, 1822), *Choromytilus chorus* (Molina, 1782), and *Choromytilus meridionalis* (Krauss, 1848) which were determined by BLAST search to be the most similar. *M. discors* is a northern European species whereas the remaining three are tropical or southern hemisphere species. Therefore it is likely that the parent species for these larvae is not on the NCBI database and so the larvae must be left at the genus level. This is shown to be *Musculus* due to *M. lateralis* being the most similar species as shown by phylogenetic analysis (Figure 5.3). It is possible that the sequence relates to that of

Musculus subpictus (Cantraine), a bivalve species formally known as *Modiolarca tumida*, but there are no sequences of this species on the NCBI database.

Larval sequences were matched to reference sequences of other mytilid species in three other cases; 2 were identified as most similar to a species of *Mytilus* and 3 were identified as most similar to *Leiosolenus obesus* by BLASTn searches. These larvae were recorded during October 2009, January 2010, and February 2010. Bootstrapping analysis shows that those larvae identified as a species of *Mytilus* cannot be resolved to a higher taxonomic level. This is not surprising as the occurrence of *Mytilus edulis* / *Mytilus galloprovincialis* hybrids in the South-west of Great Britain is well documented (Hilbish. *et al.*, 2002) and it is possible that the larvae in this survey are indeed those of a *Mytilus* hybrid. The hybrid zone is known to run from St. Ives in Cornwall to Start Point in Devon with very little larval transport from either the *M. edulis* zone east of Start Point or the *M. galloprovincialis* zone east of St. Ives, and with Plymouth Sound and Station L4 situated towards to centre of this zone, all adult and larval *Mytilus* individuals are known to be hybrids (Hilbish *et al.*, 2002).

M. edulis/galloprovincialis hybrids are known to exhibit doubly uniparental inheritance (DUI). This unique mechanism in *Mytilus* spp. is contrary to the normal uniparental mtDNA in most other animals (Śmietanka *et al.*, 2004). The female genome is usually only transmitted in the maternal lineage and is present in both males and females, while the male genome is transmitted in the paternal lineage. In DUI however, the female genome can be transmitted by the paternal lineage as it can assume the role of the male genome (Śmietanka *et al.*, 2004). This can hinder phylogenetic studies of taxa and populations (Ladoukakis & Zouros, 2001) and it is possible that this is the case in this study. There are sequences of the ribosomal 18S gene of *M. edulis/galloprovincialis*

hybrids present on the NCBI database but no homology was identified suggesting different regions of the gene had been sequenced. This, combined with the lack of matches with the COI sequences generated in this investigation, prevented further resolution of *Mytilus* individuals. *L. obesus* is an Indo-Pacific wood boring mytilid species (Owada, 2007) and it is unlikely to be the origin of the larvae collected, as the BLASTn search conducted on the larval sequences returned *L. obesus* with a maximum identity of 94% which supports this supposition.

The larvae of the genus *Kellia* were recorded during the months of October, November, and December 2009. The level of taxonomic resolution can only be reliably set at genus level as *K. jacksoniana* is an Indo-Pacific species and the known local species, *K. suborbicularis* is absent from the database. Further sequencing of *K. suborbicularis* adults would allow for these larvae to be further discriminated. Larvae whose sequences matched those of a number of species in the family Mactridae occurred in most months with the exception of October and were most common in the spring months. Each larval sequence returned a large number of BLAST hits with over 97% maximum identity when compared to the NCBI database. The phylogenetic tree also could not resolve the relationships within this family suggesting that the members of the family Mactridae share a large amount of sequence similarity between their 18S gene sequences and as such cannot be reliably resolved to the species level.

Statistical analysis of the untransformed standardised data shows the presence of two distinct groups of samples (Figs. 5.4. & 5.5). The first contains the samples taken on 26/10/2009, 08/11/2009, 09/11/2009, 01/12/2009, and 15/12/2009, and the second group contains all of the remaining samples. Hierarchical cluster analysis was used to attempt to determine any natural groupings of samples within the data that are more

similar to each other than those in different groups within the data (Clarke & Warwick, 2001). The analysis shows that these two groups are different from one another at the 10% similarity level and suggests the presence of one species group from 2009 and another from 2010. Any further grouping between the samples within these two groups was not significant.

Using individual bubble plots (Figs. 5.6. & 5.7) to show the percentage of each taxon in each sample, allows for the temporal distribution of those groups to be observed. It is clear that some of the species/genera are prevalent during the time period covered by the first sample cluster and others are prevalent during the second cluster of samples. For example, *Kellia* larvae are more abundant in the autumn and winter months of 2009 whereas the larvae of *P. pellucidus* are much more abundant in the winter and spring months of 2010. MDS and cluster analysis (Figs. 5.8. & 5.9) show the presence of two distinct groups (A & B) within the species recorded, at a similarity level of 5%. This further supports the patterns observed in the bubble plots. These groups contain the taxa shown to be prevalent at particular times in the bubble plots of abundance. Further groupings were identified in group A. However, it is not possible to reliably determine any further groupings within group B. The studies on the Station L4 time-series data from 1988-2007 and 2008-2010 showed that seasonal variation was most prevalent. These bivalve data show the group containing the 2009 samples represents larvae released in the autumn and early winter months, while the group containing the 2010 samples represents larvae released in late winter and early spring, suggesting the presence of seasonal variation.

Many of the species that were expected to have larvae present in the samples in this study were in fact absent, such as *Aequipecten opecularis* (Linneaus). Their presence as

adults at the study site has been confirmed by the benthic survey conducted concurrently by Plymouth Marine Laboratory at Station L4 (Appendix 4). Therefore, it is reasonable to have assumed that their larvae would have been recorded in this survey. The presence of the larvae of several species known to inhabit hard substrata is particularly interesting as adults of these species are absent from the benthic survey. The sea-bed at Station L4 is a mixture of sand and shell gravel and therefore we would expect to find bivalve species that inhabit these types of substrata and exhibit a burrowing life strategy. It may be that the larvae of those species that inhabit the study site as adults either exist at a depth very close to the sea-bed, a level that a horizontal tow doesn't sample, or they exhibit a high dispersal distance from the parent individual, and this is why they are absent from the samples. However, given the regularity of the sampling effort, one would have expected to record the presence of the larvae of these species.

The fact that hard-substrata species are common in the meroplankton in the autumn and winter months may be as a result of the plentiful hard substrata found around the borders of Plymouth Sound. The Plymouth breakwater may be a source of these larvae and it may be that the larvae of *H. arctica* and *B. parva* possess those characteristics suitable for a long residence time and high motility in the meroplankton, thus allowing for a greater dispersal distance from the adult to be attained. Further examination of the behavioural and morphological characteristics of these larval species would potentially allow the reasons for their abundance in the samples to be determined. The presence of high motility and strong swimming apparatus would explain how the larvae travelled the distances from local hard substrata.

The larvae of *P. pellucidus* dominate the meroplankton sampled in this study during the winter and spring months of 2010. This species is known to inhabit sandy soft-sediments (Marine Biological Association of the United Kingdom, 1957) and is recorded from the study site and so the presence of *P. pellucidus* larvae in these samples is to be expected. The number in which they occur is surprising and this may be a reflection of the physiological characteristics of the adult which cause the production of a large number of offspring or the larvae may be able to move vertically in the water column relatively freely whilst not travelling a large distance from the adults. The abundance of *P. pellucidus* in the results may also be an artefact of the methodology as PCR may be more successful in amplifying these larvae than those of other species.

Lebour (1947) described the presence and relative abundance of the larvae of *Lima hians* (Gmelin, 1791) in the meroplankton community in Plymouth Sound both inside and outside the breakwater (Lebour, 1937b). This species was known to inhabit coarse sand and gravel and can reach up to 400mm in length (Marine Biological Association of the United Kingdom, 1957). *L. hians* is completely absent from both this study and the benthic survey suggesting that it has disappeared from the Plymouth area since the 1930s and 1940s. This may be for a number of reasons and it is likely that removal due to commercial trawling or other anthropogenic impacts is the main cause (Capasso *et al.*, 2010). The dominance of the meroplankton in this study by the larvae of small-bodied species, for example *P. pellucidus*, may also be an indication that larger bivalve species are absent from the immediate area with the exception of those that inhibit hard substrata and are thus reasonably safe from fishing pressure.

The use of the 18S rRNA gene has allowed for the identification of bivalve larvae to the species level in many cases. Previously, bivalve larvae have only been recorded at the

Class level in plankton studies at Station L4. This study has allowed for the identification of a seasonal succession of sorts among the bivalve species of the Plymouth area where distinct groups of taxa and time periods have been recorded. It has also revealed certain species whose presence in the meroplankton at the study site is a surprise and other species whose absence is also of particular note.

The enhancement of the existing database of both 18S rRNA sequences and mtCOI sequences would further the understanding of the patterns revealed in this study by allowing for the rest of the larval sequences produced to be analysed. In order to further determine the seasonal patterns touched on in this study, it would be useful to continue the sampling effort over a longer time-period. This would possibly allow for those species that spawn at different times of the year to be identified and recorded.

CHAPTER 6. THE DEVELOPMENT OF A MOLECULAR TECHNIQUE TO DISTINGUISH DIFFERENT SPECIES OF MEROPLANKTONIC LARVAE.

6.1 INTRODUCTION.

The investigation detailed in this chapter outlines the development of a simple and cost-effective method to distinguish between the larvae of certain meroplanktonic species using molecular techniques without the need for performing sequencing reactions. This would save time and expenditure while still expediting the large through-put of samples involved in studies of planktonic organisms. Traditionally, the number of samples associated with large field surveys often dictates the reliance on microscopic techniques as the sample processing time associated with the use of molecular methods is often too high to provide a viable alternative (Lindeque *et al.*, 2006). Therefore, the aim was to develop a quick and simple molecular method which would allow for the greater taxonomic resolution of such planktonic samples as those from the ongoing time-series at Station L4.

As previously discussed in Chapters 3, 4, and 5, the analysis and identification of meroplankton samples to date have been determined by the restraints of the morphological characteristics present and recognisable combined with the taxonomic expertise of those analysts involved. Identification is also often hindered by the numerous similarities between the larvae of different species within a genus or family (Garland & Zimmer, 2002; Webb *et al.*, 2006). The use of molecular techniques to identify planktonic organisms including larvae is being increasingly used to overcome these problems (Lindeque *et al.*, 1999; Lindeque *et al.*, 2006).

Cytochrome *c* Oxidase subunit I is a highly conserved mitochondrial gene that appears in multiple copies within a cell making it relatively easy to amplify, and its inheritance is maternal (Gissi *et al.*, 2000; Galtier *et al.*, 2009). This causes the whole mitochondrial

genome to act as a single, non-recombining locus as all sites possess a shared genealogy (Galtier *et al.*, 2009). These characteristics have resulted in mitochondrial DNA becoming one of the most extensively used and popular markers of molecular diversity today (Yamauchi, 2005; Galtier *et al.*, 2009). The COI gene has been used in molecular studies of numerous marine organisms including: decapod crabs such as *Liocarcinus depurator* (Linneaus) (Pan *et al.*, 2008), penaid shrimp such as *Marsupenaeus japonicus* (Bate) (Palumbi, 1991; Yamauchi, 2005), porcellanid crabs (Werding *et al.*, 2001), abalone (Vadopalas *et al.*, 2006), and bivalves (Hare *et al.*, 2000). One of the reasons that the COI gene has been used so regularly in studies involving crustaceans is the early availability of robust universal primers for this gene (Folmer *et al.*, 1994; Hebert *et al.*, 2003a). Indeed, such is the usefulness of this gene that it is thought that it could provide the basis for a global bio-identification system for animals (Hebert *et al.*, 2003a). The Barcode of Life project is such an initiative and seeks to enable the rapid identification of the millions of species of life on Earth by DNA barcoding using the COI gene (Ratnasingham & Hebert, 2007). Proof of this principle has been provided by analysis of a 648 bp fragment of the COI gene among closely related species across a wide variety of phyla that shows its suitability for use in a global bio-identification system (Hebert *et al.*, 2003b).

While other genes, such as 16S mtDNA, 18S and 28S rRNA, have been used in the molecular analysis of marine organisms including crustaceans (Kim & Abele, 1990; O'Foighil *et al.*, 1995; Schubart *et al.*, 2001; Perez-Losada *et al.*, 2004; Porter *et al.*, 2005; Meyer *et al.*, 2010), the COI gene has been routinely used and shown to be particularly successful in molecular studies of a wide range of crustacean families (Werding *et al.*, 2001; Yamauchi, 2005; Pan *et al.*, 2008). Therefore, it was decided that a technique would be developed for application with crustacean species using the COI

gene. This was because the meroplanktonic larvae of crustaceans were the most abundant in both the 1988-2007 and 2008-2010 time-series from Station L4.

The use of molecular techniques to resolve the identification of meroplanktonic larvae can be expensive and time-consuming (Webb *et al.*, 2006). The performance of DNA extraction techniques and PCR reactions is costly enough before sequencing reactions are conducted. In many cases, these reactions are contracted out to external companies adding further expense to an individual study. One technique that eliminates the need for larval sequencing is PCR amplification of the conserved regions of genes such as COI, or ribosomal RNA genes, followed by Restriction Fragment Length Polymorphism (RFLP) analysis (Lindeque *et al.*, 1999). This offers a quick and reliable molecular tool which is less costly than traditional sequencing.

In order to develop a technique for the discrimination of different meroplanktonic larval species, reference sequences must be generated from adult specimens to determine the potential success of such a method. Sufficient specimens of each of the species recorded from the area are needed to provide adequate coverage of the local gene pool to account for intra-specific variation. The high abundance of crustacean larvae in the Station L4 time-series suggests that they play an important ecosystem role; therefore, three crustacean taxa were tested for their suitability for study in this investigation. Two porcellanid species; *Porcellana platycheles* (Pennant) and *Pisidia longicornis* (Linneaus), three species of the genus *Upogebia*; *Upogebia deltaura* (Leach), *Upogebia pusilla* (Petagna), and *Upogebia stellata* (Montagu), and four species of the genus *Galathea*; *Galathea intermedia* (Liljeborg), *Galathea dispersa* (Bate), *Galathea squamifera* (Leach), were known to be common as adults in the Plymouth Sound area. Their larvae were recorded regularly in the Station L4 2008-2010 data and these larvae

were unable to be resolved morphologically to the species level using light microscopy therefore making these taxa suitable candidates for selection in this investigation.

Many of the crustacean species recorded in the Station L4 time-series play an important role in the ecology of the local marine environment, for example; the mud shrimp genus *Upogebia* are important ecosystem engineers as they increase benthic respiration rates and enhance carbon and nitrogen cycling by bioirrigation (D'Andrea & DeWitt, 2009). To further understand roles such as these, it is important to understand the role of larval recruitment in structuring adult populations of these species. This can only be accomplished by identifying larvae to species which, in many cases, was impossible using traditional microscopic methods to examine morphological features. Therefore, the development of a molecular technique that allows the larvae of these species to be identified is valuable in allowing the populations of such species to be examined.

6.2. MATERIALS AND METHODS.

6.2.1. Collection of adult specimens for analysis.

Thirteen adults of *Porcellana platycheles* and adult individuals of *G. squamifera* were collected from the rocky shore at Mountbatten Bay in Plymouth Sound on 7/9/2010. Benthic sampling for adult individuals of *P. longicornis, Upogebia* sp., and *Galathea* sp. was conducted at Station L4 as described in Chapter 2.2. Seventeen *Upogebia* adults were collected: 8 x *U. deltaura*, 8 x *U. stellata*, and 1 x *U. pusilla.* Five *U. deltaura* were collected from Jennycliffe, two from the Rame Head sampling site, and one from Cawsand Bay. Two *U. stellata* were collected from Jennycliffe, two from Cawsand

Bay, and four from the L4 sampling station. The single *U. pusilla* individual was also collected at Station L4. Each adult individual was identified to species using morphological characteristics and light microscopy prior to being frozen at -20°C.

6.2.2 DNA extraction and amplification from adult specimens.

Adult specimens were subjected to the DNA extraction protocol detailed in Chapter 2.4 and DNA was quantified using the Thermo Scientific Nanodrop 1000 spectrophotometer to confirm the presence of sufficient template for amplification and to determine the appropriate level of dilution for PCR if necessary. Muscle tissue was extracted from the chelipeds of *Upogebia* adults using a scalpel and placed in homogenising solution before DNA extraction commenced.

Amplification of a partial region of the COI gene was conducted upon the extracted DNA following the protocol detailed in Chapter 2.4 using the universal COI primers LCO1490 - (forward) $^{5'}$-GGTCAACAAATCATAAAGATATTGG-$^{3'}$ - and HCO2198 (reverse) $^{5'}$-TAAACTTCAGGGTGACCAAAAAATCA-$^{3'}$ to amplify a DNA fragment of approximately 710 bp in conjunction with positive and NTC controls, as described by Folmer (1994). The PCR cycling conditions (Fig. 5.2) were taken from Webb *et al* (2006) and comprised an initial denaturing phase of 2.5 minutes at 94°C, 40 cycles consisting of a denaturing phase of 1 minute at 94°C, an annealing phase of 1.5 minutes at 52°C, and an extension phase of 72°C. Once the 40 cycles had finished, an elongation stage of 72°C for 5 minutes followed before the samples were stored at 4°C until use. The following reaction mix was used:

- DNA template 10-690 ng
- dNTPs 0.02 mM
- Promega Gotaq Flexi buffer 1x
- $MgCl_2$ 2.5 mM
- LCO 1490 5 pmol (0.5 µL)
- HCO 2198 5 pmol (0.5 µL)
- Gotaq DNA polymerase 1.25 units
- Molecular-grade water (up to a total reaction volume of 50 µL)

The resultant PCR products were subjected to gel electrophoresis on a 1% (w/v) agarose gel in 1 x TAE for 60 minutes at 90 volts. Gels were subsequently viewed on a UV transluminator to confirm the presence of a 710 base pair fragment. Failed PCR reactions were repeated using 1/10 and 1/100 dilutions with distilled water to facilitate successful PCR amplification,. Following gel electrophoresis, 10 µl aliquots of the products of successful PCR reactions were placed into separate vials and sent to LGC Genomics GmbH for sequencing. The sequences were examined using the MEGA and BioEdit software packages to construct consensus sequences for the adult specimens.

6.2.3. Enzyme digestion of adult sequences.

The consensus sequences were then uploaded to the New England Biolabs NEBcutter (Version 2.0) tool on the New England Biolabs website (http://tools.neb.com/NEBcutter2/) in order to determine suitable restriction enzymes that would cut the fragments of the different adult sequences at different locations to produce different band lengths during gel electrophoresis. The enzyme *Bpm*I was selected for use with porcellanid sequences as it will cut the 710 bp fragment belonging

to *P. platycheles* into two segments of 280 bp and 429 bp whereas it will cut the fragment from *P. longicornis* into three segments of 457, 210, and 42 bp respectively. Each enzyme used a different recognition site on the COI sequence of each species during digestion (Figs. 6.1. & 6.2).

```
230       240       250       260       270       280       290       300
TATAGCTTTCCCACGAATGAATAACATAAGATTTTGACTTCTTCCTCCAGCTTTGACTCTTCTTTTAATAAGAGGATTAGTTG
```

Figure. 6.1. The location of the recognition site of the COI gene sequence of *P. platycheles* used by the restriction enzyme *Bpm*I during RFLP digestion.

```
390       400       410       420       430       440       450       460
ATGGGAATTTTTTCTCTTCACCTGGCTGGAGTTTCTTCCATTTTAGGTGCTGTRAATTTTATCTCTACAATTATTAACATAC

580       590       600       610       620       630       640       650
ATGTTACTAACAGATCGAAATTTAAACACTTCTTTCTTTGACCCTGCTGGAGGGGAGACCCTATTCTTTACCAACATTTATT
```

Figure. 6.2. The locations of the recognition sites of the COI gene sequence of *P. longicornis* used by the restriction enzyme *Bpm*I during RFLP digestion.

The two digest reactions will produce different band lengths for each species when subjected to gel electrophoresis on a 2% (w/v) agarose gel and visualised with a UV transluminator (Fig. 6.3).

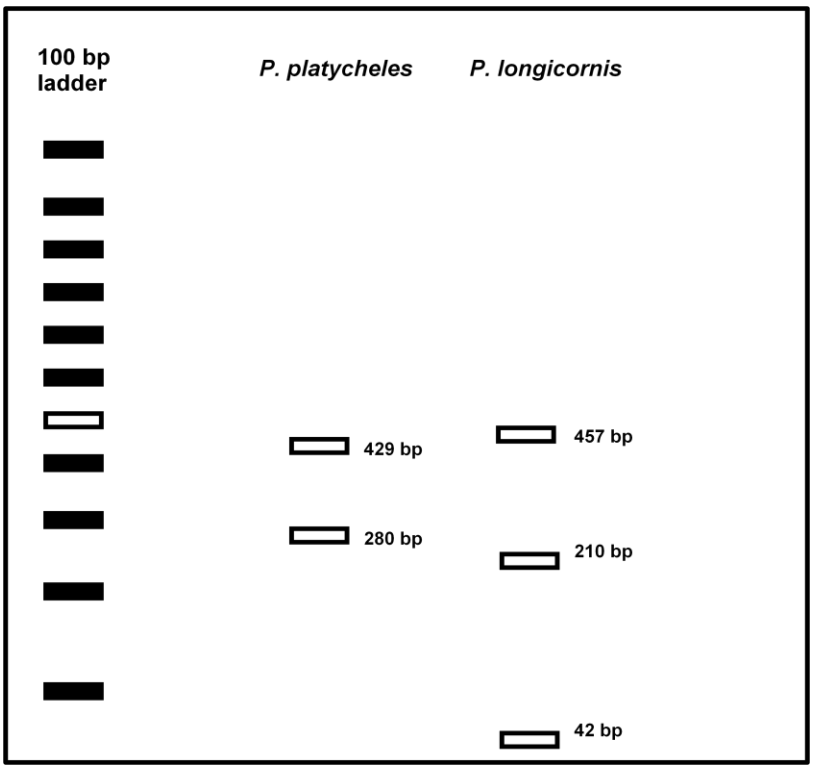

Figure. 6.3. The predicted agarose gel showing the locations of the different bands produced by restriction digest using the enzyme *Bpm*I on PCR products derived from adult specimens of *P. platycheles* and *P. longicornis.*

The enzymes initially identified as most suitable for *Upogebia* species were: *Sex*AI and *Avr*II. *Sex*AI will cut the 710 bp fragment of the COI sequence of *U. deltaura* into a 610 and 90 bp fragments and *Avr*II will cut the 710 bp fragment of the COI sequence of *U. stellata* into 452 and 258 bp fragments (Fig. 6.4). The fragment of *U. pusilla* will remain uncut.

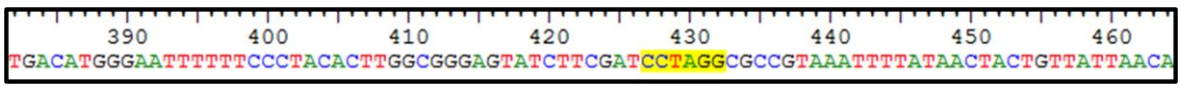

Figure. 6.4. Diagram showing the recognition site of the COI gene sequence of *U. stellata* that is used by the restriction enzyme *Avr*II during the RFLP digestion.

The PCR products of the adult individuals were cleaned up using the Promega Wizard SV Gel and PCR Clean-up System as per manufacturer's instructions. The resultant products from adult porcellanid specimens were then subjected to enzyme digest reactions using the following mix:

Reaction Mix.

- DNA template/ PCR product: 42-43 ng μL^{-1}
- *Bpm*I: 2.5 units
- NEB Buffer 3: 1x
- BSA: 0.1 mg mL^{-1}
- Molecular-grade water (to a total reaction volume of 15 µL)

The above mixture was calculated so that the final concentration of NEB Buffer 3 was 1x, and the reactions were conducted in 0.5 µl tubes and incubated at 37°C for 60 minutes before being transferred to 65°C for 20 minutes to inactivate the enzyme activity. The resulting products were run on a 2% (w/v) agarose gel in 1 x TAE buffer at 90 volts for 60 minutes to validate successful digestion. The higher percentage agarose gel was used to allow for greater discrimination between different sized DNA fragments.

The resultant products of adult *Upogebia* individuals were then subjected to enzyme digest reactions using the following mix:

Reaction Mix:

- DNA template/ PCR product: 62-72 ng μL^{-1}
- *Avr*II: 2.5 units
- *Sex*AI: 2.5 units
- NEB Buffer 4: 1x
- BSA: 0.1 mg mL^{-1}
- Molecular-grade water (to a total reaction volume of 20µL)

Bovine serum albumen (BSA) and NEB Buffer 4 were added to the reaction to provide the optimum conditions required for enzyme activity. The reactions were conducted in 0.5 mL tubes and incubated at 37°C for four hours followed by gel electrophoresis conducted on a 2% (w/v) agarose gel in 1 x TAE with 15µl of each reaction solution.

6.2.4. Collection, DNA extraction, and amplification of porcellanid larvae.

Following successful digest reactions on adults of the selected porcellanid species, reactions were conducted on porcellanid larvae. Porcellanid larvae were collected from Station L4 on 8/8/2011 using the methods described in Chapter 4.2.1 and identified to genus using light microscopy. The larvae were then homogenised using a hand-held needle homogeniser (Kontes) in 20 µL of 5x Promega PCR buffer solution using the protocol described by Webb *et al* (2006) and used in Chapter 5.3.2.1. This was conducted after test PCR reactions on unhomogenised larvae produced a low success rate. After storing the porcellanid larvae overnight at -20°C, PCR reactions were conducted on 40 individuals using the reaction mix and PCR cycling conditions detailed in section 6.2.2 in conjunction with an NTC and the DNA of an adult *P. platycheles*

used as a positive control. PCR products were then subjected to gel electrophoresis and successful reactions were purified using the Promega Wizard SV Gel and PCR Clean-up System. 18 successful reactions were recorded and digest reactions using *Bpm*I, with the reaction mix and conditions detailed in 6.2.3, were conducted. The subsequent products were run on 2% (w/v) agarose gels for 60 minutes at 90 volts after being supplemented with 6x loading dye to facilitate their retention within the wells.

6.3. RESULTS.

6.3.1. Sequencing and analysis of porcellanid adults and larvae.

Several specimens that had been preserved in 70% ethanol were used but the reactions were unsuccessful and only the frozen individuals produced satisfactory results. Of the thirteen *P. platycheles* adult specimens that produced suitable and sufficient PCR product, eleven were sequenced successfully. Of these, *P. platycheles* 3, 9, 10, 11, and 13 produced both forward and reverse sequences that allowed for the construction of consensus sequences for each individual whereas only the reverse sequences of *P. platycheles* 1, 4, 5, 6, 7, and 8 were adequate for further analysis. These sequences allowed a consensus sequence of *P. platycheles* as a species to be constructed. Three specimens of *P. longicornis* produced forward and reverse sequences that were combined to create consensus sequences for *P. longicornis* adults 1, 3, and 4. A species consensus sequence for *P. longicornis* was then constructed. Each of the above mentioned sequences can be found in the Appendix 5.

The digest reactions, conducted upon DNA extracted and amplified from adult porcellanid specimens, were successful in cutting the DNA fragments into the expected band lengths (Fig. 6.5). This proved the applicability of this technique to adults of these species. The presence of a band at 710 bp on the gel represents undigested DNA from the original PCR product.

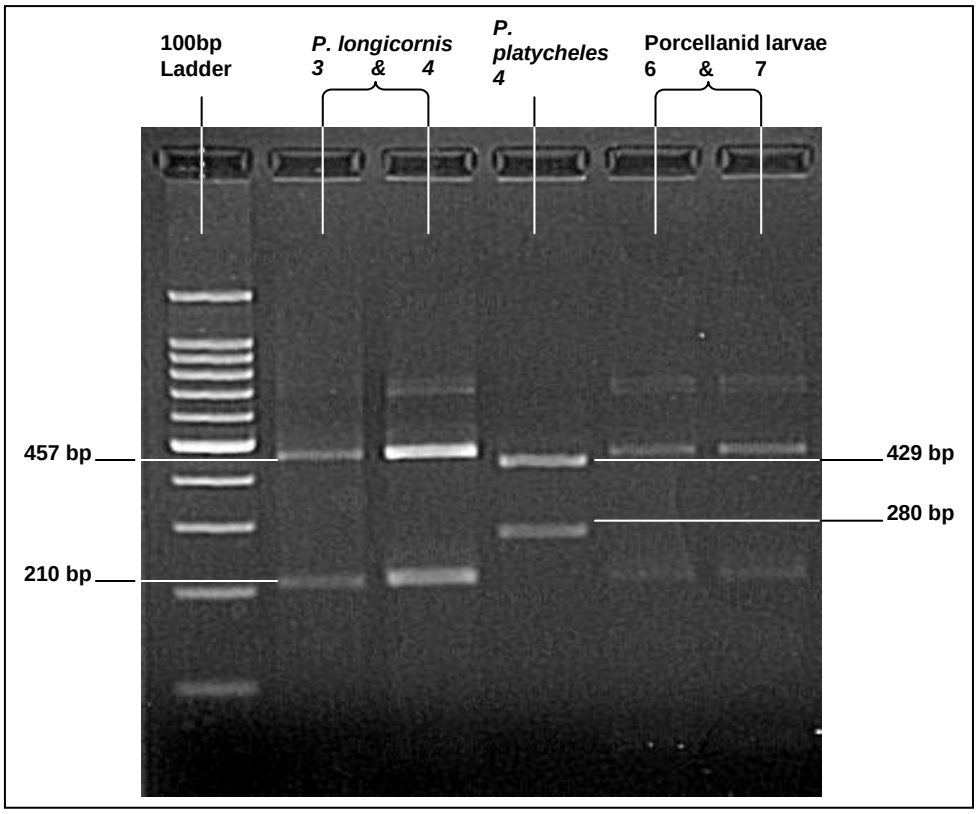

Figure. 6.5. The agarose gel produced by restriction digest using the enzyme *Bpm*I conducted on PCR products derived from adult specimens of *P. platycheles* and *P. longicornis* and two porcellanid larval samples used as test specimens.

Eighteen porcellanid larvae produced successful PCR products following homogenisation and storage in 5x buffer solution prior to PCR. This negated the need for a DNA extraction phase and enzyme digestion was conducted on these DNA fragments (Figs. 6.5. & 6.6). All of the larvae subjected to this technique were shown to be those of the species *P. longicornis.* The presence of undigested DNA was evident in

four specimens, but the expected band of 457 bp in length was present in each of the reactions, and the expected band of 210 bp was evident in sixteen specimens. The final expected band of 42 bp was present in six larval individuals. This shows that this technique worked to varying degrees of success to distinguish between different meroplanktonic larvae of the British representatives of the family Porcellanidae.

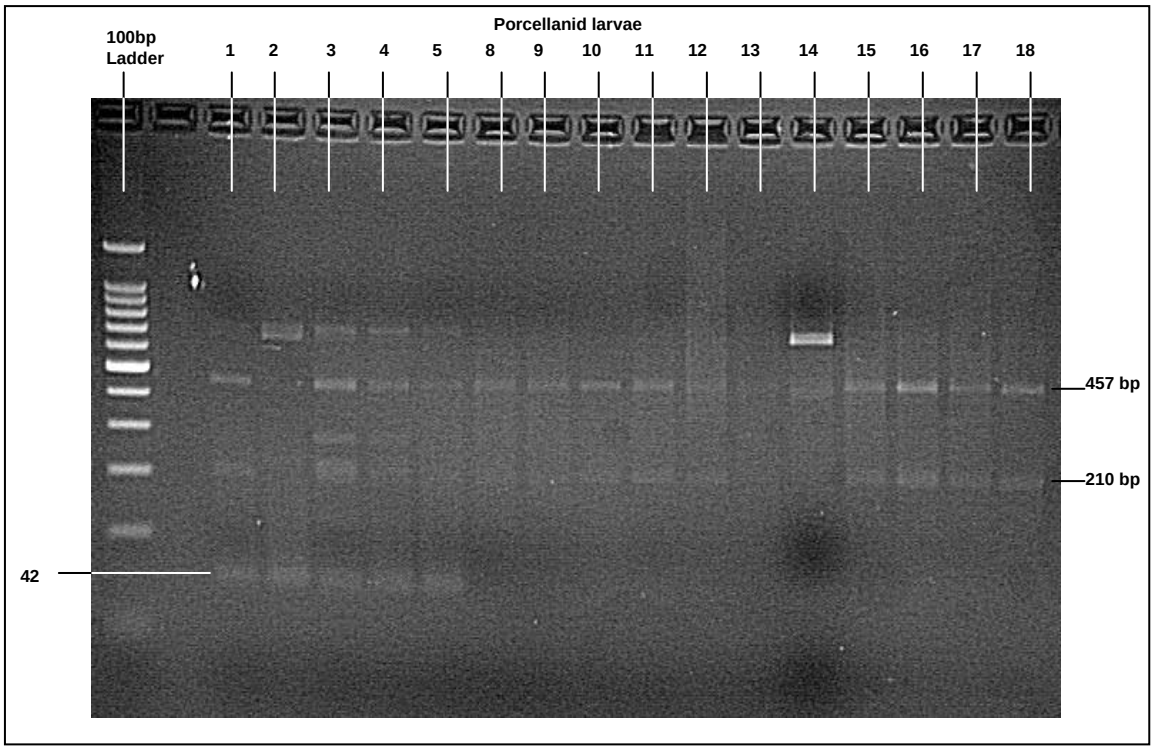

Figure. 6.6. The agarose gel produced by restriction digest using the enzyme *Bpm*I conducted on 16 PCR products derived from porcellanid larval specimens. Each larva is identified as that of *P. longicornis.*

6.3.2. Sequencing and analysis of Upogebia *adults.*

Sequences of seven *U. deltaura* adult individuals were successfully produced and these can be found in the Appendix 5. Consensus sequences of *U. deltaura* adults 1, 4, 5, and 7 were constructed. The forward sequences of *U. deltaura* 2, 3, and 6 were returned incomplete and so only the reverse sequences could be used with any degree of reliability. Using these 4 consensus sequences and 3 reverse sequences, a consensus

sequence for *U. deltaura* as a species was able to be constructed for analysis of potential restriction sites and can be found in Appendix 5. PCR products submitted for sequencing from *U. stellata* 3, 4, 5, 6, and 7 produced successful sequences. It was possible to construct consensus sequences from the forward and reverse sequences of *U. stellata* 3 and 4. Only the reverse sequences of *U. stellata* 5, 6, and 7 were suitable for analysis as the forward sequences were incomplete. A consensus sequence for *U. stellata* as a species and each forward, reverse, and consensus sequence can be found in Appendix 5. Only the reverse sequence for the single individual of *U. pusilla* was suitable for analysis and can be found in Appendix 5. It was impossible to construct either a consensus sequence for the species as a whole due to the paucity of successful sequences and available adult specimens.

The digest reactions using *Avr*II were successful in determining which individuals were those of *U. stellata*, but reactions using *Sex*AI were unsuccessful in cutting the fragments of *U. deltaura* rendering it impossible to separate *U. deltaura* from *U. pusilla* (Fig. 6.7).

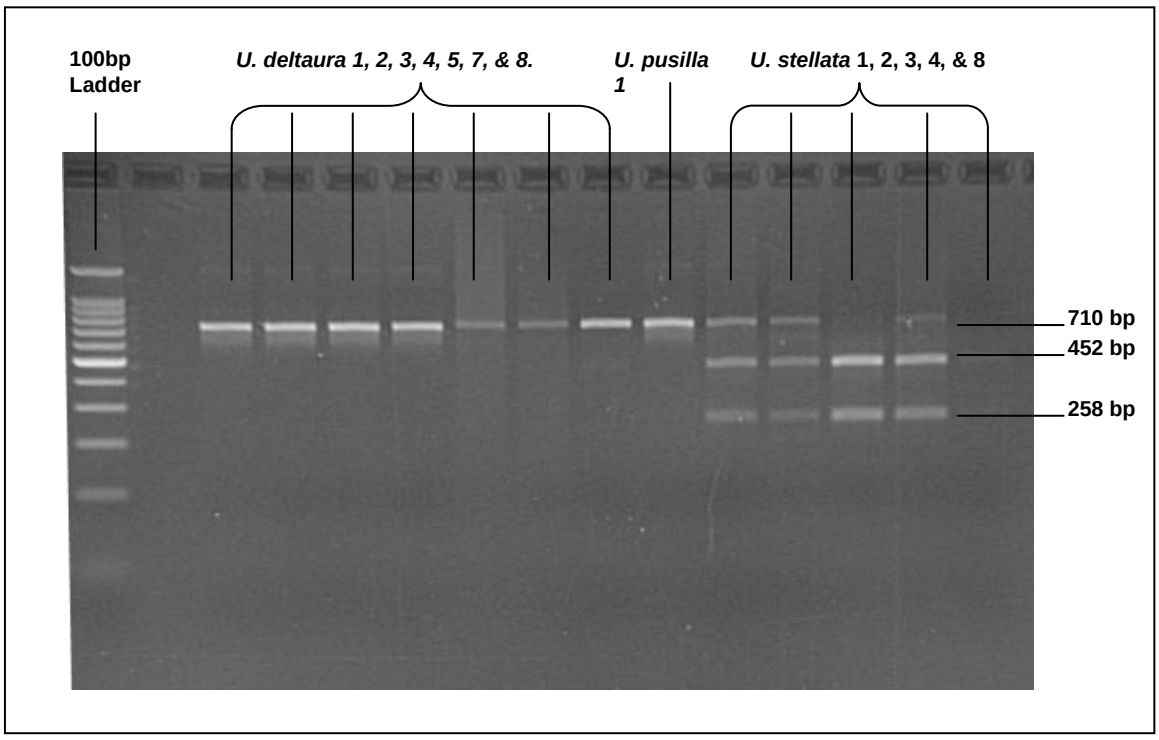

Figure. 6.7. Agarose gel showing the results of the restriction digest performed upon adult specimens of the three species of *Upogebia* present in the Plymouth Sound area.

The restriction enzyme *Sex*AI, initially identified to enable the digestion of the COI sequence of *U. deltaura* into 613 and 97 bp fragments, relied on the presence of an ambiguous site in the sequence. This site was only present in one adult sequence and did not occur in the rest of the specimens. Further examination of the consensus sequences and original sequence data confirmed this error. Therefore, the restriction enzyme was unable to cut the original sequence of this species into smaller fragments and allow for the resolution of different species as it did not recognise the region initially identified as eligible for cutting in the parent sequence. This was the only enzyme that could be identified to allow for the differentiation of either *U. deltaura* or *U. pusilla* and further examination revealed no further dissimilarities between the 710 bp COI fragments of these two species. This suggests that either there is no variation in this gene between *U. deltaura* and *U. pusilla* or that the single sequence produced from *U. pusilla* did not adequately represent the intra-specific variation present within this species. It was also noted that the presence of a failed digest could not be detected, as

the expected result for *U. pusilla* would be an undigested 710 bp fragment thus resulting in failed digest being mistakenly identified as this species. Therefore, it was not possible to successfully develop a molecular technique to discriminate between the *Upogebia* species during this study.

6.3.3. Analysis of Galathea *adults.*

The PCR reactions using DNA extracted from *Galathea* adults were not always successful and this lower success rate, combined with the difficulties in sampling adequate numbers of each species in the genus *Galathea*, led to the decision to abandon this avenue of research as only individuals of two species could be collected. Therefore, it was not possible to successfully develop a molecular technique to discriminate between the *Galathea* species during this study.

6.4. DISCUSSION.

Several genera were considered to be candidates for use in the development of a molecular identification technique, and the British representatives of the family Porcellanidae were selected as the most suitable. The selection of a crustacean species was determined by the considerable abundance and dominance of the crustacean larvae recorded throughout the year in the meroplankton samples of the 2008-2010 Station L4 time-series described in Chapter 4. This abundance of co-occurring species would mean such a technique could be implemented and provide ample larvae for use in this study thus removing the potential hindrance caused by insufficient or infrequent specimens to this investigation. Of these crustacean larvae, brachyuran larvae were the most common

but incorporated too many species known to inhabit the Plymouth Sound area (Marine Biological Association of the United Kingdom, 1957) to be suitable for the development of the type of technique detailed in this chapter. The time required to provide a sufficient number of adult specimens of these species would be too high for the constraints of this investigation.

Two species belonging to the family Porcellanidae were common in the Plymouth Sound area and surrounding environment. These were: *Porcellana platycheles*, and *Pisidia longicornis*. They were easy to identify using morphological characteristics alone and their larvae were readily identifiable as belonging to the family Porcellanidae (Lebour, 1943; Dos Santos & Gonzalez-Gordillo, 2004). They were the only taxa examined that were able to be sampled in adequate adult numbers for this investigation, and it was these factors that led to their being the final choice of species in attempting to develop a technique for identifying meroplanktonic larvae using molecular tools. The digest reactions conducted upon adult specimens showed that the enzyme *Bpm*I, identified for use with these species using the NEBcutter tool, was able to distinguish between them, thus proving the applicability of the RFLP technique.

Subsequent experimentation upon porcellanid larvae sampled from Station L4 on 8/8/2011 was successful and revealed that the larvae were all those of *P. longicornis*. This is the first time since the commencement of the Station L4 time-series in 1988 that larvae originally identified as decapod larvae in the 1988-2007 time-series, and porcellanid larvae in the 2008-2010 time-series, were able to be resolved to the species level.

The British species of *Upogebia* initially represented ideal test subjects until inadequate sampling of sufficient adult specimens from the study area prevented full coverage of the three species required. Secondly, the COI gene fragment derived from the single specimen of *U. pusilla* showed no dissimilarity with those of the adult specimens of *U. deltaura*, preventing the application of an enzyme digest that could be used to distinguish between either species. This suggested that there is either no variation in the COI gene between the two species, or that they are not in fact two separate species at all. The morphological differences used to identify them as two distinct species may merely represent two different polymorphs of the same species. Further study is needed to ascertain the taxonomic status of these two species. A greater number of *U. pusilla* specimens is required to allow for the sequencing of the COI gene in the species and, should this fail to reveal any differences with that of *U. deltaura*, the examination of different genes such as 16S would be the next route of study. Limitations in the sampling of sufficient numbers of the different *Galathea* species precluded their use and resulted in their ultimate dismissal from this study as suitable target organisms.

Individual larvae that had been homogenised and frozen in 5x PCR buffer produced the most successful results in this study as use of larvae that had been preserved in 70% ethanol rarely resulted in successful PCR reactions. This may be due to any residual ethanol inhibiting the PCR reaction, despite rehydrating prior to any reactions being initiated. The process of freezing in 5x PCR buffer was adapted from the protocol detailed by Webb *et al* (2006); however, tests of this method did not produce the required success rate within porcellanid larvae. The inclusion of a homogenisation stage facilitated further release of DNA from the cells and led to higher rates of successful PCR reactions. Homogenisation and subsequent freezing of larval specimens proved so

successful that the normal DNA extraction phase prior to PCR, as was utilised on the adult specimens, was not needed in this case.

The removal of a DNA extraction procedure when using molecular techniques to identify a given species can vastly reduce the time and expenditure normally required. Fewer reagents and a lower processing time will allow for the increased through-put of marine larvae from large surveys, thus ameliorating the typical costs of any given study. The consequences of such improved efficiency will allow for the increased application of molecular protocols in the identification of meroplanktonic larvae thereby reducing the reliance upon traditional microscopic examination of morphological characteristics and its inherent limitations. However, this technique is not intended to replace microscopic methods but instead to augment the identification procedure as larvae require identification to Family level before this molecular technique can be used. In the case of Porcellanidae, larvae can be readily identified to family using light microscopy but cannot be reliably separated to species due to the lack of definitive morphological differences between *P. platycheles* and *P. longicornis.*

The development of a diagnostic RFLP technique in this study has proven to be successful in distinguishing between the meroplanktonic larvae of the target species. This success shows that this technique has the potential to reduce the time and cost normally associated with the use of molecular methods for taxonomic resolution. The removal of a sequencing phase in the molecular procedure for marine larval identification allows this technique to be applied to samples collected at sea whilst onboard in a relatively rapid manner without the need for storage and preservation of samples for further analysis upon return to land. The use of the COI gene in this instance has allowed the larvae of two species; *P. platycheles* and *P. longicornis*, to be

identified confidently to species level which will promote the further understanding of the life-cycles and seasonal patterns of abundance and distribution of these two organisms in the Plymouth Sound area.

The implications of the technique developed in this study are to provide a simple and relatively cost-effective method to be used in conjunction with traditional microscopic techniques for the analysis of meroplanktonic samples from large field surveys. The development of a simple molecular technique will allow for the discovery of patterns of seasonality amongst certain meroplanktonic species that have hitherto been estimated or attained using laboratory experimentation. This will further the understanding of the complex life-cycles and seasonal patterns of abundance of these species at Station L4 by allowing for the increased taxonomic resolution of previously unidentified specimens. Future work to extend such a technique should allow for the meroplanktonic larvae of further species to be identified and this was only prevented by the time constraints and sampling limitations of this study.

CHAPTER 7. OVERALL DISCUSSION AND CONCLUSIONS.

7.1. SUMMARY OF INVESTIGATIONS.

The aims of the studies detailed in this thesis were to examine the dynamics of the meroplankton assemblages in a coastal ecosystem by focussing on the larvae of benthic species. A review of the literature highlighted the numerous factors affecting the survival and success of meroplanktonic larvae and identified a gap in the understanding of meroplanktonic life-cycles. Increasing the level of understanding would help to determine the subsequent effects upon benthic community composition through benthic-pelagic coupling caused by these factors acting upon meroplanktonic organisms (Pechenik, 1999; Kirby *et al.*, 2007; Weersing & Toonen, 2009). Analysis of the meroplankton abundance data from the Western Channel Observatory Station L4 time-series of the years 1988-2007 was conducted to examine and identify any seasonal and long-term patterns of meroplankton community change (Chapter 3).

The study of meroplanktonic larval dynamics has generally been overlooked in the past 50 years in favour of holoplanktonic organisms. This is also true of Plymouth Sound and its surrounding areas, which have been largely overlooked for a number of years with the focus of the 1988-2007 Station L4 time-series being mainly upon holoplanktonic organisms such as copepods (Southward *et al.*, 2004). Previous studies upon meroplanktonic larvae had been limited to the qualitative observations from the 1920s to 1940s (Lebour, 1928a; 1936; 1937a; 1947) and those of the Plymouth Marine Fauna (Marine Biological Association of the United Kingdom, 1957), thus highlighting a need for more current knowledge of the seasonal and inter-annual patterns of community composition and abundance.

Increasing the present understanding of meroplankton dynamics has the potential to further advance current knowledge of the life-cycles of benthic organisms. Further understanding the organisms which have important ecosystem and commercial roles would be of considerable value for aiding their conservation and that of their habitats so that healthy populations could be maintained. This would also aid the preservation and maintenance of those species which have important commercial value either through direct fisheries such as scallops and mussels, or providing an important link in marine food webs that commercially-fished species rely upon. The development of renewable technology could also benefit from this information in terms of the location of future developments and their potential impact upon benthic communities.

The study of meroplanktonic larvae is often limited by the level of taxonomic resolution applied to the organisms sampled. This led to the investigation of the 1988-2007 Station L4 time-series data detailed in Chapter 3 being restricted to a broad taxonomic level. As such, the decision was taken to conduct a further time-series from Station L4 with the aim of increasing the taxonomic resolution of the meroplanktonic larvae sampled in an attempt to identify seasonal patterns of abundance and species composition that were not evident in the previous time-series. By identifying meroplanktonic larvae to higher level using traditional microscopic methods, it was possible to reveal the seasonal patterns of those species that were previously included in larger groups of organisms. Continuing the Station L4 time-series has allowed for the comparison with the patterns revealed in the 1988-2007 dataset, and with the historical data provided by Lebour (1947), to assess the presence of any variation in species composition and whether similar patterns of seasonal and inter-annual variability to those identified in the investigation in Chapter 3 could be seen today. This study is detailed in Chapter 4.

Taxonomic resolution of meroplanktonic individuals by traditional microscopic methods is often limited by the expertise of the analyst, the time constraints and numbers of samples generated by large field surveys, and the meroplanktonic larvae of different species routinely displaying very similar morphological characteristics at early developmental stages (Garland & Zimmer, 2002). Whilst microscopic techniques remain the most popular method for larval identification, the need for a technique that allows higher taxonomic resolution has been identified. The use of molecular methods has been feted as a possible solution (Hare *et al.*, 2000; Webb *et al.*, 2006; Le Goff-Vitry *et al.*, 2007a; Pan *et al.*, 2008) and the investigation detailed in Chapter 5 examined the possibility of utilising such a technique to identify bivalve larvae sampled from Station L4 that have hitherto only been possible to resolve to the Class level.

The investigation described in Chapter 5 highlighted the applicability of molecular methods for the identification of meroplanktonic organisms to the species level; however, these techniques are often expensive and time-consuming and as such are limited in their usefulness in processing samples from large-scale studies. Whilst such techniques cannot wholly replace traditional microscopic methods of identification (Lindeque *et al.*, 2006), the integration of molecular methods with those of traditional microscopy has been examined and the need for simpler, more cost-effective techniques for use in conjunction with microscopy has been highlighted. The investigation detailed in Chapter 6 attempted to develop a technique which would streamline the typical molecular process by removing the need for the usual sequencing phase, ultimately reducing the cost per sample in a given survey.

This overview chapter aims to summarise the key findings from the studies on meroplanktonic larvae described in each of the experimental chapters and discuss the

wider implications of the results of this thesis. The requirements of future work and its detail will then be discussed before overall conclusions from the analyses conducted in this thesis will be drawn with particular reference to the seasonal and annual patterns of meroplanktonic organisms identified at the study site and the methods employed to further the understanding of meroplankton as a whole.

7.2. SEASONAL DYNAMICS OF THE MEROPLANKTON ASSEMBLAGE AT STATION L4 DERIVED FROM THE 1988-2007 TIME SERIES.

The presence of considerable seasonal variation in species composition of the meroplankton community at Station L4 was identified in every year of the 1988-2007 time-series. The groups examined (Cirripedia, Decapoda, Echinodermata, Bivalvia, and Polychaeta) each showed distinctly different average seasonal cycles of abundance with periods of peak numbers and decline being evident. Cirripedia accounted for the greatest proportion of the meroplanktonic larvae recorded over the duration of the time series with a massive explosion in numbers occurring in response to the spring phytoplankton bloom that occurs every year in March at Station L4 (Boalch, 1987; Aiken *et al.*, 2004; Southward *et al.*, 2004). Historical studies show that many species of Cirripedia undergo their main settlement phase in March and April, (Marine Biological Association of the United Kingdom, 1957) with some, such as *Semibalanus balanoides*, initiating spawning upon direct contact with phytoplankton cells (Starr *et al.*, 1991). It is the presence of the spring phytoplankton bloom that drives the release of cirripede larvae in the Plymouth Sound area as the diatom *Skeletonema costatum*, a species that contributes heavily to the spring bloom, acts as a trigger for cirripede species to release

the larvae they have been storing in their mantle cavity (Barnes, 1962; Gaston *et al.*, 1997).

Cirripedia larvae occur in such high numbers at Station L4 during the spring months that the small but significant increase recorded in their numbers over the time series was directly responsible for the corresponding small but significant increase observed in total meroplankton numbers over the same time period. Cirripede larvae were the only group studied that showed any significant, albeit small, change in abundance over the time-series with the largest variation in numbers generally being observed on a seasonal basis. Adult cirripedes are not found at the Station L4 site (see benthic survey data - Appendix 4) as the substrate is a mixture of sand and gravel (Pingree, 1980; Southward *et al.*, 2004). Therefore it is expected that the cirripede larvae sampled at Station L4 are spawned by adults inhabiting the hard substrate habitats in the Plymouth Sound area in large numbers (Southward, 1991) and are carried to L4 by the prevailing currents and estuarine outflow from Plymouth Sound. It is known that the abundance of cirripede species is linked to environmental temperature (Southward, 1991) and therefore, the small increase in sea surface temperature (0.6°C per decade) observed over the time-series may have contributed to the small increase seen in cirripede larvae.

Decapod larvae were shown to occur in much lower numbers than cirripede larvae, with the periods of highest abundance being observed during the spring and summer months. The occurrence of decapod larvae in most months is likely to be a consequence of the large number of different species known to inhabit the Plymouth Sound area (Marine Biological Association of the United Kingdom, 1957), all contributing to those larvae recorded as Decapoda. Historical records show that these different species exhibit different spawning times, thus ensuring that decapod larvae are present for most of the

year (Lebour, 1928a; Marine Biological Association of the United Kingdom, 1957). The pattern observed at Station L4 over the last 20 years in this study suggests that the overall seasonal cycle of Decapoda has remained the same, and there is no evidence of a trend in changing abundance over this period. The lower numbers of decapod larvae than Cirripedia are liable to be due to their larger overall size and thus larger relative energetic costs of production (Thorson, 1946). Another explanation for the larger numbers of cirripede larvae recorded in the time-series is the considerably larger adult cirripede populations than adult decapods in the surrounding habitats (Southward, 1991; Marine Biological Association of the United Kingdom, 1957) thus spawning a higher number larvae as a result. A primary reason for this disparity in adult numbers is that decapods occupy a higher trophic level than Cirripedia and therefore will occur in lower numbers as a result of the relative energy costs of occupying such a position (Menge & Sutherland, 1976; Egerton, 2007).

The highest numbers of polychaete larvae were also observed during the spring and summer months, with a similar seasonal cycle to that of decapod larvae being exhibited. The wide variety of different species of polychaete and the associated life history strategies displayed is likely to have contributed to the presence of polychaete larvae in most months, with the onset of phytoplankton blooms and subsequent increase in the populations of plankton grazers determining the release of polychaete larvae into the water column during spring and summer (Thorson, 1946).

Echinoderm larvae were recorded in the highest numbers in the summer and early autumn months and this was likely to have been related to the corresponding phytoplankton population at the study site and the highest sea surface temperatures of the year. While the abundance of echinoderm larvae has been correlated to sea surface

temperature (SST) in previous studies (Kirby *et al.*, 2007; Kirby *et al.*, 2008), the increase in overall SST at Station L4 recorded over the time-series of 0.6°C per decade (Smyth *et al.*, 2010) has not appeared to result in any significant associated increase in echinoderm larval numbers.

Bivalve larvae exhibited a massive increase in numbers on an annual basis during the time-series in the autumn months, which is most likely related to the autumn phytoplankton bloom recorded at the study site every year (Lebour, 1938; Boalch, 1987; Edwards & Richardson, 2004; Southward *et al.*, 2004). The release of bivalve larvae occurs in autumn as water temperature is known to govern gonadal development in marine bivalves (Loosanoff & Davis, 1963; Chávez-Villalba *et al*, 2002). The higher water temperatures associated with the summer months allow bivalve adults to assimilate energy by feeding on the phytoplankton in the blooms during the summer in order to produce gametes for release in conjunction with the autumn bloom. Lower competition for a ready food source with other species, decreased rates of predation by other planktonic organisms, and preference for the particular phytoplankton species found in the autumn bloom may all be contributing factors to this massive abundance of bivalve larvae during these months.

While significant seasonal variation occurred at Station L4 during the time-series, there was little, albeit significant, inter-annual variability, with the seasonal patterns of abundance and community composition being shown to occur at regular yearly intervals. Multivariate analysis confirmed that the majority of variation shown in community composition was indeed a direct result of this seasonal variation rather than any long-term change throughout the time-series. Other studies have described similar patterns of long-term variability in meroplankton populations where seasonal variation

accounted for the patterns of community composition observed while inter-annual variation remained relatively constant in terms of species diversity (Pitois *et al.*, 2009). The seasonality observed at the study site is likely to be a result of the different spawning times of each of the groups which drive the apparent succession in terms of community composition which in turn are driven by environmental cues including phytoplankton abundance and sea surface temperature (Andreu & Duarte, 1996; Kirby *et al.*, 2007; Kirby *et al.*, 2008).

Cirripedia abundance corresponded with the spring diatom bloom which has remained relatively constant and is stimulated by nutrient availability and day-length or light intensity (Edwards & Richardson, 2004). The timing of larval release is correlated with photoperiod duration (Macho *et al*, 2005) as cirripedes are known to release larvae during daytime hours in large numbers to induce larval aggregation which, through swamp effect over predators, increases larval survival. Therefore the increasing day-length during spring combined with an ample food source provided by the phytoplankton bloom enhances the chances of larval survival (Macho *et al*, 2005). Lunar-related tidal cues trigger spawning events in marine organisms. The cirripede, *Chthamalus*, is known to release larvae during spring tides which occur in conjunction of full and new moons, and certain decapod larvae that are spawned during the largest amplitude lunar tides are transported by ebb currents to deeper waters where predation is reduced thus increasing chances of survival (Christy, 1986).

Sea surface temperature does appear to play a role in determining the onset of spawning in several of the groups studied, with the main increases in abundance occurring when sea surface temperature is at its maximum, as is the case with echinoderm and bivalve larvae in response to the autumn bloom. This bloom is associated with nutrient release

caused by increased vertical mixing and the breakdown of stratification in the water column as a result of the onset of cooling and increased wind action (Findlay *et al.*, 2006). Light levels remain high enough not to inhibit photosynthesis and phytoplankton grazer populations are diluted by the vertical mixing, thus promoting the onset of the autumn bloom. At Station L4, however, this is correlative evidence through observation and there is no direct mechanistic evidence supporting this theory. Higher sea surface temperature is known to reduce larval development time thus increasing the likelihood of survival (Hoegh-Guldberg & Pearse, 1995; Reitzel *et al.*, 2004) and, in the case of cirripedes, is known to be linked directly to species abundance (Southward, 1991). Other factors such as food availability, predation, and various oceanographic processes are all likely to play a role in determining the optimum spawning time of meroplanktonic species (Eckman, 1996; Todd, 1998; Bhaud, 2000).

The value of using long-term data-sets to examine the seasonal and annual dynamics of meroplanktonic larvae is considerable. They provide a valuable insight into meroplanktonic life-cycles and allow for the important role that meroplankton play in coastal pelagic ecosystems to be examined. Meroplankton can, at certain times of the year, be the dominant members of the larger zooplankton community in terms of numbers and this can have considerable influence in the marine ecosystem as a whole. During the 1988-2007 time-series, the average meroplankton contribution in terms of numbers to the zooplankton community was 13%, but this was sometimes as high as 42.5%. They act as both planktonic predators thus determining both the population dynamics of other meroplanktonic and zooplanktonic organisms, and as a food source themselves providing a valuable resource for other marine species such as fish (Beaugrand, 2005). Changes in meroplankton abundance can therefore have considerable effects upon adult fish populations and subsequently fisheries as well, and

this can have important commercial consequences. Given the important role that meroplanktonic organisms are known to play in coastal pelagic ecosystems, it is important to continue to study and understand the trends in abundance and seasonality so that estimates can be made of possible changes in response to various environmental triggers, such as those caused by climate change (Greve *et al.*, 2005). Long-term datasets can also be used to monitor the effects of pollution events and the recolonisation of affected areas, the appearance and spread of introduced species, and the effects of temperature increases through climate change.

7.3. SEASONAL DYNAMICS OF THE MEROPLANKTON ASSEMBLAGE AT STATION L4 2008-2010.

The study described in Chapter 3 highlighted the value of using long-term datasets to assess patterns of meroplankton community change over a given time period to further understand the complex life-cycles exhibited by meroplanktonic organisms. The investigation detailed in Chapter 4 encompassed the continuation of the meroplankton time-series with the focus on increasing the taxonomic resolution applied to the organisms sampled. This was in order to identify further patterns of community composition that were not evident at the broad taxonomic level of the data from the 1988-2007 time-series.

Similar seasonal cycles of abundance to those seen in the 1988-2007 time-series were observed in the five groups in the 2008-2010 data-set, further supporting the conclusion that seasonal variation is responsible for the changes in density and community composition observed with little inter-annual variation being recorded. Brachyura zoeae

were the most common meroplanktonic organism recorded over the time-series which differs to the patterns seen in the 1988-2007 time-series where cirripede larvae were by far the most abundant. This may be a result of the different mesh sizes used, with the finer mesh of the 1988-2007 time-series sampling a greater number of cirripede larvae than that of the medium mesh in the 2008-2010 survey. By increasing the taxonomic resolution it was possible to ascertain the seasonal cycles for several species previously aggregated together as Decapoda larvae. For example, *Ebalia* zoeae were most common during the winter and spring months and porcellanid zoeae were present in their highest numbers in late spring and early summer.

The presence of a higher proportion of early developmental stages of cirripede and decapod larvae in the 2008-2010 samples is possibly a result of dispersive migration due to the development of stronger swimming abilities and more advanced morphological features. This would lead to an increase in dispersal potential as larvae develop (Lindley, 1987; Lee *et al.*, 2004). Decapod megalopae are known to regulate their position in the water column via selective tidal stream transport (STST) when returning to estuaries for settlement (Metaxas, 2001; Forward *et al*., 2003; Lee *et* al., 2005). This comprises a vertical migration pattern where megalopae move in and out of the water column in relation to tidal state in order to facilitate their movement further up the estuary (Forward *et al*., 2003b).

By travelling in the water column during flood tides and migrating to the bottom during ebb tides, decapod megalopae are able to return to the estuaries to settle (Tankersley *et al.*, 2002) whilst zoeae are transported from the spawning sites during ebb-tides (Forward *et al*., 2003b). Therefore, the tidal state at the time of sampling will have played a role in which developmental stages were sampled by a horizontal tow net as

the other stages were occupying a different depth than that of the tow net (10m). This would naturally result in lower numbers of megalopae than zoeae being sampled. Another reason may be the high mortality rate experienced by earlier developmental stages predominantly resulting in lower numbers of subsequent life-stages (Eckman, 1996).

Differences in the sampling methodology utilised during each time-series may have had effects on the variation recorded among certain groups and species. The 200 µm mesh of the WP2 haul net used in the 1988-2007 Station L4 time-series, as opposed to the larger mesh size of the horizontal tow net of 335 µm used in the 2008-2010 study, is likely to have sampled a greater proportion of smaller meroplanktonic larvae such as those of Bivalvia and Cirripedia. This is liable to skew the data in favour of those larger organisms such as brachyuran larvae that were the dominant group in the more recent survey. Previous studies (Clark *et al.*, 2001; John *et al.*, 2001; Pitois *et al.*, 2009) assessing the variation in samples of different mesh sizes concluded that, while there was an effect upon the size and number of species sampled, mesh size was not the sole determinant of the differences observed between the various surveys examined. Possible explanations include active avoidance of sampling gear and passive avoidance due to hydrodynamic factors (Clark *et al*, 2001).

By sampling every depth at the study site, the vertical haul of the 1988-2007 time-series is likely to have sampled those larvae that exhibit diel migration or do not travel far from the seabed (Colebrook *et al.*, 1961; John *et al.*, 2001) and is liable to have encountered newly spawned larvae earlier and settling larvae later than those sampled by the horizontal tow (Pan *et al.*, 2011). This would then lead to variation in the observed peaks of density for species present in each survey. The greater volume of

water sampled by the horizontal tow as a result of a larger distance covered combined with the known patchiness of meroplankton distribution, means that the horizontal tow net is liable to encounter greater numbers of individuals and therefore species than that of the vertical haul (Wiebe & Holland, 1968; Clark *et al.*, 2001).

The effects of the different sampling time-scales between the two time-series are liable to have caused considerable variation in the number and density of the individual species sampled. Regular weekly sampling (1988-2007 time-series) is more likely to have detected large increases in abundance due to spawning events than a monthly sampling effort (2008-2010 survey), which may miss them due to larval dispersal, stage duration, mortality, behaviour, and settlement (Eckman, 1996). However, the samples of both time-series were collected at approximately the same time of day, thereby ameliorating the possible effects of diel migration upon the species sampled (Pitois *et al.*, 2009).

Statistical analysis revealed the presence of four groups within the samples of the 2008-2010 data. Two groups (spring and late-summer/autumn) contained the greatest numbers of species and larvae, and represent the times of the year when food supply is plentiful and most larval spawning occurred. A degree of seasonality was evident with different species comprising the two main groups observed. The other two groups were in winter and early spring where fewer larvae were recorded. The patterns observed in the 2008-2010 study represent a general continuation of those seen in the previous time-series with seasonal variation being the driving factor in changes in community composition and abundance. It is possible that a degree of autocorrelation is present within the data with the same larval cohort potentially being sampled in consecutive

months. This could lead to apparent seasonal patterns of abundance being interpreted as more pronounced than was actually the case (Beaugrand & Reid, 2003).

Comparison with averaged historical data yielded little evidence of major variation during the intervening years at the broad scale used due to the qualitative nature of the earlier records. The degree of cyclicity observed in the historical data is much more pronounced than that of the 2008-2010 time-series as a result of the earlier records representing average abundance as opposed to the often single monthly samples present in the recent study. The degree of cyclicity in the historical records is more evident in the offshore data, and the greater diversity in the numbers and types of species recorded in the offshore data resulted in higher variation amongst samples.

The lower level of taxonomic discrimination applied to the 2008-2010 data-set compared to that of the historical data may have prevented the detection of any species disappearances during the intervening years. Any differences in the abundance of certain species may be either an artefact of converting the two data-sets into a broadly comparable abundance scale, or due to the normal seasonal and annual fluctuations in adult fecundity, larval dispersal, and survival due to various oceanographic processes, tides, currents, or wind action rather than an indicator of any long-term change (Queiroga *et al.*, 2006). The observed increase in sea surface temperature since 1988 may have contributed to such variation, but the absence of empirical sea surface temperature data corresponding with the historical data limits the conclusions that can be drawn.

Linking the meroplankton data with benthic surveys at the study site reveals the loss or reduction in the abundance of species such as *Lima hians* that are most probably the

result of disturbance by demersal fishing activities in the area, such as beam trawling and scallop dredging (Capasso *et al.*, 2010). The potential consequences of the loss of such important species from the area upon the meroplankton community and wider zooplankton community as a whole are liable to have been serious (Lindley & Batten, 2002). Such consequences have been reported from the North Sea (Kirby *et al.*, 2007; Kirby *et al.*, 2008) and are likely to continue until a population is revived by individuals from a nearby viable population. This, however, is dependent on a variety of pre- and post-settlement processes such as those previously listed (Todd, 1998; Pechenik, 1999).

While it has been shown that the spring diatom bloom has remained relatively constant and is presumably related to day-length or light intensity (Edwards & Richardson, 2004) and the link between the spring bloom at Station L4 and the spawning of meroplanktonic larvae will continue, it is important to consider the possible effects of trophic mismatch. Certain organisms that rely on temperature to stimulate physiological development or release larvae have been shown to considerably advance their seasonal cycles in response to temperature increase. The level of this response varies considerably in different organisms and has led to a mismatch between subsequent trophic levels in terms of the timing of primary, secondary, and tertiary production (Edwards & Richardson, 2004).

Variations in the timing of phytoplankton blooms in response to interannual thermal shifts have been recorded (Genner *et al*., 2010) with earlier blooms related to warmer waters. This has lead to asynchrony between phytoplankton blooms and peak abundance of zooplankton grazers, which can result in later larval spawning events being affected by changes in the phytoplankton dynamics of a given area (Edwards & Richardson, 2004). The analysis of long-term datasets can help to reveal any changes in

the timing of phytoplankton blooms and associated spawning events in response to thermal changes, and understand the effects on the population dynamics of ecologically and commercially important marine species (Genner *et al*., 2010).

7.4. SEASONAL DYNAMICS OF BIVALVE LARVAE AT STATION L4 AND THE MOLECULAR METHODS USED TO FURTHER THEIR IDENTIFICATION.

The identification and understanding of the seasonal cycles of abundance of the various meroplanktonic organisms that inhabit the study site has been limited so far, by the taxonomic expertise of the analysts involved and the morphological similarities inherent in the early developmental stages of different meroplankton species. In the 1988-2007 time-series Bivalvia larvae were identified to just 'bivalve larvae' and while the large increase in these larvae observed every year in late September and early October, and their general abundance during the winter months allowed for certain patterns to be revealed, the species that contributed to these observations remained unknown. Samples of bivalve larvae taken during the 2008-2010 study were subjected to PCR reactions and subsequent sequencing before being compared with known adult sequences on the NCBI database and through the use of a phylogenetic tree.

The presence of *Phaxas pellucidus* larvae amongst the samples was to be expected given the species' known presence as adults in the benthic substrata at the study site. However, the absence of many of the species that exhibit a burrowing life history strategy known to inhabit the soft sediments of Station L4 was surprising and this absence can only be explained by the supposition that their larvae have a high dispersal

potential from the adults, or that they inhabit a region very close to the seabed and are thus not collected by the sampling apparatus. Despite this, the relative frequency of the sampling effort would lead to the expectation of recording these species at least once.

Mytilus larvae were recorded, but were unable to be reliably resolved to the species level. This was not unexpected due to the documented presence of *Mytilus edulis*/*Mytilus galloprovincialis* hybrids in the South-west of Great Britain (Hilbish. *et al.*, 2002) and it is probable that the larvae recorded in this study are indeed those of a *Mytilus* hybrid. Species of *Mytlius* are known to exhibit a mechanism contrary to the normal uniparental mtDNA in most other animals, known as doubly uniparental inheritance (DUI) (Śmietanka *et al.*, 2004). In DUI, the female genome can be transmitted by both the maternal and paternal lineages as it can assume the role of the male genome, which can hinder phylogenetic studies of taxa and populations and is likely to have been the case in this study (Ladoukakis & Zouros, 2001; Śmietanka *et al.*, 2004). There are sequences of the ribosomal 18S gene of *M. edulis/galloprovincialis* hybrids present on the NCBI database but no homology was identified suggesting different regions of the gene had been sequenced. This, combined with the lack of matches with the COI sequences generated in this investigation, prevented further resolution of *Mytilus* individuals.

The most surprising results from the study are the presence and relative abundance of the meroplanktonic larvae of several boring species of lamellibranch such as *Hiatella arctica* and *Barnea parva*. Adults of these species are not recorded in the associated benthic survey due to their predisposition to inhabit hard substrata, although the abundance of suitable hard substrata habitats in the Plymouth Sound area may be the reason for their presence in the meroplankton community at Station L4. The Plymouth

Breakwater, for example, may be the source of these larvae but their presence at the study site would require a long residence time and high motility in the meroplankton to facilitate the dispersal distance needed from the adults. A detailed analysis of the local circulation patterns would help to determine the source of these larvae (Hilbish *et al*, 2002).

Two distinct groups within the data were identified during the study. The first group comprised five samples from the latter part of 2009, which represented those larvae spawned in the autumn and winter months, and the second comprised those remaining samples from 2010, which represented those larvae spawned in late-winter and early spring. The prevalence of certain species in one group over another does allow for a type of seasonal progression to be identified with some species appearing to spawn in autumn and others in the winter months. This highlights the value of increased taxonomic resolution in identifying patterns of abundance and species composition of meroplanktonic communities. The use of molecular techniques to facilitate this increased resolution, and the value of such methods in studies of meroplanktonic organisms is considerable, but several limitations were identified during the course of this investigation.

Despite the small size of the bivalve larvae negating the need for a distinct DNA extraction phase, and the costs both in time and reagents associated with such a stage, the efficiency of the technique was 37.6%. This resulted in many of the larvae sampled remaining unidentified due to the destructive nature of the procedure. As such, the patterns observed in species composition and abundance may be determined by those species that, for whatever reason, are more amenable to this process. This is possibly the reason for the notable absences of several species from the meroplankton that are

known to occur as adults at the study site. The limitations imposed by the existing bivalve sequence database may also have resulted in several species not being resolved to the desired taxonomic level. The absence of several known local species from the database led to several specimens not being identified further than the family or genus level. A more comprehensive database would also have allowed for the possible identity of *Mytilus* hybrids to be revealed as well as those larvae tentatively identified as most similar to the Indo-Pacific species *Leiosolenus obesus* and *Lyrodus pedicellatus* to be confirmed.

The success rates of the 18S rRNA gene were higher than those PCR reactions using the COI gene that were conducted concurrently. This, and the lack of BLASTn hits for the COI sequences of bivalve species from the database, precluded their use. The relative merits of the 18S gene for use in studies of bivalve larvae are well documented (Bell & Grassle, 1998; Garland & Zimmer, 2002; Giribet & Wheeler, 2002; Webb *et al.*, 2006; Taylor *et al.*, 2007) and it has proven useful for the purposes of this investigation. The 18S rRNA gene examined in this investigation is known to be more suitable for studies of genera and families while the mitochondrial COI gene is useful for examining populations and species (Garland & Zimmer, 2002). This may mean that bivalve taxa would be able to be successfully identified to the species level should sufficient usable COI sequences be obtained allowing the resolution of those individuals in this study that could only be reliably identified to family or genus level.

Further trouble-shooting of the technique described, in terms of pre-treatment methods and PCR conditions, may serve to increase the efficiency of using the 18S gene for the purposes of such studies. This would therefore allow for the identification of species and detection of patterns of abundance and seasonality that were previously unknown;

however it is clear that the results detailed in Chapter 5 highlight the potential benefits and applicability of the use of molecular techniques in furthering the taxonomic resolution of meroplanktonic species.

7.5. THE DEVELOPMENT OF A MOLECULAR TECHNIQUE TO DISTINGUISH DIFFERENT SPECIES OF MEROPLANKTONIC LARVAE.

The value of using molecular techniques in conjunction with traditional microscopic examination of morphological characteristics has been demonstrated in the study detailed in Chapter 5 and discussed in previous studies (Lindeque *et al.*, 2006); however, the slow and expensive nature of employing such techniques often precludes their application in large field surveys. The removal of one or more of the stages involved in the molecular identification of larval samples greatly reduces the time and expenditure of such a procedure. This increases its potential as either a viable alternative to traditional microscopy, or as a valuable aid when used in conjunction with these methods.

The development of an RFLP technique for the differentiation of meroplanktonic larvae will hopefully allow for the identification of organisms that cannot be resolved to species morphologically. A crustacean family was considered to be the most suitable candidate given the dominance in the Station L4 meroplankton samples by several co-occurring groups of Crustacea, meaning that such a technique could be implemented and providing a ready supply of larvae for experimentation. A crustacean species was selected as they were known to be common as adults in the local benthos, thus able to provide sufficient specimens for adequate coverage of the local gene pool to account for

intra-specific variation, their larvae were known to be common at Station L4, the high abundance of these larvae suggested that crustacean species played an impotant ecosystem role, and these larvae were unable to be resolved morphologically to the species level using light microscopy therefore making a crustacean taxon a suitable candidate for selection in this investigation.

Several different organisms were trialled with the species of the family Porcellanidae ultimately selected as most suitable as they sufficiently met all the criteria required. The two British species, *Porcellana platycheles* and *Pisidia longicornis*, were known to be common in the Plymouth Sound area, and porcellanid larvae were routinely sampled at Station L4 and easily distinguishable from other meroplanktonic larvae to Family level by morphological characteristics (Lebour, 1943; Dos Santos & Gonzalez-Gordillo, 2004).

The three British representatives of *Upogebia* were initially successful with specimens of each species providing sequences; however, the lack of any dissimilarity between the COI sequences of *Upogebia deltaura* and *Upogebia pusilla* prevented the identification of suitable restriction enzymes despite the successful digest and resolution of *Upogebia stellata* using the enzyme *Avr*II. This lack of differentiation between two of the *Upogebia* species may be an indicator of the presence of two polymorphs of the same species rather than two historically separate species based on morphological characteristics alone or merely a representation of the lack of variation in the COI gene. The species of the genus *Galathea* were rejected due to insufficient numbers of adult specimens being obtained.

Numerous studies on a wide variety of crustacean species supported the use of the COI gene in this investigation (Palumbi, 1991; Werding *et al.*, 2001; Pan *et al.*, 2008) and its conserved nature and presence in multiple copies within the cell made it easy to amplify in a PCR reaction (Galtier *et al.*, 2009). These characteristics have facilitated the development of the robust universal primers used in the technique developed in this chapter (Folmer *et al.*, 1994; Hebert *et al.*, 2003a; Yamauchi, 2005).

Use of the restriction enzyme *Bpm*I to target specific regions of the 710 base pair fragment of the COI gene has allowed the development of a diagnostic RFLP technique to differentiate between the larvae of the two British porcellanid species. Testing the method on material from field samples revealed that all of the porcellanid larvae were in fact those of *P. longicornis*. Further refining of the technique to increase its efficiency would encompass the initial pre-treatment of the larvae which produced varied results. Those organisms stored in 70% ethanol were unable to produce successful PCR reactions and subsequent digestion. This may have be a result of residual ethanol inhibiting the process, or the undetected presence of other PCR inhibiting compounds within the target organism. All the successful reactions were derived from larvae that had been mechanically homogenised and frozen in 5x buffer solution. *P. longicornis* individuals were considerably more difficult to produce successful results from than those of *P. platycheles*. Earlier studies have assessed the success rates of different pre-treatment methods for use in PCR reactions and the freezing of larvae in a buffer solution was determined to be suitable for use in this study (Webb *et al.*, 2006; Sawada *et al.*, 2008). The addition of mechanical homogenisation of the sample specimens prior to PCR produced a much more successful outcome.

The successful development of a diagnostic RFLP technique during this investigation highlights the potential for the use of molecular methods in resolving the identification of meroplanktonic larvae to a finer taxonomic level. The identification of porcellanid larvae to species will allow for the further understanding of the complex life-cycles and the seasonal patterns of abundance and species composition of meroplanktonic organisms at Station L4 and the wider meroplankton community. This technique, when used in conjunction with traditional microscopic methods, will reduce the normal expenditure associated with the analysis of samples from large-scale field surveys, and the removal of a sequencing phase increases the applicability of molecular methods to meroplankton identification. For instance, such a technique could now be employed on surveys at sea eliminating the need for the storage and preservation of samples for analysis upon return to the laboratory.

The technique developed in this investigation highlights the considerable usefulness of the COI gene in allowing the identification of meroplanktonic larvae in long-term field surveys. Mitochondrial DNA has become one of the most extensively used and popular markers of genetic diversity today (Yamauchi, 2005; Galtier *et al.*, 2009). The COI gene has been used in molecular studies of numerous marine organisms (Palumbi, 1991; Hare *et al.*, 2000; Werding *et al.*, 2001; Yamauchi, 2005; Vadopalas *et al.*, 2006; Pan *et al.*, 2008) and such is its usefulness that it has been used as the basis of the Barcode of Life project (Hebert *et al.*, 2003a). This project is attempting to enable the rapid identification the millions of species of life on Earth by DNA barcoding using the COI gene (Ratnasingham & Hebert, 2007).

7.6. POSSIBLE DIRECTIONS FOR FUTURE RESEARCH.

The investigations reported in this thesis have yielded important information towards understanding of the role that meroplanktonic organisms play in coastal pelagic ecosystems. However, the conclusions drawn have been based on data collected over a period of 23 years and have shown the driving factor in species abundance and community composition to be seasonal variation, with little inter-annual change observed. In order to identify patterns occurring over longer time-periods, it is important to continue collecting such time-series data from sites such as Station L4 and to compare it with data from the benthic communities. This would allow the temporal variability in meroplanktonic species to provide an insight into their long-term life-cycles. It would also allow the monitoring of the possible effects of climate change and such phenomena as the northward expansion and southern contraction of species' ranges, which are being observed elsewhere in the world today (Greve *et al.*, 2005).

The analysis of data on sea surface temperature, chlorophyll content, trace nutrients, salinity etc, in conjunction with meroplankton sampling would allow for further light to be shed on the factors affecting meroplanktonic individuals allowing studies on growth rates and reproductive output to be conducted. The effects of trophic mismatch in response to temperature changes could be observed to determine the potential knock-on effects on benthic populations and their larvae. This would enable further understanding of their seasonal and inter-annual cycles of abundance and community composition.

Studying the factors known to affect meroplankton communities such as adult fecundity and fertilisation success, growth and larval stage duration, mortality, behaviour, dispersal (Bhaud, 2000), settlement (Eckman, 1996), predation, inter- and intra-specific

competition for food or space, disease, parasites, and various physiological stresses such as temperature (Kirby *et al.*, 2008), and salinity (Eckman, 1996; Todd, 1998), will allow for the further understanding of the complex life histories of these organisms and the important role they play in the marine environment.

As the investigation into the 2008-2010 time series data-set has shown, increasing the taxonomic resolution applied to meroplanktonic larvae has revealed previously unknown information regarding those species that comprise the broad taxonomic groups detailed in the 1988-2007 time-series. This has allowed comparisons with historical data from the early to mid-twentieth century to be conducted on a broad scale that have revealed little change over this time period. By increasing the taxonomic resolution in future surveys and in the ongoing time-series at Station L4, the influence of benthic-pelagic coupling on meroplanktonic communities can be further understood by examining individual taxa rather than groups of taxa. This would allow for the prediction of the effects on either the benthic or planktonic communities caused by anthropogenic activities such as fishing, and suitable monitoring and policy-making steps to be taken.

The development and refinement of molecular techniques for use in the identification of meroplanktonic larvae will allow for the seasonal and annual patterns of individual species to be revealed. The techniques described in this thesis provide an indication of what is possible and, with further time and effort spent on collecting adult and larval specimens to facilitate the development of these methods, it should be possible to enable the identification of additional larval species. The study on bivalve larvae showed which patterns can be revealed by utilising these methods and a concentrated effort to supplement the existing 18S and COI sequence databases would allow for

further species to be reliably identified. Refining these methods to maximise their efficiency, while maintaining the associated costs through either the pre-treatment, PCR, and RFLP phases of the process would allow for a greater throughput of samples than was previously possible.

The overall efficiency of the techniques described in this thesis was relatively low with only 37.6% of bivalve larvae being successfully identified. Increasing the length of the mechanical homogenisation applied before the larvae are frozen in 5x buffer solution may cause further disruption to cell membranes and allow for the release of greater quantities of DNA for subsequent amplification. Further study to confirm the presence of certain unidentified PCR inhibiting compounds and the effects they have on these processes is required before their effects can be ameliorated.

7.7. CONCLUSIONS.

- Meroplanktonic organisms play an important role in coastal pelagic marine ecosystems. At certain times of the year they can comprise a considerable percentage of the total zooplankton community in terms of numbers. This is usually in conjunction with phytoplankton blooms, where they contributed 42.5% on one occasion at Station L4.

- Seasonal variation is the driving factor in determining patterns of species abundance and community composition at Station L4 with little inter-annual variability being observed.

- The use of long-term data-sets in revealing the long-term patterns of community composition and abundance of meroplanktonic larvae plays an important role in understanding of the complex life-histories of these species and should be continued to identify those patterns not evident over the time-period studied.

- Increasing the taxonomic resolution applied to meroplanktonic organisms facilitates the further understanding of the seasonal cycles of species at the study site that were previously unknown.

- The use and development of molecular techniques provide a valuable tool in aiding the taxonomic resolution of meroplanktonic larvae and therefore allowing for previously unknown patterns of species diversity and richness to be ascertained.

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