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The biological and ecological impacts of hypoxia on coastal benthic communities

by

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ABSTRACT

Traditionally, hypoxia has been defined as the situation where DO levels have fallen below 2.0 mg O_2 L^{-1}, but increasing evidence suggests that this low level of DO is inadequate to describe the onset of hypoxia impacts for many organisms. Consequently, there is a need for a greater understanding of how 'moderate' alterations in DO levels will affect ecosystem processes and functionality, specifically through behavioural and physiological alterations at the organism and community level. This thesis reports on mesocosm experiments which were conducted to examine the effects of moderate (> 3.0 mg O_2 L^{-1}) hypoxia on firstly, a key ecosystem engineer, the brittlestar *Amphiura filiformis*, and secondly, on the Station L4 infaunal macrobenthic community. Station L4 is a longstanding marine biodiversity and MSFD reference site and forms part of the Western Channel Observatory. At the organism level, short-term (14 d) exposure to moderate hypoxia significantly reduced oxygen uptake rates, oocyte diameter and oocyte development in *A. filiformis*. However, these physiological affects occurred irrespective of brittlestar population density. Additionally, moderate hypoxia reduced brittlestar activity, in terms of bioturbation behaviour, consequentially having an effect on ammonium and silicate fluxes. These observations were only detected when brittlestar population density was high. It was concluded that denser populations of *A. filiformis* may therefore exhibit the greatest changes in behaviour and shifts in ecosystem function as competition for resources and oxygen heightens. The benthic community at Station L4, displayed considerable tolerance to medium-term (6 wk.) exposure to moderate hypoxia, in terms of structure, diversity and bioturbatory behaviour, but these results may be different if exposure was longer or more severe. Alterations in nutrient fluxes were detected, but there was little evidence to suggest these changes were due to macrofaunal behavioural alterations. Additionally, results from this study revealed

that bringing complex natural communities into the mesocosm caused a substantial loss of individuals and species, mainly due to translocation and disturbance effects. This important insight into the effects of bringing community assemblages into the mesocosm confirms that even with a loss of diversity, the L4 community maintained functionality and was resilient to alterations in DO. This suggests that the L4 benthic community does not depend on any one specific species for the provision of important ecosystem processes, resulting in considerable functional resilience within the L4 system. However, vulnerability to benthic systems may increase if functionality is dominated by species such as *A. filiformis*. Consequently, moderate hypoxia may not immediately affect benthic communities in terms of structure and diversity, but the physiological effects on individuals, especially to reproductive development, may cause alterations in the quality and quantity of planktonic propagules supplied by benthic species to the pelagic environment. This could affect benthic community diversity and functionality in the long term if repeated hypoxic events occur.

"If a child is to keep alive his inborn sense of wonder, he needs the companionship of at least one adult who can share it, rediscovering with him the joy, excitement, and mystery of the world we live in."

Rachel Carson, 1907 - 1964

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AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes, and several relevant training courses were undertaken. Papers prepared for publication are listed at the beginning of each chapter.

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  "Ocean Acidification: the next chapter"

- Poster presentation to UK Ocean Acidification Research Programme, Exeter, UK, April 2012
  "Ocean Acidification: the next chapter"

- Oral presentation to Plymouth Marine Laboratory, Plymouth, UK, August 2012
  "The impact of dead zone expansion on biodiversity and ecosystem function: project overview"

- Oral presentation and organising committee member for Making Waves: 5th Annual Plymouth Marine Science Education Fund Conference, Plymouth, UK, December 2012
  "The impact of hypoxia on the burrowing brittlestar Amphiura filiformis: experimental design, methods and data collection"

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  "The effects of hypoxia and population density on respiration and reproductive development in the Ophiuroid brittlestar, *Amphiura filiformis*"

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CHAPTER 1: GENERAL INTRODUCTION

1.1. INTRODUCTION

Oxygen is a fundamental requirement to all aerobic organisms for survival and normal physiological function. Thus, it is considered by some, as the most important environmental variable that supports life on Earth (Diaz 2001). Within the oceans, dissolved oxygen (DO) concentrations vary considerably. Seawater is oxygenated by the influx of oxygen gas across the air-sea surface (ventilation) and also through the process of photosynthesis. Physical mixing and biological processes draw oxygenated water down through the water column to create a hospitable oxygenated environment for pelagic and benthic organisms. However, regions with extreme oxygen deficit are not uncommon and have occurred throughout geological time (Chen et al. 2007, Zhang et al. 2010). These hypoxic or anoxic areas are often termed 'oxygen minimum zones' or 'dead zones' and are formally classified as such, when DO levels are between 0.0 mg O_2 L^{-1} (anoxia) and 2.0 mg O_2 L^{-1} (hypoxia) (Diaz 2001, Rabalais et al. 2002) . Naturally occurring hypoxia has long been found in the bottom waters of silled basins and fjords, areas with restricted water circulation or in regions where upwelling of oxygen-depleted sub-surface water occurs (Middelburg & Levin 2009). In addition to these bathymetric and physical influences, DO concentrations are also governed by several biological and biogeochemical processes, (Zhang et al. 2010) making DO within the marine realm a topic of considerable interest (Diaz & Rosenberg 2011).

1.2. HYPOXIA IN RECENT DECADES

Hypoxia in the coastal oceans has spread exponentially since the 1960s (Diaz & Rosenberg 2008). Over 400 systems, affecting more than 245,000 km^2 are reported to be affected by hypoxia with the majority situated in coastal zones

(Fig. 1.1) (Diaz & Rosenberg 2008, Rabalais et al. 2010). Whilst there have always been naturally occurring marine hypoxic habitats, the recent global increase in the frequency and severity of coastal hypoxia is causing alarm, most notably because it can be attributed to specific anthropogenic activities (Diaz & Rosenberg 2008, Rabalais et al. 2010, Zhang et al. 2010). Marine hypoxia is now recognised as a global threat to ecosystem health due to its capacity to alter key ecological, biological and physiological functions (Nilsson 2000, Nilsson & Rosenberg 2000, Cheung et al. 2008, Weissberger et al. 2009).

In 1985 an article titled, "Eutrophication – the Future Marine Coastal Nuisance?" (Rosenberg 1985) was one of the first to suggest that atmospheric deposition and nutrient enriched riverine outflows were causing the onset of eutrophication in the Baltic and Kattegat Seas which, as a result, were severely affected by low DO levels. Rosenberg (1985) stated that there was enough evidence to suggest that the continued use of fertilizers and the venting of nitrogen oxides from fossil fuels will, in the near future, cause widespread eutrophication that will become a common hazard in marine coastal areas across the world (Rosenberg 1985). Unfortunately, this remarkably accurate prediction is now reflected in global data sets compiled over the 30 years since Rosenberg's initial report was published (Diaz et al. 2011).

It is now recognised that human activities such as the application of fertilizer, land clearing, discharge of human waste, animal production and fossil fuel combustion, supplement surface and ground waters with the nutrient elements nitrogen (N) and phosphorous (P) (Nixon 1995, Cloern 2001, Gray et al. 2002). This increase in flux of N and P into the hydrological system, which eventually

flows into coastal waters, acts as a 'marine fertilizer', stimulating plant growth and disrupting the balance between production and metabolism of organic matter in the coastal zone (Cloern 2001). As a result, there is a clear causal link between anthropogenic sources of nutrients and the presence of eutrophic indicators in coastal waters (McQuatters-Gollop et al. 2009). Moreover, with an ever increasing global population, the demand for food and resources is expected to heighten. This will increase the need for intensive anthropogenic practices to meet the demands of a growing 21st century population and inevitably, nutrient enrichment and eutrophication in coastal areas is predicted to worsen.
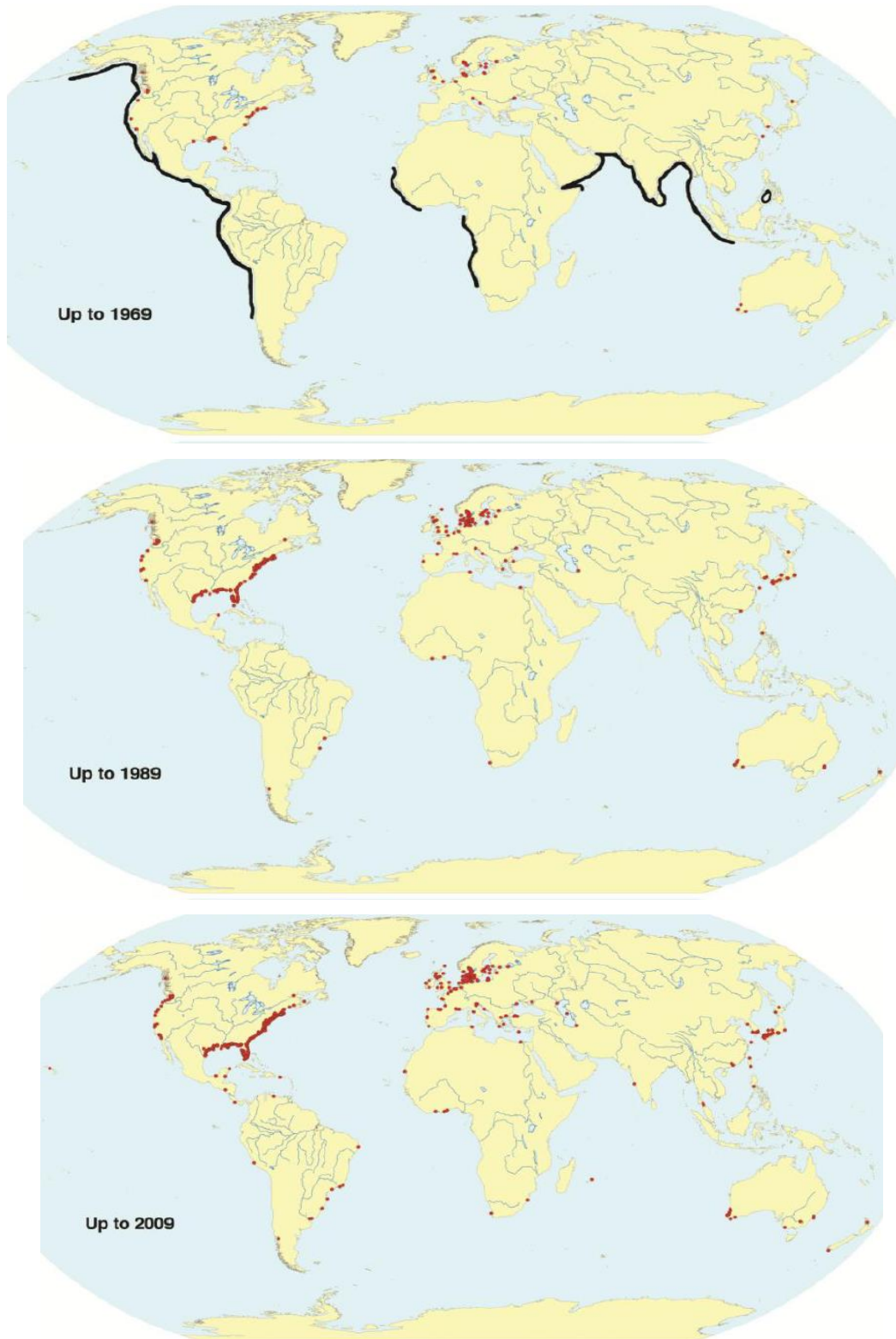
Figure 1.1. Global development of coastal hypoxia over recent decades, • = documented hypoxic event,)= upwelling areas, modified from Rabalais et al. (2010).

1.3. FROM EUTROPHICATION TO HYPOXIA

The excessive supply of nutrients to a coastal system triggers an increased growth of algae and plants, i.e. primary production (Dugdale & Goering 1967, Rosenberg 1985, Gray et al. 2002). During the preliminary stages of intensified primary production, DO levels within the water column may increase due to the upturn in production of photosynthetic organisms. However, as the yield from this primary production accumulates, it exceeds the amount consumed by grazers, and begins to sediment out and decomposes. At this point, the DO levels can rapidly decrease as the biological oxygen demand (BOD) within the water column and at the benthos increases due to microbial decomposition (Bishop et al. 2006, Zhang et al. 2010). Benthic organisms are particularly vulnerable to a surge in BOD and the consequential decrease in DO, because the large majority of the excess organic matter produced settles out and can smother the benthos.

Bottom water oxygen depletion is often exacerbated or prolonged when the water column in question is stratified or when renewal of oxygenated water is limited (Diaz & Rosenberg 2008, Levin et al. 2009). Stratification can result from strong thermal or saline gradients, particularly where freshwater run off is excessive, or where strong seasonal cycles in rainfall and temperature occur (Levin et al. 2009). In addition, tidal cycles and local currents may limit the amount of oxygenated seawater that is reintroduced within a given space and time. These common physical characteristics of coastal areas, together with the aforementioned anthropogenic pressures, make coastal regions prime

candidates for the onset of eutrophication and a concomitant decrease in DO, particularly at the benthos.

Although the causal link between nutrient enrichment and eutrophication is widely accepted, the cause-effect relationships are far more complex because coastal ecosystems respond to nutrient loading in many different ways (McQuatters-Gollop et al. 2009). For example, some of the most productive fisheries in the world are associated with nutrient inputs either from land or upwelling (Diaz & Rosenberg 2011), hence there is a delicate balance and a strong economic interest into the monitoring and management of nutrient loadings in coastal areas. Despite this interest, hypoxia is now prominent on a global scale. Over the last three decades, reports indicate an annual increase of 5.5 % in the number of coastal sites suffering from hypoxia (Vaquer-Sunyer & Duarte 2008) and there is sufficient evidence to propose that this worldwide increase in extent and frequency of hypoxia is linked to anthropogenic activities (Cloern 2001, Gray et al. 2002, Rabalais et al. 2002, Diaz & Rosenberg 2008, Howarth et al. 2011). Consequently, the most serious threat resulting from excess nutrients and the subsequent onset of the eutrophication process in coastal zones is the unseen decrease in DO levels in bottom waters (Diaz & Rosenberg 2008).

1.3.1. The importance of coastal zones

Coastal ecosystems are among the most productive and diverse areas on Earth (Mann 1988, Struyf et al. 2004, Meadows et al. 2012). They are characteristically complex, both ecologically and physically. They encompass a broad range of habitat types and harbour a unique wealth of biodiversity,

providing resources and services that are vital for human health and survival (Burke et al. 2001).

It is estimated that these marine habitats, from the intertidal zone out to the continental shelf break, provide over US$ 14 trillion worth of ecosystem goods (e.g. food and raw materials) and services (e.g. disturbance regulation and nutrient cycling) *per* year (Costanza et al. 1997, Harley et al. 2006) making them regions of considerable economic and ecological interest. As a result, healthy and productive coastal ecosystems signify a unique life support system for marine life and humans alike, where any degradation in environmental status is recognised to hold far reaching effects (IPCC 2007). However, over recent decades, human actions have profoundly changed the extent, condition and capacity of most major ecosystem types across the world (Barbier et al. 2008). Subsequently, the impacts of anthropogenic activities on coastal ecosystems, both direct (e.g. nutrient enrichment, pollution, fishing) and indirect (e.g. increasing temperatures and climate change) is a pressing issue that requires considerable attention (Harley et al. 2006).

1.4. THE ROLE OF BENTHIC COMMUNITIES

Given the importance of coastal ecosystems and the vulnerability of benthic habitats to hypoxia, the effects of decreasing DO on benthic organisms and communities is of major concern, particularly because benthic communities contribute to a number of vital processes that can influence the provision of ecosystem goods and services, in addition to maintaining ecosystem function.

Soft sediments dominate coasts and estuaries (Thrush et al. 2006) and harbour vast amounts of biological diversity, both within (infauna) and on (epifauna) the benthos. The species residing in these communities are particularly vulnerable to changes in environmental conditions due to their sessile nature. Previous work has concluded that meiofauna tend to be less affected by hypoxia than macrofauna and megafauna (Josefson & Widbom 1988, Diaz & Rosenberg 1995), however, it is not surprising that some benthic species can exhibit a degree of tolerance to reduced oxygen concentrations, as the very nature of their life-mode requires them to burrow and reside within sediments that often become anoxic beneath the superficial layers. Meiofauna tend to have a lower oxygen demand, a greater surface area to volume ratio with some species able to undergo anaerobic metabolism. They are also exposed to the surrounding sediment pore-water, low in free oxygen which enables some meiofauna species to survive hypoxia / anoxia for extended periods of time (Wetzel et al. 2001). However, not all meiobenthic communities respond uniformly to hypoxia. Data from the Gulf of Mexico shows that meiobenthic nematodes emigrate into the water column in high numbers where they survive hypoxic events until normoxia is re-established (Wetzel et al. 2001). Benthic macrofauna that reside in burrows or tubes facilitate the transport of more oxygenated water into their burrow systems, *via* bioirrigation processes. For certain species, the initial response to hypoxia involves behavioural adaptions such as raising respiratory structures above the sediment-water interface and increasing ventilation of burrow structures by peristaltic pumping or beating of appendages, to access faster moving water with more oxygen (Levin 2003). However, if hypoxia is severe or persistent, these survival mechanisms may not prevail.

It is estimated that benthic habitats can supply approximately 50 - 80 % of the nutrients required for primary production in coastal seas, with nitrogen availability being a critical factor (Herbert 1999, Dale & Prego 2002, Lohrer et al. 2004). This process is tightly linked to the transport of materials mediated by fauna living in, or on, the seabed, *via* the biogenic mixing of the sediment, a process known as bioturbation (Canfield & Farquhar 2009, Shull 2009). Through the creation of pits, mounds and burrows, sediment ingestion and excretion, in addition to the bio-irrigation of sub-surface burrow structures, benthic infauna play an important role in mediating many chemical and physical reactions. Bioturbation can enhance nutrient fluxes across the sediment-water interface, leading to increased rates of primary production, alter pH and redox gradients, affect sediment geochemistry, and help structure sediment porosity and permeability through counteracting the effects of sediment compaction (Herbert 1999, Shull 2009). Furthermore, bioturbation activities extend the oxic sediment-water interface, increasing the surface area available for sediment-water exchange processes and influencing the creation of chemically important micro-niches for a diverse array of microbes (Rosenberg 2001, Rosenberg & Ringdahl 2005).

1.4.1. Influence of bioturbation on nutrient fluxes and microbial communities

Microbial communities within sediments play a key role in the oxidation of organic compounds and the regeneration of nutrients, and are essential for sustaining primary production (Herbert 1999). Shallow coastal sediments are responsible for up to 90 % of sedimentary remineralisation of organic matter with the transformations principally mediated by bacteria (Gattuso et al. 1998).

However, as mentioned earlier, through the processes of bioturbation and bioirrigation, infaunal invertebrates have significant influence over many physical and chemical sedimentary processes in addition to affecting the structure and diversity of microbial communities (Laverock et al. 2010, Queirós et al. 2013).

Specialised groups of microorganisms play a key role in the specific transformations of nutrient elements that lead to their regeneration. Bacterial communities within sediment burrow structures can be significantly different to the bacterial community inhabiting surface sediments (Laverock et al. 2010). In addition, bacterial abundance and activity has been shown to be 10-fold higher in burrow walls compared to the surrounding sediment (Papaspyrou et al. 2005). These microbial processes are often governed by oxygen and the subsequent redox potential of the sediment (Herbert 1999, Dale & Prego 2002). For example, the marine nitrogen cycle involves several steps of oxidation / reduction reactions that take place both within the oxic and anoxic sediment layers (Herbert 1999). Within the oxic layers ammonium is oxidised to nitrite and nitrate *via* nitrification, and within the anoxic environment reduction reactions take place such as denitrification and anammox. Products of these transformations can either be released into the overlying water or adsorbed and buried into the sediment layers (Herbert 1999). Generally, in oxic environments, nutrient cycling is considered to be more efficient due to populations of enzymatically mediated microbes undertaking aerobic respiration (Dale & Prego 2002). During low levels of DO, remineralisation processes can be reduced (Kemp et al. 1990) especially if anaerobic digestion becomes the dominant pathway (Bianchi et al. 2000) and alternative e^- acceptors, if present, have to be

used. Consequently, benthic areas that host abundant infaunal communities, with a larger number of burrow or sediment structures and high bioturbation potential, are likely to have a greater role in the recycling and remineralisation of nutrients, affecting fundamental processes that contribute to the overall functioning of the ecosystem (Naeem et al. 1994, Cardinale et al. 2000).

1.5. RESPONSES TO HYPOXIA

1.5.1. Individual level responses

The responses of marine organisms to hypoxia at the individual level have been studied in greater detail than community level responses, with the wealth of our knowledge about physiological adaptations to hypoxia originating from studies conducted on intertidal organisms, which are regularly subjected to hypoxia during isolation from the water column at low tides (Diaz & Rosenberg 1995). Organism response to hypoxia is species-specific and dependent on the duration and severity of the hypoxic event. A report in 2008 conveyed that the majority of investigations referred to a value of 2.0 mg O_2 L^{-1} or lower to define hypoxia, but organisms tend to exhibit several sub-lethal physiological and behavioural modifications to hypoxia before reaching this value (Gray et al. 2002, Vaquer-Sunyer & Duarte 2008). For example, previous research has documented that growth rates could be affected at oxygen concentrations between 6.0 and 4.5 mg O_2 L^{-1} (Chabot & Dutil 1999, Gray et al. 2002), whilst other aspects of metabolism can be affected between 4.0 and 2.0 mg O_2 L^{-1} (Nilsson & Sköld 1996, Nilsson 1999, Rosas et al. 1999, Calder-Potts et al. 2015), with mortality not occurring until oxygen concentrations are below 2.0 mg O_2 L^{-1} (Wang & Widdows 1991, Baker & Mann 1992, Vistisen & Vismann 1997). Behavioural alterations also occur when oxygen concentrations are reduced,

and act as an important link between individual responses and population change (Boyd et al. 2002). For example, behavioural responses such as shallower burrow depths and elongated siphons may alter predator-prey relationships (Sturdivant et al. 2012), whilst altered locomotion may affect food finding behaviour (Boyd et al. 2002). Subsequently, behavioural changes at the individual level can hold direct and indirect consequences to individual fitness, with cascading responses across several levels of biological organisation, including effects to community composition and ecosystem function (Fig. 1.2) (Solan et al. 2004a, Zhang et al. 2010, Riedel et al. 2014).
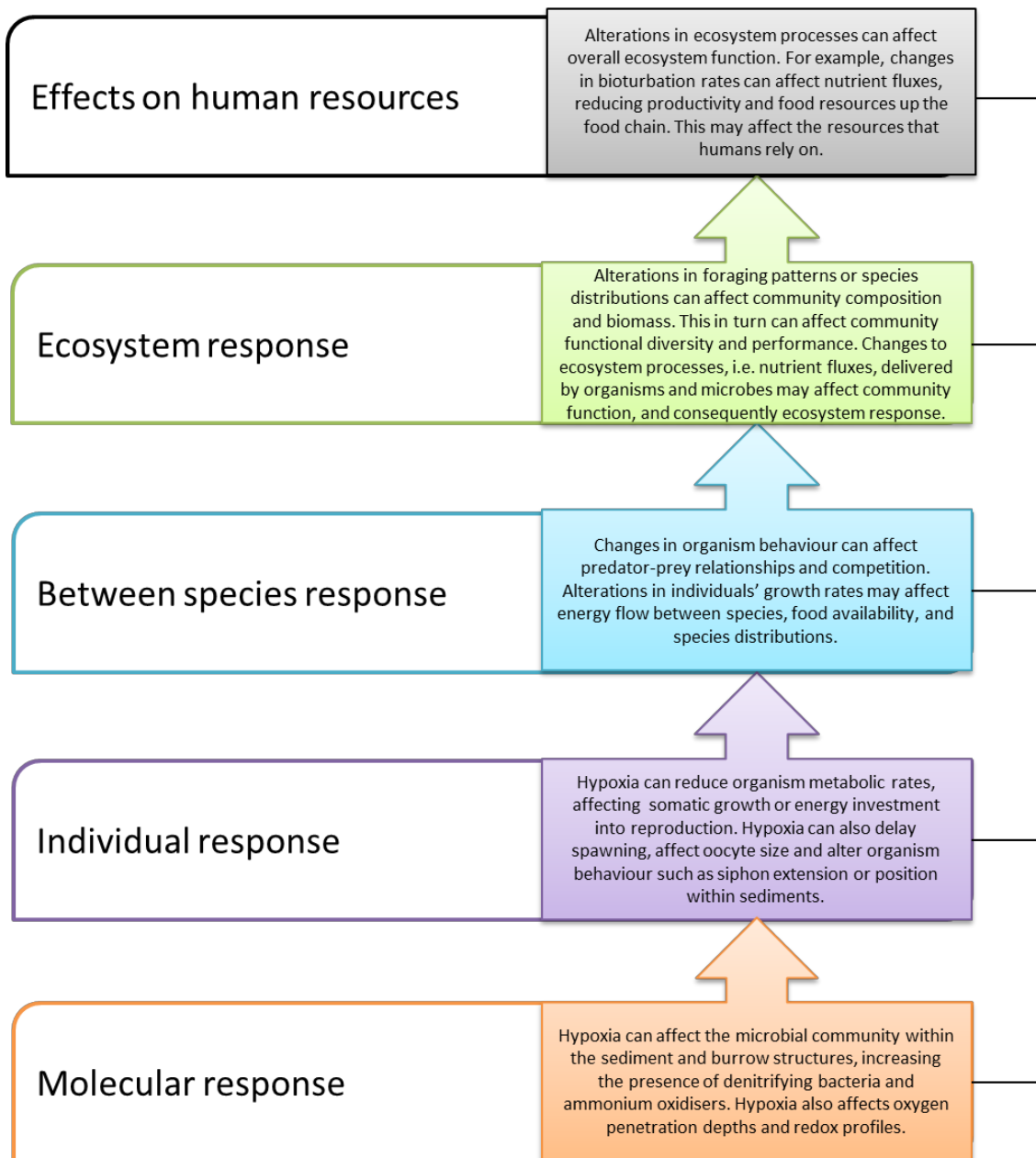
Figure 1.2. Potential effects of hypoxia at different organisational levels.

1.5.2. Community level responses

The effects of hypoxia at the individual level can be nested within and complexly related to the effects occurring at the community level, (Fig. 1.2) that may impact organism abundance, community structure, functional diversity, energy flows, food web dynamics and intra-specific competition. These modifications within benthic communities have the potential to disturb the day-to-day

processes that modulate ecosystem function (Breitburg et al. 2009). Villnäs et al. (2012) found that in natural communities exposed to hypoxia, the disturbance mediated change in community composition was an important explanatory variable for changes in sediment oxygen and nutrient fluxes, with variability in ecosystem function also being directly related to the duration of hypoxia as well as the benthic community. The number of species within a community and their identities are an important factor in assessing the consequences of disturbance for ecosystem functioning (Villnäs et al. 2012), however, other factors, such as functional diversity and the non-random order of species loss, also has a significant role on ecosystem functioning (Solan et al. 2004a, Villnäs et al. 2012). Species extinction within a community is generally considered a non-random process (Srivastava 2002) with risk determined by life-history traits such as rarity, body-size, consumer pressure and sensitivity to disturbance (Solan et al. 2004a). Consequently, some species may eventually become locally extinct with the onset of hypoxia, while others may be opportunistic and thrive (Vaquer-Sunyer & Duarte 2008). This extreme variance in tolerance to decreasing DO, partially explained by life-history traits, holds interesting consequences for population dynamics and community structure, both during and after a hypoxic event (Lim et al. 2006).

Consequently, the community level responses observed during hypoxia are difficult to predict as they depend on many different compounding aspects that are often specific to a particular habitat or site, and related directly to the population exposed. For example, reproductive state, the amount of food availability or population density of a particular species may influence the survivorship of that species during hypoxia, offering it considerable advantages

(or disadvantages) over competing species. For example, Llansó (1991) found that the timing of hypoxia relative to recruitment was critical to survivorship, and documented a decline in summer recruitment peaks for two species of polychaete with the onset of a hypoxic event.

Studies conducted in Cheasapeake Bay, U.S.A., show that higher DO levels result in a greater community biomass, until a DO threshold of approximately 4.5 mg L^{-1} (Seitz et al. 2009). Community biomass is a parameter that can often be positively correlated to primary production, but in Cheasapeake Bay this is not always the case (Kemp et al. 2005), giving reason to suggest that the benthos has been severely degraded by regular hypoxic events and there has been long-term conditioning of the community, which is dominated by opportunistic or tolerant species.

Several other factors also influence survivorship during hypoxia, such as the cause, length and severity of the event and the possibility of interacting effects of other environmental variables that organisms are exposed to, e.g. contaminated sediment or pollution. In the York River, U.S.A., periodic hypoxia has been found to be less severe than hypoxic events in similar habitat areas, due to the level of hypoxia, its short duration and relatively small area covered (Diaz & Rosenberg 1995). Consequently, the magnitude of response to a hypoxic period is species-specific, but also site-specific, making predictions about the ecological and environmental penalties problematic.

1.6. BIODIVERSITY AND ECOSYSTEM FUNCTION

Since the launch of the Convention on Biological Diversity (CBD) at the 1992 UN Earth Summit, the relationship between biodiversity (the variety of life at multiple levels) and ecosystem function has emerged as a central issue within ecological and environmental sciences, especially since the recognition that species have many important functional roles within ecosystems (Bengtsson 1998, Loreau 2000). It is now generally accepted that the variety of species within ecosystems collectively determine, in part, the occurrence and rates of key biogeochemical cycles that help to regulate global scale systems that support life on Earth (Loreau 2000). Understanding the role that biodiversity has in establishing, driving or maintaining ecological processes will help to identify the mechanisms that underpin ecosystem function and consequently enrich our knowledge about what secures the provision of goods and services.

It has been identified in terrestrial research that species richness may enhance ecosystem productivity and the more connections that are present within a food web, the greater the stability of the community (Naeem et al. 1994). However, this work fails to distinguish that species have varying levels of functions and roles within habitats and ecosystems. Recognition of species functional diversity has been an important milestone that has enhanced our understanding about the mechanisms behind biodiversity and ecosystem functioning, but has also created lively debates about the conservation of biodiversity. For example, is species diversity loss acceptable if the presence of certain key functional groups can support the continuation of vital ecosystem processes? Many argue that even when species diversity is not critical for maintaining ecosystem

processes, it may nevertheless, be essential to provide greater resistance to changing environmental conditions (Loreau et al. 2001).

Global biodiversity has recently been declining at a rate faster than that calculated from historic fossil records (Millennium Ecosystem Assessment, 2005), and this rapid reduction is thought to be a direct consequence of anthropogenic activities, such as harvesting, habitat destruction and pollution, but also due to human-induced global climate change (Bulling et al. 2010). This alarming loss in biodiversity has motivated scientific enquiry into how ecosystem processes will be affected by species loss (Caliman et al. 2007).

1.6.1. Functional diversity and ecosystem performance

As discussed in section 1.4., the biogenic mixing of benthic sediment exhibits significant influence over many sedimentary chemical and physical reactions. Benthic infauna mix and move sediment and particulate materials during foraging, feeding and burrow maintenance behaviour, but depending on life-history traits, such as feeding modes, sediment reworking modes, body size and mobility, species exhibit different functional traits associated with sediment mixing. These functional traits can be combined with biomass and abundance data to create a metric known as 'Bioturbation Potential (BP)', first described by Solan et al (2004a). Although not a direct measure of bioturbation, BP for a specific community (BP_c) can provide an estimate of the potential of a community to bioturbate. Therefore, due to the complex biogeochemical interactions between benthic infauna, sediment mixing and processes such as nutrient fluxes, ecosystem performance may depend more on the presence of key functional types, rather than species richness itself (Lohrer et al. 2004,

Thrush et al. 2006). For example, *Amphiura filiformis* is an active and well-studied bioturbator that is a dominant species in many coastal and shelf areas of the NE Atlantic (Solan & Kennedy 2002, Solan et al. 2004a, Queirós et al. 2006). In a 'random extinction' event simulation study focused on the North Sea, the biogenic mixing depth (BMD), an indicator of bioturbation, was dependent on whether *A. filiformis* was among the survivors (Solan et al. 2004a). Additionally, communities exposed to fishing pressure in the Irish Sea demonstrated reduced community biomass and production following the loss of the dominant *A. filiformis* (Queirós et al. 2006). Therefore, although species richness within a community is important, contributions to ecosystem function are not equal among community members.

Bellwood et al. (2003) described how one species of parrot fish (*Bolbometopon muricatum*) was responsible for almost all of the bio-erosion occurring on oceanic tropical reefs. In its absence, reefs displayed marked changes in calcification rates, coral growth rates, colony shapes, colony fitness and coral distributions, in addition to changes in erosional activity and an enhanced potential for echinoid invasions (Bellwood et al. 2003). This one specific species had a domineering functional effect, with its potential loss causing major shifts in ecosystem dynamics.

The reduction or loss of just one key species within a community can cause important ecosystem processes to become vulnerable. Therefore, the need to consider both species richness and functional diversity is a necessary requirement to ensure functional elements in marine ecosystems are protected and conserved (Bellwood et al. 2003), in addition to enhancing our knowledge

about the biodiversity-ecosystem-function relationship. In order to fully comprehend the ramifications of increasing coastal hypoxic events on biodiversity and ecosystem function, information that details how hypoxia affects key functional species, population dynamics, community assemblages, and ecosystem processes is a high priority. In doing so, knowledge can be gained about the biological and ecological mechanisms that cause changes at the physiological and behavioural level, that may underpin community and ecosystem level responses.

1.7. FUTURE PREDICTIONS FOR MARINE HYPOXIA

Although management strategies exist to monitor and reduce nutrient loadings into coastal areas, it is predicted that marine hypoxia is set to worsen, both within coastal regions and in the open oceans (Diaz & Rosenberg 2011).

Within the open oceans, studies show that DO appears to be declining in both the central North Pacific Ocean and the tropical oceans worldwide (Whitney et al. 2007, Keeling et al. 2010, Falkowski et al. 2011). Evidence suggests that the oxygen decline regimes currently being observed in the open oceans could be representative of natural cyclical processes or a non-periodic trend related to climate change and global warming due to the reduced solubility of oxygen at higher temperatures (Matear et al. 2000, Diaz & Rosenberg 2008, Falkowski et al. 2011, Gnanadesikan et al. 2011). It is probable that both of these situations are occurring, but the proportion attributed to climate change is difficult to quantify and is coupled with other key factors such as decreased ventilation, increased stratification at high latitudes and changes in biological respiration rates (Falkowski et al. 2011). As a result, ocean models have predicted a

decline of 1 - 7 % in global ocean oxygen concentrations over the next century (Keeling et al. 2010). This potential increase in oxygen minimum zones is worrying as it may have a profound effect on oceanographic processes in addition to the biology and ecology of marine organisms and ecosystems (Falkowski et al. 2011).

1.7.1. Multiple stressors

In addition to reductions in DO, marine ecosystems are being exposed to other "stressors" that can be altered by human activities (Breitburg et al. 2015). Variations in temperature, acidity, DO and disease can all occur simultaneously, affecting all levels of biological organisation between physiological and ecological scales in unpredictable directions (Breitburg et al. 2015). Understanding the effects of multiple stressors is particularly important, but also difficult, as large knowledge gaps still exist when comprehending the effects of single stressors. Ocean warming and climate change is likely to increase stratification within the upper ocean due to alterations in seawater density between temperature gradients. Increased ocean stratification holds implications for ocean productivity, nutrient and carbon cycling and marine habitats (Keeling et al. 2010), as nutrient rich, colder, denser water remains separated from warmer near-surface waters. Furthermore, deoxygenation is closely linked to ocean acidification through several mechanisms. The factors most directly responsible for acidification are increasing atmospheric carbon dioxide levels and high rates of respiration fuelled by excess inputs of nutrients to coastal waters (Breitburg et al. 2015). Climate change factors such as alterations in temperature, ocean acidification, sea-level rise, precipitation, and storm frequency may exacerbate the effects of nutrient loadings, run-off, water

column stratification and primary productivity, which will likely contribute to an observable increase in hypoxic areas (Altieri & Gedan 2015).

Furthermore, human activities such as the burning of fossil fuels and concrete production will undoubtedly continue. This lasting contribution to greenhouse gas emissions will add to global scale alterations related to climate change and global warming, resulting in warmer seawater temperatures, stronger stratifications and increased inflow of freshwater and nutrients into coastal areas as weather patterns are altered (Rabalais et al. 2009). It is also feasible to suggest that as the human population increases, the anthropogenic pressures placed on coastal systems in the future may be severely magnified and more complex than present (Rabalais et al. 2009).

Consequently, the number and intensity of stressors acting on our coastal and oceanic waters is increasing. Some evidence is available that suggests the effects of climate are sufficiently strong enough to further increase the severity of hypoxia, even if rates of eutrophication are stabilised or reduced (Villate et al. 2013, Carstensen et al. 2014, Altieri & Gedan 2015). Therefore, a fundamental goal of global change biology is to diagnose the cause of ecological changes and predict what species or populations are under threat (Gunderson et al. 2016). With an increase in predictive understanding of the responses to anthropogenically induced changes, informed decisions can be made on how to manage marine habitats and protect the functional performance of ecosystems.

1.7.2. Rationale for investigating 'moderate' hypoxia

Within Sect 1.1, hypoxia was introduced and referred to as a reduction in DO to a level of 2.0 mg O_2 L^{-1}. Defining hypoxia by a set DO concentration can compel researchers to constrain their investigations to within this 'generally accepted' limit, with most reports (55 %) referring to and using the value of 2.0 mg O_2 L^{-1} (Vaquer-Sunyer & Duarte 2008). This threshold is related to a generalised lethal level of low DO leading to the collapse of fisheries (Diaz 2001, Rabalais et al. 2002). However, ample experimental evidence exists to challenge the notion that this level of reduced DO is inadequate to describe the onset of hypoxia impacts for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel & Childress 2013). Additionally, the onset of coastal hypoxia is a process, and, therefore, a range of reduced DO may occur over a period of time, causing alterations in organism behaviour and functionality, prior to migration or mortality. Additionally, there is growing evidence that indicates hypoxic effects on organisms can occur at much higher DO levels, indicating the need for new definitions of 'hypoxia' and new criteria for establishing O_2 tolerance thresholds based on measures of physiological or ecosystem performance (Seibel & Childress 2013). Seibel (2011) suggested that many 'hypoxia' studies are not biologically relevant, and recommends abandoning all terms except *hypoxia*, meaning relativity low oxygen, and *anoxia*, the complete absence of oxygen (Seibel 2011). Under future global change scenarios, nutrient inputs and eutrophication may not be the primary determinant governing coastal hypoxia. Therefore the severity, intensity and duration of hypoxic events are likely to be unpredictable, and could range from mild deviations from 'normoxia' for short periods of time to moderate or severe reductions in DO. Consequently, from this point forward, for clarity and consistency throughout this thesis, the term

'hypoxia' will refer to any DO level lower than 'normoxia'. The experiments conducted within this thesis were set at a hypoxic level above the traditional 2.0 mg O_2 L^{-1} threshold and are sometimes referred to as 'hypoxic', 'moderately hypoxic' or 'reduced DO'. When discussing previous work and other examples, actual DO levels will be stated whenever possible.

As mentioned in Sect. 1.5, the organism response to alterations in DO is species specific and is initially represented through changes in organism behaviour and physiology, with mortality being the end-point (Grieshaber et al. 1994). Reductions in DO far above the 'classic' threshold of 2.0 mg O_2 L^{-1} have been shown to affect organism growth, reproduction, locomotion and feeding (summarised in Gray et al. 2002), causing complex direct and indirect responses across several levels of biological organisation, from individual fitness to community composition (Fig. 1.2) (Riedel et al. 2014). These complex alterations and intimately nested responses, may affect important processes that contribute to ecosystem functioning, yet the impact of reduced DO (i.e. at a level above 2.0 mg O_2 L^{-1}) on nearshore marine and estuarine communities is not well understood (Froehlich et al. 2015). Furthermore, even less is known about the how 'moderate' reductions in DO will affect ecosystem processes and ecosystem functioning through behavioural and physiological alterations at the organism and community level.

Therefore, monitoring and managing hypoxic events and reductions in DO, in terms of species loss or mortality is not sufficient, and there is a need for greater understanding of when ecosystems approach critical points in relation to 'moderately' reduced DO concentrations and the functioning of important

processes. This calls for identifying perturbations at an earlier stage, with recognition given to alterations in organism behaviour and physiology, and how this may impact ecosystem processes. Consequently, the investigations undertaken here have utilised a comparatively 'moderate' level of hypoxia (> 3.0 mg O_2 L^{-1}) above that of the classic definition of 2.0 mg O_2 L^{-1}.

1.8. THESIS AIMS AND OBJECTIVES

The aims of this thesis are to (a) understand the effects of population-level processes (e.g. organism density) on individuals' biological and ecological responses to moderate hypoxia, and (b) determine the sensitivity of a typical U.K. coastal benthic community to moderate hypoxia and measure the potential ecological effects.

This thesis specifically addresses these aims by pursuing the following activities and objectives:

Firstly, a controlled mesocosm exposure experiment was conducted in which individuals of the key bioturbating species *Amphiura filiformis* were held at different densities and exposed to either normoxic or hypoxic conditions. From this experiment the impacts of hypoxia on the physiological performance of *A. filiformis* were investigated, whilst also examining if population density resulted in positive species interactions that enhanced survivorship **(Chapter 2)**.

Individuals from the above exposure experiment where then also used to investigate how hypoxia affected the bioturbatory behaviour of *Amphiura filiformis* and, subsequently, its ability to maintain nutrient fluxes as a proxy for

ecosystem function. Again these data were analysed to examine if denser populations displayed greater behavioural resilience to hypoxic stress supporting the provision of ecosystem function **(Chapter 3)**.

Given that organisms do not naturally reside in a monospecific environment and that, despite the importance of certain key species, the overall provision of ecosystem function can be considered an output of the entire community, a second mesocosm experiment was then conducted to examine and document the sensitivity and community response (in terms of structure and diversity) of a typical benthic infaunal community to hypoxic stress **(Chapter 4)**.

Furthermore, key behavioural (bioturbation) and functional (nutrient fluxes) data were recorded from this community mesocosm experiment. These data are interpreted in light of the results presented in Chapter 4 to improve our understanding of the overall ecological responses to hypoxia, and consequences for ecosystem function, of a coastal infaunal benthic community **(Chapter 5)**.

Finally, this thesis considers if there are common themes between the individual-level biological and ecological responses to hypoxia and the community-level biological and ecological responses, and assess' if the responses measured can be used to predict ecosystem level alterations to potential hypoxic events **(Chapter 6)**.

CHAPTER 2: PHYSIOLOGICAL AND HISTOLOGICAL RESPONSES OF BRITTLESTARS TO HYPOXIA

A mesocosm study investigating the effects of hypoxia and population density on respiration and reproductive biology in the brittlestar, *Amphiura filiformis*

2.1. INTRODUCTION

Coastal hypoxia has been documented in over 400 systems worldwide, affecting in excess of 245,000 km^2 of the World's oceans (Diaz & Rosenberg 2008), and seems to be a growing problem (Zhang et al. 2010, Zhang et al. 2013). As global warming and eutrophication continue to exacerbate hypoxia, there is an increasing view that reduction in dissolved oxygen (DO) is a key environmental stressor within marine ecosystems and that this stress has the potential to define benthic community composition and modify biogeochemical cycles (Rosenberg et al. 2001, Wu & Or 2005, Meire et al. 2012, Zhang et al. 2013). Depending on the severity and duration of a hypoxic event, impacts on organisms and communities can vary from short-term behavioural changes, medium-term transitory or lasting physiological alterations, to long-term species absences through interruptions to reproductive success, species migrations or local extinctions (Rosenberg et al. 2001). Consequently, there is growing concern over the long-term impacts of regular or seasonal hypoxia on the sustainability and biodiversity of coastal ecosystems (Diaz & Rosenberg 1995, Wu 2002, Thomas et al. 2007, Zhang et al. 2010, Bijma et al. 2013).

Most impact studies of hypoxia in the coastal zone have focused on commercially important species, e.g. larval development in mussels (Wang & Widdows 1991), survival and avoidance behaviour of penaeid shrimp (Wu et al. 2002), settlement and growth in oysters (Baker & Mann 1992, David et al. 2005), and food consumption and growth in fish such as bass, cod and flounder (Chabot & Dutil 1999, Kimura et al. 2004, Brandt et al. 2009, Herbert et al. 2011). A number of studies have examined the effects of hypoxia on benthic community structure and recovery, (Diaz & Rosenberg 1995, Lim et al. 2006,

Van Colen et al. 2010, Fleddum et al. 2011) but much less information exists on how hypoxia and changes in DO affect reproductive biology, a trait imperative for species survival, community recovery and biodiversity security. Life cycle events of species are greatly dependant on factors such as DO and temperature, and include reproductive output, recruitment and post-recruitment development, and larval transport and settlement, all of which play an important role in benthic community structure, diversity and functioning (Birchenough et al. 2015). Moreover, the majority of hypoxic events and alterations in DO occur during the summer months, often after spring blooms (Diaz & Rosenberg 2008) which is a key reproductive period for many benthic invertebrate species. The paucity of data on the effects of reduced DO and hypoxia on reproductive biology in marine benthic species makes it difficult to predict the ecological consequences of field population resilience, their ability to recover, and ultimately if these impacts have implications for ecosystem health and function in the long-term (Wu 2002).

The few studies conducted on the effects of hypoxia on reproductive or embryonic development suggest that hypoxia can cause uncoupling of growth and morphogenic processes in mussel larvae (Wang & Widdows 1991), reduced settlement and inhibited growth in oyster larvae and juveniles (Baker & Mann 1992), delayed embryonic development in gastropods (Chan et al. 2008) and delayed spawning in the brittlestar, *Amphiura filiformis* (O.F. Müller, 1776) (Nilsson & Sköld 1996). The ability of organisms to maintain their metabolic rates and thus energy assimilation for important life-history traits, such as reproductive output, is vital when exposed to environmental stress such as

hypoxia, and potentially holds consequences for an organism's distribution and abundance (Wu 2002, Spicer 2014).

The burrowing brittlestar, *Amphiura filiformis* is abundant in European waters (O'Reilly et al. 2006) and often aggregates to form population densities ranging from 280 indiv. m^{-2} (Sköld et al. 1994) to 2,250 indiv. m^{-2} (Rosenberg et al. 1997). Geographic, bathymetric and environmental parameters help explain these vast differences in population density, especially when discrete aggregations coincide with fine sediment characteristics as described by O'Connor et al. (1983). However, it is not known if dense aggregations of *A. filiformis* results in any positive species interactions (i.e. the ecological concept of facilitation), such that survivorship can be positively related to population density (Bruno et al. 2003).

Amphiura filiformis is primarily a suspension feeder that mostly remains buried below the sediment surface and protrudes one or more arms into the water column. This species actively undulates its arms and pumps its disc for respiratory gas exchange, burrow ventilation, collection of food and the transportation of sediment and waste (Vopel et al. 2003). This species is particularly well studied due to its importance as an ecosystem engineer (Solan & Kennedy 2002), with its capacity to modify the local environment and improve oxygenation within the sediment (Vopel et al. 2003).

Consequently, we hypothesised that *Amphiura filiformis* may benefit from being within dense aggregations, especially when exposed to moderate hypoxia, due to its ability to increase the oxygenation and movement of the surrounding

sediment and water, possibly resulting in lower metabolic costs of living, (i.e. reduced arm undulations and pumping of the disc) allowing for energy investment into other important traits such as reproductive output. Investigating if there are any potential links between population density, aerobic metabolism and reproductive investment of *A. filiformis* may help define which populations are most at risk when moderate hypoxia occurs.

To answer these questions, a 14-day mesocosm experiment was conducted to quantify the impacts of moderate hypoxia on the aerobic metabolism and reproductive biology of the brittlestar, *Amphiura filiformis* across a range of organism densities. Rates of oxygn uptake within normoxic and moderate hypoxic conditions were measured as a proxy for aerobic metabolism. In addition, the oxygen uptake of organisms exposed to moderate hypoxia was re-assessed when brittlestars were returned to normoxic conditions. This may provide insight into whether moderate hypoxia can cause longer lasting disruptions to physiological activity or if *A. filiformis* can exhibit metabolic plasticity when environmental parameters change. Reproductive development was assessed by measuring the mean feret diameter in developed (late-vitellogenic) oocytes and by quantifying the variation in the number of the three main oocyte developmental stages.

2.2. MATERIALS AND METHODS

2.2.1. Sediment collection

On 25th May 2012 sediment was collected at a water depth of approx. 10 m from an area of 'very fine sand' with an overlaying surface layer of 'clay / silt' in Cawsand Bay, Plymouth, U.K. (N 50°21.998'; W 4° 07.961) using a 0.1 m^2 box

core. Once retrieved the surface layers of sediment (10 - 15 cm) were placed into bags and transported to the Plymouth Marine Laboratory (PML, Plymouth, U.K.) mesocosm facility where these were sieved (2 mm) in filtered seawater (10 µm diam. Hydrex filters). The sieved sediment was placed into a holding tank and allowed to settle for 48 h in order to capture the fine fraction. Post-settlement, excess water was carefully drained off and the sediment homogenised by mixing. Fifty experimental glass aquaria (20 cm wide x 5 cm deep x 30 cm high) were filled with sediment up to a depth of 19 cm (± 1 cm) producing 11 cm of overlying water.

Each aquarium was connected to a flow-through seawater system that delivered aerated, twice filtered (10 µm and 1 µm diam. Hydrex filters) seawater from a 450 L header tank (DO = 8.19 ± 0.49 mg O_2 L^{-1}, S = 34.50 ± 0.17, T = 15.20 ± 0.66 °C, pH = 8.08 ± 0.05, mean ± 95% C.I.) (Table 2.1) *via* a peristaltic pump (323E, Watson Marlow, Falmouth, U.K.) set at a rate of 20 ± 0.5 mL min^{-1}. Water inlet pipes were connected to each aquarium 1.5 ± 0.5 cm above the sediment surface. The average water volume held within each aquarium was 1,100 cm^3, resulting in an approximate water renewal rate every 55 min. Aquaria were kept under these conditions for a further 21 d, to allow the sediment to settle and for biogeochemical processes and gradients to re-establish. Aquaria that showed any visual signs of bioturbation during this time were removed from the experimental set up.

Table 2.1. Summary of seawater treatment conditions throughout the experiment. Values are means ± 95 % confidence intervals, and the range of values is given in brackets (min – max). Dissolved oxygen (DO) measurements are corrected for atmospheric pressure and temperature.

	Normoxic header	Normoxic aquaria	Hypoxic header	Hypoxic aquaria
DO ($mg\ O_2\ L^{-1}$)	8.19 ± 0.49	8.09 ± 0.06	3.13 ± 0.21	3.59 ± 0.04
	(6.90 – 9.42)	(6.92 – 9.00)	(2.59 – 3.87)	(2.95 – 4.57)
DO (%)	98.98 ± 5.92	97.77 ± 0.68	37.82 ± 2.54	43.34 ± 0.45
	(83.47 – 113.93)	(83.71 – 108.81)	(31.38 – 46.79)	(35.62 – 55.34)
Temperature (°C)	15.20 ± 0.66	12.30 ± 0.10	14.78 ± 0.89	12.41 ± 0.10
	(12.50 – 16.50)	(10.90 – 14.50)	(12.50 – 16.90)	(10.50 – 14.40)
Salinity (ppt)	34.50 ± 0.17	34.32 ± 0.07	34.46 ± 0.16	34.25 ± 0.04
	(34.23 – 35.90)	(33.80 – 36.60)	(34.23 – 35.90)	(33.59 – 35.41)
pH (NBS)	8.08 ± 0.05	8.13 ± 0.02	8.08 ± 0.08	8.16 ± 0.01
	(8.00 – 8.21)	(8.07 – 8.32)	(7.83 – 8.22)	(8.07 – 8.32)

2.2.2. Brittlestar collection

Individuals of *Amphiura filiformis* were collected from the same site as the sediment (12th -14th June 2012). Specimens were handpicked to avoid damage (such as arm loss) and carefully washed with fresh seawater. Only individuals with a disc diameter > 4 mm, based on the size at which adults reach sexual maturity (O'Connor et al. 1983), plus five intact arms were placed into containers (vol. = 250 mL, three indiv. *per* container) containing freshly collected seawater and transported to PML within 3 h of collection. As large numbers of individuals were required, collection was conducted over three consecutive days. After each collection day individuals were placed in a large (vol. = 50 L) aerated seawater storage tank (DO = 8.07 ± 0.05 $mg\ O_2\ L^{-1}$, S =

34.20 ± 0.42, T = 12.39 ± 0.15 °C, pH = 8.08 ± 0.03, (mean ± 95 % CI) that contained sieved (2 mm) sediment approx. 7 cm deep. After the final collection day, individuals were maintained unfed in the storage tank for a further four days. Following this, brittlestars were carefully extracted from the sediment and only specimens that appeared undamaged with five intact arms were used in the experiment.

Before haphazard assignment to the experimental aquaria, individuals were carefully blotted dry and weighed using a precision balance (Sartorius, R200-D, Göttingen, Germany) (± 0.01 mg). The wet mass of the brittlestars were used to calculate an appropriate feeding dose *per* aquaria based on the total biomass within each aquaria. Aquaria were dosed with a marine microalgae shellfish diet (1800 Instant Algae® Marine Microalgae Concentrated Shellfish Diet, Reed Mariculture, CA, U.S.A.), a mixture of *Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii* and *Tetraselmis* sp. (cell count 2 billion mL^{-1}, 8 % dry-mass). Dose *per* aquaria was calculated as 1.5 % of the total ash-free dry mass (AFDM) of brittlestars *per* aquaria, which was estimated using a wet mass (WM) to AFDM conversion factor from Ricciardi & Bourget (1998) (Ophiuroidea: AFDM/WM = 7.4 %). Once brittlestars had been added to the experimental aquaria a five day settling period under normoxic conditions commenced. Feeding began during this phase and was repeated daily for the duration of the experiment. The correct amount of 'Instant Algae mix' for each aquarium was prepared daily and delivered *via* syringe. Algae was released into the water column approximately 2 cm above the sediment surface and evenly dispersed over the entire sediment area. Water flow to each aquarium was ceased for 1 h after the addition of algae. Ingestion rates were not measured, however visual recordings

of active feeding behaviour were noted. At each time point aquaria were not fed for 48 h prior to sampling, so that brittlestars' digestion processes would not significantly affect rates of oxygen uptake.

2.2.3. Experimental design and set up

Of the 50 sediment aquaria prepared, 42 were selected for use within the experiment. Aquaria were set up to produce a regression experimental design, in which the effects of individuals' density and time were set as independent variables against which to assess any response. Aquaria were haphazardly assigned to one of two oxygen levels (normoxia = 8.09 ± 0.06 mg L^{-1} or moderate hypoxia = 3.59 ± 0.04 mg L^{-1}) and one of six organism density levels (0, 5, 9, 13, 17, 21 indiv. *per* aquaria, equating to 0, 500, 900, 1300, 1700, 2100 indiv. m^{-2} respectively). However, for Chapter 2, the zero brittlestar density treatment was not required, and was only included in the results as a 'control' for bioturbation investigations within Chapter 3. Consequently, for the remainder of this chapter, the 'zero' density treatments are omitted. Time point T0 marked the start of the experiment where five aquaria were haphazardly removed and sampled to create 'pre-exposure' data. At T6, T10 and T14 time intervals, five normoxic aquaria and five hypoxic aquaria were haphazardly sampled, again including all density levels. Sacrificial sampling of experimental aquaria occurred throughout the experiment.
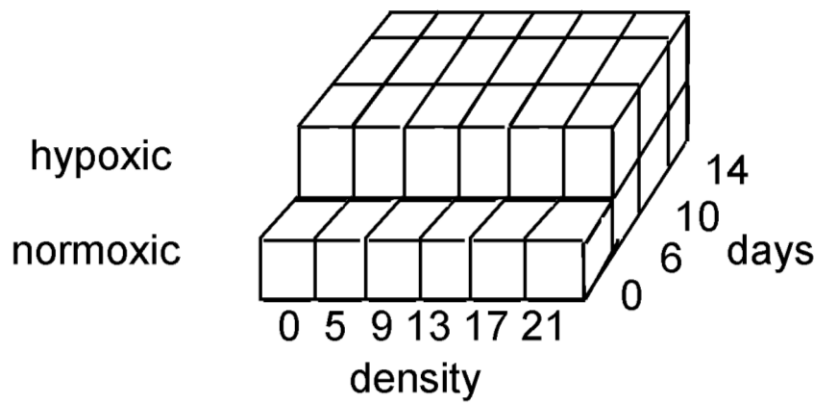
Figure 2.1. Diagram of experimental design. Each box represents a single experimental aquarium, which was designated one of six brittlestar density treatments (0, 5, 9, 13, 17, 21), one of four time treatments (0, 6, 10, 14 days), and one of two water treatments (normoxic or moderately hypoxic). Aquaria were removed from the experiment after their designated sampling day.

Figure 2.2. Image of the experimental system showing the two header tanks, perisaltic pumps and individual aquaira within a flow through system. Sediment appears green due to the addition of luminophores for bioturbation investigations documented in Chapter 3.

2.2.4. Seawater manipulations

Within the mesocosm, moderately hypoxic and normoxic seawater was held in two separate header tanks (vol. = 450 L each). To produce moderately hypoxic seawater, DO levels were modified using a computerised control system (Webmaster Series, Walchem Holliston, MA, U.S.A), which regulated the addition of oxygen free nitrogen gas. This was a modification of the feedback system used by Widdicombe and Needham (2007) to create acidified seawater. In the modified system nitrogen gas was finely bubbled through aquaria air stones whilst seawater DO concentrations were monitored using a submersible DO sensor (DO6441-T, Sensorex, Los Angeles, CA, U.S.A.). Once the DO had fallen to the desired level the supply of nitrogen was halted, *via* an automated feedback relay system. The replenishment of fresh seawater caused the DO in the hypoxic header tank to increase, which triggered the addition of nitrogen until the DO sensor detected the pre-set value. Within the header tank small plastic spheres floated on top of the moderately hypoxic water to reduce gas exchange with the atmosphere, whilst seawater in the normoxic header tank was kept aerated with a continuous air supply bubbled through aquaria air stones. Both header tanks contained submersible pumps to aid water circulation and mixing, and both were shielded with a hard top cover. Each header tank was connected to its respective experimental aquaria, *via* a peristaltic pump, which supplied a constant supply of seawater. As the volume of water within each header tank decreased, seawater was replenished from a large reservoir (vol. = 15,000 L), which was periodically restocked with seawater collected approx. 14 km offshore from Plymouth, UK.

At time point 'T0' the water supply feeding half of the experimental aquaria was transferred from the normoxic header tank to the moderately hypoxic header tank; it took approximately 24 h for the water in these aquaria to reach the desired experimental levels (an approx. decrease of 0.19 mg O_2 L^{-1} h^{-1}). The seawater within both header tanks and the experimental aquaria were monitored daily for DO, temperature, salinity and pH (Table 2.1) using a multiprobe (9828, Hanna Instruments, Woonsocket, RI, U.S.A.).

2.2.5. Quantifying metabolic rates

Rates of oxygen uptake (as a proxy for metabolic rate) of *Amphiura filiformis* were measured using a 'closed-respirometry' technique. At T0, five aquaria (one from each density treatment) were haphazardly removed for analysis. These aquaria formed the 'pre-exposure data'. On the remaining sampling days (T6, T10, T14) five aquaria receiving moderately hypoxic water (one from each density treatment) and five aquaria receiving normoxic water (one from each density treatment) were haphazardly removed from the experiment. Brittlestars were carefully extracted from the sediment and gently rinsed with filtered seawater over a 2 mm sieve. Four individuals from each aquarium were then haphazardly selected for use in the oxygen uptake trials.

Individuals were carefully blotted dry, weighed using a precision balance (R200-D, Sartorius, Goettingen, Germany) (± 0.01 mg) and placed in a numbered mesh basket, containing eleven uniform glass marbles (1.3 cm diam.) which provided an inert structure for the brittlestars to hide and bury within. From then onward, specimens were kept submerged in filtered (0.22 μm diam.) seawater that contained a DO concentration corresponding to their previous experimental

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water treatment. Whilst submerged, the baskets were placed into individual blackened glass chambers (vol. = 50 mL). Each chamber was left open to the surrounding water in the holding tank for 60 min to allow brittlestars to settle. During this time DO concentrations in each holding tank was monitored and adjusted accordingly to replicate the water conditions experienced within the experiment. Subsequently, a 10 mm magnetic stirrer was placed within each chamber, separated from the organism by the mesh basket. Chambers were then sealed, ensuring no trapped air bubbles. The sealed chambers were placed within a recirculating chilled water bath (T = 11.5 °C) under which, lay three stirring plates (MIX 15 eco, 2mag, München, Germany) set to 200 rpm. Water velocity within the chambers was not recorded but preliminary experimentation established that 200 rpm was sufficient to mix the water in the chambers and not cause stress to the brittlestars. Five blank control chambers containing moderately hypoxic water and no brittlestars were run simultaneously.

The decline in oxygen within each closed chamber was determined using a non-invasive optical oxygen analyser (5250i, OxySense, Dallas, TX, U.S.A) as detailed in Calosi et al. (2013). This technique uses a fibre optic reader-pen that contains a blue LED and photodetector to measure the fluorescence characteristics of an oxygen sensitive dot which was previously placed inside the glass chambers. For the first hour, measurements were taken every 15 min, and then every 30 min for the next 4 h. During this time, the reductions in oxygen levels within the chambers were monitored to ensure that the incubation itself did not impose a respiratory stress on the brittlestars. Oxygen levels within

the chambers did not fall more than 13.40 % ± 1.30 (mean ± 95 % CI) of the starting value during the course of the oxygen uptake experiments.

Immediately after the first oxygen uptake trial, all individuals that were within moderately hypoxic water began a second experiment but with refreshed oxygenated seawater (8.09 mg O_2 L^{-1}) being used instead of the hypoxic water. Additionally, five new blank chambers were also refreshed with normoxic water. However, due to logistical limitations, all 'control' individuals that were kept within normoxic water during the experimental period and the first oxygen uptake trial, were not subjected to a second trial. Consequently, the results from this second trial were interpreted separately and with some caution. In summary three sets of oxygen uptake data were collected; (1) Nt / No (normoxic experimental treatment and normoxic oxygen uptake), (2) Ht / Ho (hypoxic experimental treatment and hypoxic oxygen uptake) (3) Ht / No (hypoxic experimental treatment and normoxic oxygen uptake), a recovery trial in oxygenated water after exposure to hypoxia. For clarification, the term 'hypoxic' water or treatment refers to a 'moderate' level of hypoxia above that of the traditionally defined 2.0 mg O_2 L^{-1}.

Small amounts of background oxygen uptake (< 10.40 % of measured values) were detected in the normoxic and hypoxic blank control pots. Therefore, background respiration was accounted for when calculating the rates of oxygen uptake by individual brittlestars. All remaining individuals from the experimental aquaria were preserved in Bakers Formal Calcium solution for subsequent analysis of the gonads.

2.2.6. Measurement of oocyte diameter

Individuals from time points T0 and T14 were removed from Bakers Formal Calcium solution, and their arms excised close to the disk. Each disk was placed into a small glass vial (vol. = 20 mL) and dehydrated using a sequence of increasing ethanol concentrations (30 min in each 50, 70, 95 % (repeated) and then 95 % ethanol/monomer (1:1)). Disks were left in 2-hydroxyethyl methacrylate monomer overnight and then embedded in monomer with activator before being left to set for 24 h. Subsequently, casts were removed from the moulds and air dried for 12 h. A glass knife was used to cut transverse sections through the disk (5 µm thick), which were then mounted on glass slides and left to air dry. Sections were stained using the Periodic Acid Schiff Method (PAS). Due to the unknown distribution of females within each aquaria, and time restraints hindering the sectioning of every individual within the experiment, the above procedures were repeated until a total of 33 female individuals were sectioned, i.e. 11 from T0 (normoxia), 11 from T14 normoxic treatment and 11 from T14 hypoxic treatment. In order to assess the effects of organism density on the reproductive parameters measured, females from every aquarium covering each density treatment were sectioned. Only one aquaria (hypoxic treatment, indiv. *per* aquarium = 5, time point = T14), was excluded from this analysis because it contained all male specimens.

The plane of the disk section that intersected the greatest number of ovary sections was chosen for each female, and a series of images were captured under low power (x 10) magnification using a microscope (Reichert Polyvar, Leica, Wien, Austria) through a mounted camera system (Coolpix 995, Nikon, Tokyo, Japan) that documented the entire brittlestar section. Photos from each

image series were selected at random and oocyte feret diameter of every late-vitellogenic oocyte displaying a nucleus in each photo was measured until 100 oocytes from each individual had been measured. In total 3300 late-vitellogenic oocytes were measured. Image analysis was completed using the software Image-Pro Plus (v4.5 Media Cybernetics Inc., MD, U.S.A). Oocyte feret diameter is the greatest diameter measurement through the oocyte, based on the assumption that oocytes are not always spherical. Two basic rules were followed to select oocytes that were measured: (1) oocytes must be in the late-vitellogenic stage; (2) the nucleoli must be visible (Bowmer 1982, Brogger et al. 2013).

2.2.7. Estimating gonad maturation

Counts of oocyte developmental stages were calculated using the same images taken for oocyte diameter assessment. Using Image-Pro Plus, 20 ovaries were selected randomly from each individual and the total number of late-vitellogenic, mid-vitellogenic and pre-vitellogenic oocytes within each ovary were counted. Across 33 indiv. a total of 35,275 oocytes were examined. The staining method used during slide preparation allowed for distinct colour differences between these three major developmental stages which account for the different proportions of carbohydrate macromolecules present.

2.3. STATISTICAL ANALYSES

Statistical analyses were completed using the software package MINITAB 16 (ver. 16.2.4.4) and PRIMER 6 (ver. 6.1.18). Normality and the homogeneity of variances of the data were examined using Anderson-Darling and Levene's tests respectively. Assumptions for normal distributions of the data and

homogeneity of variance were met for all parameters examined, apart from the late-vitellogenic oocyte stage. However, after an Ln transformation these data met assumptions for normality.

Regression analysis was completed on 'oxygen uptake', 'oocyte diameter data' and 'oocyte developmental stage data' to examine the effects of hypoxia against organism density and exposure time. These tests revealed that organism density within the aquaria had no effect on any parameters measured and analyses were re-run, excluding density as a factor. An example of these regression analyses examining the effects of brittlestar density is incorporated in Section 2.4.1. The lack of a 'brittlestar density' effect resulted in turning the original 3-factor design into a 2-factor design, pooling density treatments together, to compare the effects of experimental time and water treatment. In order to avoid comparing independent and repeated measures in one analysis, (for the oxygen uptake data in particular) data were examined using 2-sample t-tests. For continuity, the remaining data sets were also examined using the same methods. Rates of oxygen uptake data from the Ht / No data set (recovery trial) were examined separately using a paired t-test with the corresponding data from the Ht / Ho trial.

2.4. RESULTS

2.4.1. Rates of oxygen uptake

Regression analysis revealed that 'organism density' within the aquaria had no significant effects on 'oxygen uptake rates' (F range 0.030 - 7.840, *p* range 0.068 – 0.879) (see Table 2.2 for examples). The multivariate test PERMANOVA was also completed to assess the effects of 'density', 'water

treatment' and 'exposure time' on 'oxygen uptake rates' and again, density of organisms in aquaria had no effect on the results (Pseudo-F = 0.339, p(perm) = 0.849).

Table 2.2. Representative examples of regression analyses examining the effects of 'brittlestar density' on oxygen uptake rates. Brittlestar density had no effect on any parameters measured and was pooled for further analysis. * Significant p values (to 95 % significance level).

Time & Treatment	F-value	R-Sq	t-value	p-value
T6 normoxic	0.03	0.9 %	0.16	0.879
T6 hypoxic	0.57	16.1 %	-0.76	0.504
T14 normoxic	7.84	72.3 %	-2.80	0.068
T14 hypoxic	0.05	1.5 %	0.21	0.845

2.4.2. Normoxic treatment, Normoxic oxygen uptake (Nt / No)

The mean rate of oxygen uptake from specimens of *Amphiura filiformis* pre-exposure (T0) was 0.823 O_2 h^{-1} g^{-1} wet mass. After 6 and 10 d experimental exposure (T6 and T10), the average oxygen uptake rate of organisms kept within normoxic water decreased slightly compared to the rate expressed at T0, but not significantly (Fig. 2.3, Table 2.3). After 14 d (T14) the mean rate of oxygen uptake increased significantly compared to all previous readings in the same treatment (Table 2.3), indicating that there may be an experimental time effect.
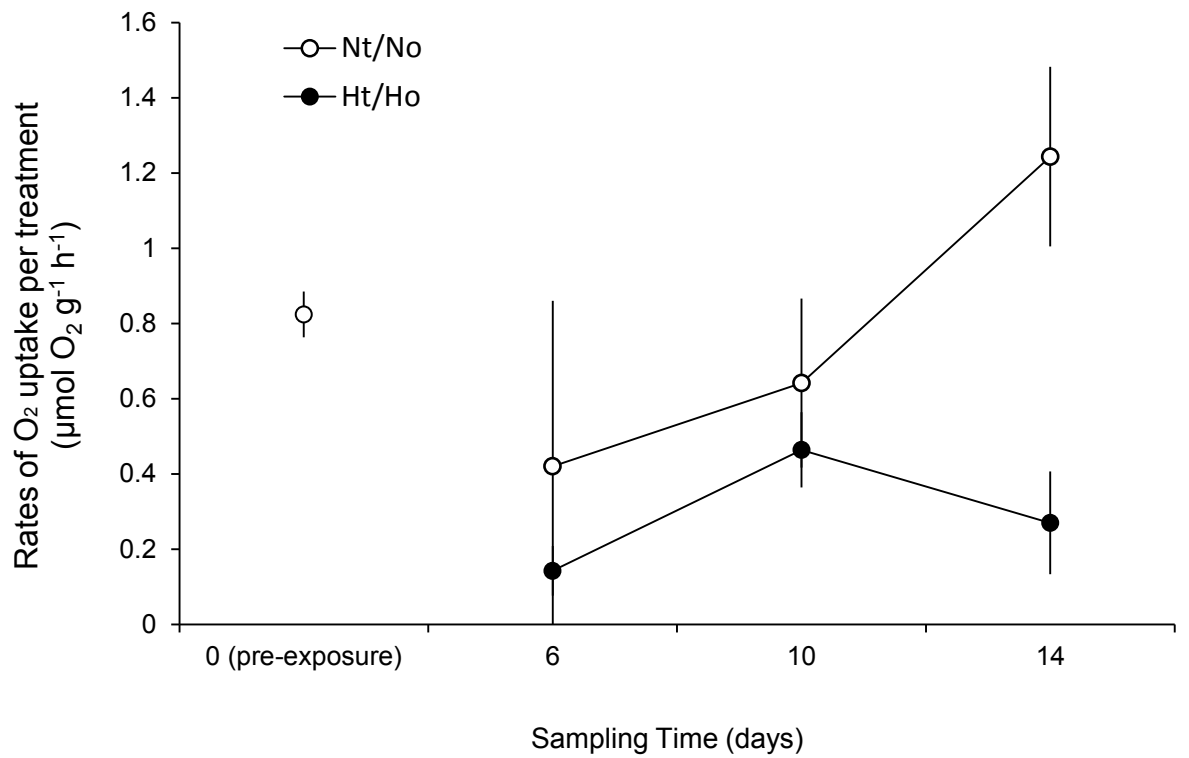
Figure 2.3. Rates of oxygen uptake (μmol O_2 g^{-1} wet mass h^{-1}) for *Amphiura filiformis* within Normoxic treatment / Normoxic oxygen uptake (Nt / No) and Hypoxic treatment / Hypoxic oxygen uptake (Ht / Ho) trials. Data expressed as means $\pm$ 95 % confidence intervals, n = 5.

Table 2.3. Two-sample t-tests on rates of oxygen uptake data from normoxic set (Nt / No) and hypoxic set (Ht / Ho). * Significant p values (to 95 % significance level); n = 5.

Time	Nt / No		Ht / Ho		Nt / No vs. Ht / Ho	
	t-value	*p*-value	t-value	*p*-value	t-value	*p*-value
T0 *vs.* T6	1.780	0.150				
T0 *vs.* T10	1.540	0.199				
T0 *vs.* T14	3.340	0.029*				
T6 *vs.* T10	0.880	0.420	5.250	0.002*		
T6 *vs.* T14	3.220	0.018*	1.650	0.160		
T10 *vs.*T14	3.600	0.009*	2.250	0.059		
T6					1.220	0.289
T10					1.420	0.216
T14					6.950	<0.001*

2.4.3. Hypoxic treatment, Hypoxic oxygen uptake (Ht / Ho)

Organisms subjected to the Ht / Ho trial exhibited a mean oxygen uptake of 0.142 O_2 h^{-1} g^{-1} wet mass at T6 which significantly increased to 0.463 O_2 h^{-1} g^{-1} wet mass at T10 (Fig. 2.3, Table 2.3). This increase in oxygen uptake rates from T6 to T10 shadows that of the organisms within the Nt / No trial (Fig. 2.3). After 14 d (T14) exposure to hypoxic water, oxygen uptake rates were slightly reduced compared to T10 rates, but did not alter significantly. The only significant difference that occurred between Nt / No trial and Ht / Ho trial was at T14, where organisms kept within normoxic water (Nt / No) showed a marked increase in their oxygen uptake rates, but organisms within hypoxia (Ht / Ho) did

not (Fig. 2.3). This may be a reflection on experimental effects and acclimation to laboratory conditions.

2.4.4. Hypoxic treatment, Normoxic oxygen uptake (Ht / No)

Organisms that were exposed to the second oxygen uptake trial, Ht / No, (recovery trial) showed no significant differences in their oxygen uptake rates compared to their paired data points collected during the Ht / Ho trial (Table 2.4) meaning that, once returned to oxygenated water, oxygen uptake rates did not immediately increase in a significant manner.

Table 2.4. Paired two sample t-test on rates of oxygen uptake data from hypoxic set (Ht / Ho) and recovery set (Ht / No). * Significant p values (to 95 % significance level); n = 5.

	Ht / Ho *vs.* Ht / No	
Time	t-value	*p*-value
T6	1.220	0.289
T10	2.660	0.057
T14	0.930	0.405

2.4.5. Oocyte feret diameter

Feret diameter of developed (late-vitellogenic) oocytes were measured in individuals at T0 (pre-exposure) and at T14 only. Regression analysis showed 'density' of organisms within aquaria had no effect on oocyte feret diameter (F = 0.100, p = 0.754). Initially, at T0, mean oocyte diameter measured 102.80 ± 2.08 µm (95 % C.I. n = 5). After 14 d, brittlestars that had been exposed to

normoxic water exhibited a significant increase in mean oocyte diameter (114.00 ± 1.40 μm, 95 % C.I. n = 5) compared to individuals measured at T0 indicating growth over the experimental period (t = 8.070, *p* < 0.001). Specimens exposed to the hypoxic treatment for 14 d (T14) exhibited a mean oocyte diameter of 105.85 ± 1.36 μm (95 % C.I. n = 4), which is a slight increase in diameter compared to individuals at T0, but not significant (t = 2.320, *p* = 0.081). Comparing between water treatments, oocyte diameter was significantly smaller in specimens that were exposed to 14 days of hypoxia compared to specimens exposed to 14 days of normoxia, (t = 10.130, *p* = 0.001) indicating reduced oocyte growth within the hypoxic water treatment over the experimental period.

2.4.6. Estimating gonad maturation

Comparing the pre-exposure measurements taken at T0 under normoxic conditions to the oocyte developmental stages, also under normoxic conditions, 14 d later, there was clear evidence of developmental progression; the number of late-vitellogenic oocytes increased significantly, with a concomitant decrease in pre-vitellogenic oocytes (Fig. 2.4, Table 2.5). However, individuals that had been kept in hypoxic water for 14 days showed no significant differences in late-vitellogenic and mid-vitellogenic oocyte numbers when compared to the pre-exposure measurements (T0), indicating a significantly reduced amount of developmental progression.

For individuals within the normoxic and hypoxic treatments measured at T14, the amount of time for oocyte development was the same, yet individuals exposed to hypoxia had significantly less late-vitellogenic oocytes, and a

greater number of mid and pre-vitellogenic oocytes compared to individuals kept in normoxic water at T14 (Fig. 2.4, Table 2.5). Exposure to hypoxia may have disrupted oocyte development resulting in less oocytes maturing and growing into the late-vitellogenic stage (Fig. 2.5).
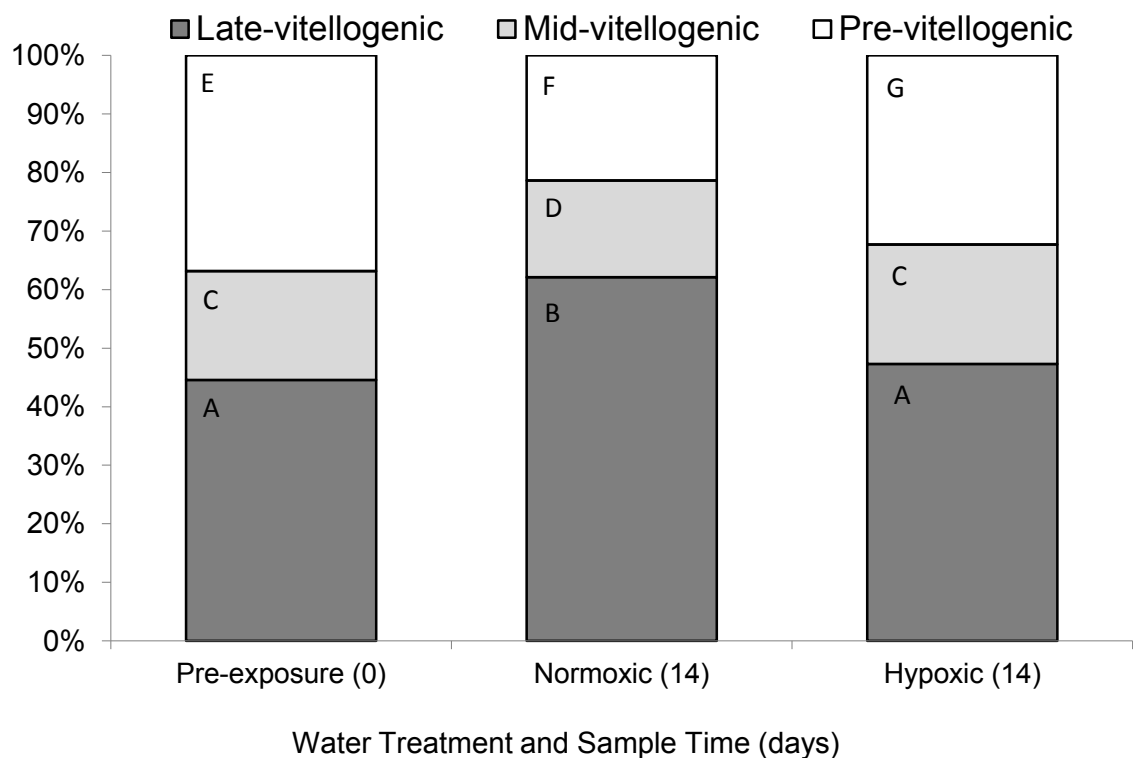
Figure 2.4. Reproductive ratios (%) for oocyte development stage within *Amphiura filiformis* pre-exposure (T0) and normoxic and hypoxic treatments at T14. Data are means (95 % confidence intervals are not shown, but average at 1.82, with a range = 0.91 - 3.97). Different letters on graph represent significant differences within the same oocyte developmental stage, but across the treatment groups (T0 and T14 normoxic n = 5, T14 hypoxic, n = 4).

Table 2.5. Two-sample t-test on *Amphiura filiformis* oocyte developmental stage data. Comparisons between T0 (n = 5), T14 N (normoxic treatment) (n = 5) and T14 H (hypoxic treatment) (n = 4). * Significant p values (to 95 % significance level).

Time / Treatment	t-value	p-value
	Late-vitellogenic	
T0 *vs.* T14 N	4.970	< 0.001*
T0 *vs.* T14 H	0.990	0.339
T14 N *vs.* T14 H	5.240	< 0.001*
	Mid-vitellogenic	
T0 *vs.* T14 N	1.180	0.252
T0 *vs.* T14 H	1.580	0.130
T14 N *vs.* T14 H	2.730	0.014*
	Pre-vitellogenic	
T0 *vs.* T14 N	7.010	< 0.001*
T0 *vs.* T14 H	2.600	0.018*
T14 N *vs.* T14 H	5.360	< 0.001*

Normoxic (T14)	Hypoxic (T14)
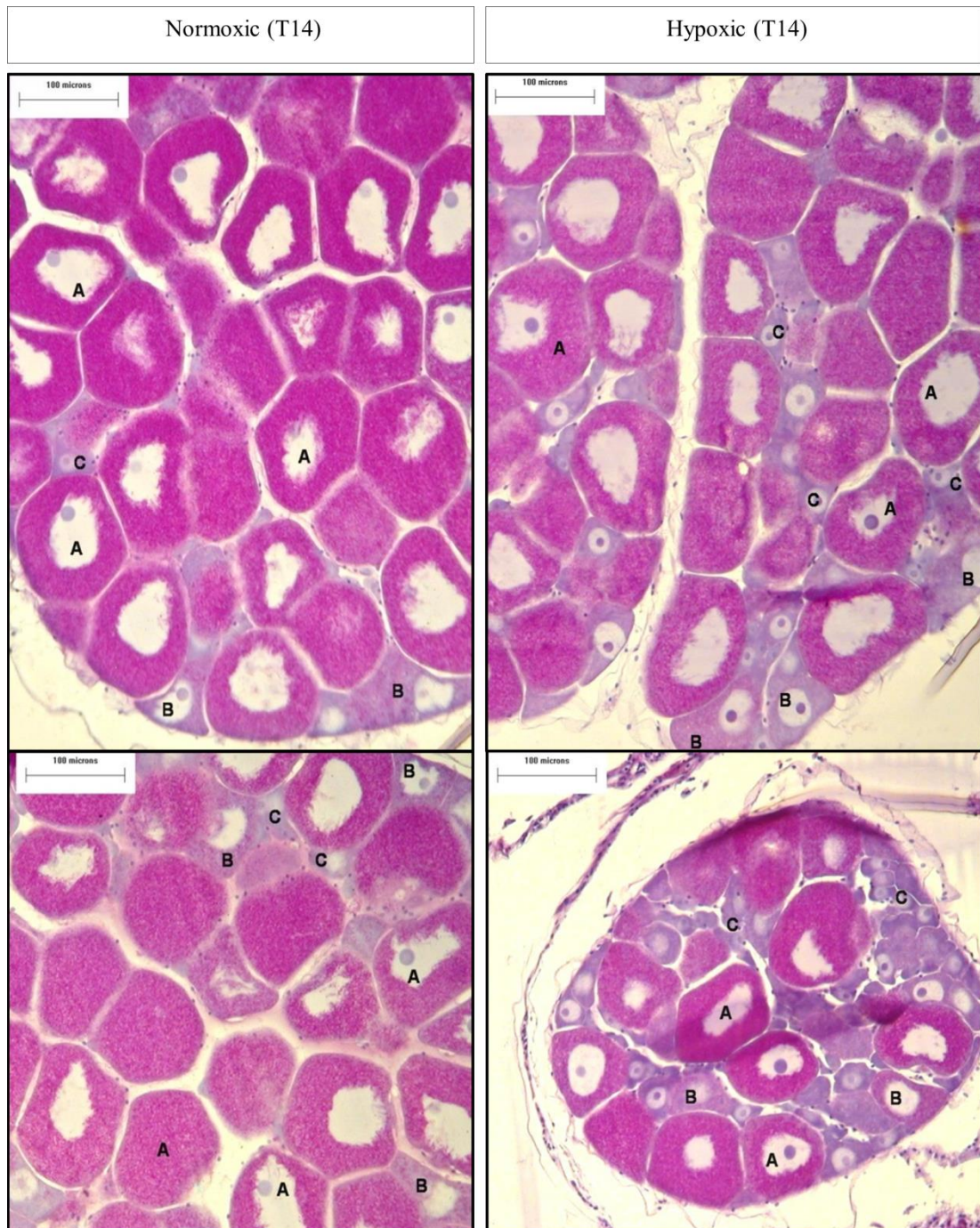
Figure 2.5. Representative examples of *Amphiura filiformis* oocytes from individuals within normoxic (T14) and hypoxic (T14) treatments. Note that the organisms exposed to hypoxia have significantly less late-vitellogenic oocytes and more pre-vitellogenic oocytes present compared to the normoxic treatment. A = late-vitellogenic, B = mid-vitellogenic, C = pre-vitellogenic. Scale bar = 100 microns.

2.5. DISCUSSION

Exposure to 14 days of moderate hypoxia reduced the aerobic metabolism of the brittlestar *Amphiura filiformis* and disrupted its reproductive development to an extent where oocyte diameter was reduced by 7.15 % and where there are 14.83 % fewer oocytes that reached the late-vitellogenic stage. However, these effects occurred irrespective of population density. Consequently, the fact that the number of individuals *per* aquarium had no significant effect on any of the parameters measured here, suggests that in the current experiment individual brittlestars do not appear to gain energetic advantage from being in dense aggregations.

2.5.1. Effects of density

Organism aggregations are a regular feature of natural ecosystems and occur for many reasons including social and spawning activities or to provide benefits to individuals such as thermal advantages (Chapperon & Seuront 2012). Here, it was hypothesised that aggregation may benefit *Amphiura filiformis, via* increased aeration and movement of the overlying water, aiding burrow ventilation and reducing energetic costs. However, in this study, the density of *A. filiformis* did not affect any of the parameters that were investigated. One possible explanation for the lack of density-dependent effects could be due to individuals being confined within glass aquaria, where there is reduced laminar flow and potential edge effects. Although brittlestars were collected from an area of low flow and shear stress (Uncles & Torres 2013) previous studies have shown that water flow rate has positive effects on *A. filiformis* somatic growth (arm regeneration rates) even when subjected to hypoxia, possibly due

to the water pressure gradients created during flow that help to ventilate the burrow (Nilsson 2000). Under low flow and low shear stress (i.e. this experiment) the capacity to irrigate their burrows requires active pumping using two arms. Therefore, irrespective of organism density, active burrow ventilation would still be a requirement to prevent the accumulation of toxic substances, possibly decreasing the energy yield that could be utilised in other important functions such as growth and reproduction. No direct measurements of burrow ventilation were recorded here, although aquaria were checked daily and protruding arms from the sediment were always present, typically indicating burrow ventilation and foraging behaviours.

Although our results suggested no advantages to *Amphiura filiformis* when in dense aggregations, there were also presumably no disadvantages. Vevers (1952) suggested that densely populated patches of the brittlestar *Ophiothrix fragilis* occurred within the Plymouth region as a result of a consistent, reliable food source supplied by tidal streams. Therefore, while aggregation benefits, in terms of lowering energetic costs to the organism, may not occur, there are other factors such as food supply which could possibly control distribution patterns for certain populations in their natural habitats. Food supply is an important factor governing growth and energetics within organisms, and may sustain growth rates even in hypoxia (Nilsson 1999). In our experiment, food was supplied daily with the dose of food proportional to the biomass within each aquarium. It may be that a large proportion of the energy gained from food ingestion was invested into maintaining burrow ventilation, especially within the hypoxic treatment. During hypoxic events, there is often an associated increase

in ammonia and hydrogen sulphide (Wu 2002), hence the consequences of allowing toxins to accumulate within the burrow could be more hazardous than exposure to hypoxia itself.

2.5.2. Effects of hypoxia on oxygen uptake

Our results are consistent with the view that *Amphiura filiformis,* in common with a number of other brittlestars has a tendency to be an 'oxyconformer', as it reduces its metabolic rates with declining pO_2 (partial pressure of O_2) in the surrounding seawater (Binyon 1972, Shick 1983). This was reflected in our results, with brittlestars exposed to hypoxia for 6 to 10 days showing a comparable trend (Fig. 2.3) of metabolic rates to that observed for individuals exposed to normoxic water, but showing consistently reduced rates.

Where there is evidence of oxyregulation in echinoderms, it can be a passive consequence of a small amount of biomass in a comparatively large fluid filled space (Johansen & Vadas 1967, Mangum & Van Winkle 1973, Spicer 1995), as in echinoids, due to active perfusion of respiratory structures (Johansen & Petersen 1971), or due to the presence of an extracellular respiratory pigment like haemoglobin. For example, the burrowing brittlestar *Hemipholis cordifera* was able to maintain its metabolic rate over a broad range of pO_2 (Christensen & Colacino 2000) because of the presence of haemoglobin within red blood cells contained in the water vascular system (Christensen et al. 2003). *Amphiura filiformis* has a relatively small fluid filled space, does not appear to have a respiratory pigment or targeted mechanisms for perfusing putative gas exchange surfaces, and shows little evidence of oxyregulation (Spicer *pers. obs.*).

Brittlestars exposed to Nt / No (normoxic treatment / normoxic oxygen uptake) in the current study exhibited oxygen uptake rates broadly comparable to those recorded by Vopel (2003), but lower (even allowing for temperature differences) than those found by Buchanan (1964) for the same species. The reduced rates of oxygen uptake at T6 and T10, although not a significant change from pre-exposure rates (T0), may be representative of experimental stress and adjustment to mesocosm conditions (Fig. 2.3). The significant increase in rates of oxygen uptake at T14 could be a sign of acclimation and recovery from the initial stress of collection and laboratory conditions. Although these rates are elevated compared to all other time points recorded for the same water treatment, the values were not dissimilar to those recorded for other brittlestar species (Binyon 1972). Acclimation time was accounted for in the experimental set up, but it is difficult to show exactly what effects are caused to organisms when removed from the field and how long recovery to a 'normal' physiological state would take. If this is the case for organisms exposed to normoxic seawater, our results suggests that exposure to hypoxia has hindered this acclimation process and possibly caused metabolic depression.

Furthermore, results from the Ht / No (hypoxic treatment / normoxic oxygen uptake) trial (i.e. recovery), although viewed with caution, indicate that *Amphiura filiformis* showed no increase in its rates of oxygen uptake when re-introduced to oxygenated water. This continued reduction of metabolic rate levels even upon return to fully oxygenated water could be interpreted in a number of different ways, none of them mutually exclusive: (1) Active metabolic reduction triggered by >10 days exposure to moderate hypoxia. A number of

marine animals respond to hypoxic exposure by conserving energy through an active down regulation of certain enzymatic processes (Wu 2002) and thus it is reasonable to assume that our data here represents metabolic reduction due to exposure to hypoxia and the inability to increase rates of oxygen uptake when re-introduced to oxygenated water; (2) recovery of pre-hypoxic rates of oxygen uptake is a slow process and could have not be detected over the time frame of our experiment (i.e. within 4 h of the respiration experiment); (3) sign of pathological damage, however this is unlikely as organisms appeared healthy and mortality rate was 0 %; and / or (4) artefact of the experimental design, our data being potentially affected by the fact that brittlestars were potentially exposed to elevated levels of stress due to the undertaking of a second oxygen uptake trial, especially when the data cannot be compared to a second trial for the specimens exposed to normoxia. However, rates of oxygen uptake within the recovery trials remained similar throughout the experiment and at time point T6, almost match the rates of oxygen uptake of the organisms within normoxic water, reducing the possibility that experimental stress may be overriding the effect of the factors of interest.

If the reduction in oxygen uptake by *Amphiura filiformis* exposed to hypoxic conditions is mainly a result of reduced metabolic rates, it could be that assimilated energy may be redirected into other processes. Cheung et al. (2008) found that rates of oxygen uptake by the scavenging gastropod, *Nassarius festivus* were reduced as environmental oxygen tension decreased, with fewer egg capsules produced at lower oxygen tensions. At 3.0 mg O_2 L^{-1}, Cheung et al. (2008) documented a 48 % reduction in energy allocation to growth and reproduction compared to organisms kept at 6.0 mg O_2 L^{-1}, yet

investment into shell growth continued, indicating that under stressful conditions this species preferentially allocate energy into a trait that would enhance survival. In the case of *A. filiformis* investigated here, energy savings as a result of recourse to reduced metabolic rates could be invested into burrow ventilation (locomotion), to reduce toxins and increase burrow oxygenation. This may be the behaviour / trait that would most enhance survival under experimental hypoxic conditions when laminar flow is reduced and could explain, in part, the delay in reproductive development as observed here.

2.5.3. Effects of hypoxia on reproduction

Amphiura filiformis has a discrete, relatively short breeding period occurring largely in late summer / autumn with the fastest growth often occurring in May - June (Bowmer 1982). This experiment was timed prior to spawning, and possibly encapsulated a period of rapid growth. Our results indicate exposure to 14 days of moderate hypoxia significantly delayed reproductive development, both in terms of oocyte diameter and the number of fully developed oocytes present at the end of the experimental period. Although, it is difficult to predict what ecological effect these results may hold, they may provide an insight into why spawning was delayed for this species when exposed to hypoxia in the study conducted by Nilsson & Sköld (1996). However, it still remains unclear if this delay in oocyte development could be beneficial to the organism, where gametogenesis would resume normal development when the extrinsic environmental conditions become favourable, or if aerobic energy assimilation whilst exposed to hypoxia was so limited, it restricted energy investment into reproductive output. In previous experiments, *A. filiformis* could allocate a greater fraction of its energy budget in arm regeneration instead of disk growth

(Nilsson 2000) in order to rehabilitate its full capacity for capturing food after sub-lethal predation. Therefore, as mentioned above it is possible that energy was strategically allocated to metabolic processes and locomotory movements i.e. burrow ventilation, to increase chances of survival during hypoxic stress. Furthermore, our experiment could not take into account the effects of sub-lethal predation occurring in brittlestars' natural environment, i.e. somatic growth measured as arm regeneration. O'Connor et al. (1986) report estimates of energy flow within *A. filiformis* of 77.4 % for respiration, 16.0 % for arm regeneration and 6.6 % is allocated to gonad output. The number of arms being regenerated affects regeneration rates and energy allocation from the disk, including the gonads (Dobson et al. 1991, Nilsson & Sköld 1996) and therefore, the energy required for arm regeneration during a hypoxic event in the field may impede reproductive development to an even greater extent than measured here. Additionally, studies investigating future scenarios of warmer, more acidic oceans have also shown that these environmental parameters can affect energy allocation in brittlestars. Wood et al. (2010) found that ocean acidification and increased temperature may indirectly affect the fitness and survival of *Ophiura ophiura*, causing a slower recovery from arm damage. In earlier work, Wood et al. (2008) demonstrated that *A. filiformis* can increase its metabolism and net calcification when exposed to acidified water, but at a substantial cost (arm muscle wastage). The possible interactive effects of hypoxia, acidification and temperature on brittlestars' energetics and long term survival are currently unknown.

2.6. CONCLUSIONS

Exposure to moderate hypoxia for 14 days reduces aerobic metabolism and delays reproductive development in the ecologically important brittlestar *Amphiura filiformis*. More broadly, there is already evidence to suggest that moderate hypoxia can severely affect the reproductive and endocrine systems in a number of other functionally important marine animals (Wang & Widdows 1991, Baker & Mann 1992, Nilsson & Sköld 1996, Chan et al. 2008). However, research in this area is still in its infancy (Wu 2002). In the long term, recurring hypoxic events may have major implications for benthic and pelagic population dynamics by indirectly affecting metabolic processes and reproduction, possibility resulting in reduced diversity and functionality within these communities. Currently, the dynamics between benthic reproductive outputs, planktonic larvae recruitment and the repercussions for juvenile and adult populations when exposed to hypoxia are still poorly understood (Birchenough et al. 2015). Further investigations of the effects of hypoxia on reproduction and development of other functionally important benthic taxa would provide important insights into the long term effects to biodiversity in areas where hypoxia is going to be increasingly common and more intense.

CHAPTER 3: IMPACT OF HYPOXIA ON ORGANISM ACTIVITY AND IMPLICATIONS FOR SEDIMENT PROCESSES

Moderate hypoxia affects *Amphiura filiformis* surface bioturbation activities with consequences for nutrient fluxes.

3.1. INTRODUCTION

Continental margins account for ~ 7 % of the surface of the global oceans (Gattuso et al. 1998) with approx. 80 % of these areas occurring at < 200 m depth (Liu et al. 2010). However, despite their modest global surface area, continental margins are responsible for as much as 90 % of sedimentary re-mineralisation of organic matter (Gattuso et al. 1998). In near-coast shallow (< 25 m depth) shelf seas, light penetration and intense nutrient recycling lead to substantial near seabed primary production that can double the total carbon fixation. This process is tightly linked to the transport of materials mediated by fauna living in or on the seabed, both over short and long time scales (Canfield & Farquhar 2009, Boyle et al. 2014).

Benthic infauna are responsible for the biogenic mixing of the sediment, a process known as bioturbation, which directly or indirectly affects sediment matrices (Shull 2009, Kristensen et al. 2011). Through the creation of pits, mounds and burrows, sediment ingestion and excretion, as well as the bio-irrigation of subsurface burrows, benthic infauna play a significant role in mediating the rate and depth of many chemical and physical reactions. This ultimately drives carbon and nitrogen cycling, establishes oxygen, pH and redox gradients, determines sediment porosity and permeability, and sets microbial activity rates and diversity (Herbert 1999, Shull 2009, Laverock et al. 2010, Bertics et al. 2013).

However, changes in biodiversity, species composition and alterations to ecosystem function are predicted to increase as humans continue to affect the marine environment, especially in coastal areas (Törnroos et al. 2015). One

such disturbance to coastal ecosystems is a significant increase in events of low dissolved oxygen (DO) conditions, i.e. hypoxia, which is now recognised as a key environmental stressor and is predicted to increase in coastal areas as global warming and human-induced eutrophication intensify (Diaz & Rosenberg 2008, Vaquer-Sunyer & Duarte 2008, Howarth et al. 2011).

The response of any individual infaunal organism to hypoxia is highly variable and dependent on the severity and duration of the hypoxic event (Spicer 2014). When severe hypoxia occurs (usually defined as < 2.0 mg O_2 L^{-1}) species mortality or mass migrations can occur (Vaquer-Sunyer & Duarte 2008). However, not only is the onset of coastal hypoxia a process, resulting in the decline of DO through time, it does not always result in such severe reductions in DO. Additionally, there is ample evidence that this threshold is inadequate to describe the onset of hypoxia impacts for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel & Childress 2013), and valid arguments exist for establishing oxygen tolerance limits based on measures of physiological or ecological performance (Seibel & Childress 2013). When DO levels are reduced, before mass mortalities or species migrations are observed, organism responses to hypoxia are often initially expressed through changes in organism physiology and behaviour (Grieshaber et al. 1994). Documented changes include reduced growth in oyster larvae and juveniles (Baker & Mann 1992), delayed embryonic development in gastropods (Chan et al. 2008) and reduced metabolic rates and oocyte growth in brittlestars, as described in Chapter 2 and Calder-Potts et al. (2015). Behavioural responses include elongated bivalve siphons, abandonment of burrows and reduced burrowing depths and activity of infauna (Sturdivant et al. 2012). Importantly, behavioural data may provide a

link between individual response and population change, especially if the behaviour alters the structure and function of the community (Boyd et al. 2002).

Ecosystem engineers are defined as species that modify, maintain and create habitats and, through their actions, modulate the availability of resources to other species (Lawton 1994, O'Reilly et al. 2006). One such species, the brittlestar *Amphiura filiformis* (O.F. Müller, 1776), is an active and well-studied bioturbator (Solan & Kennedy 2002, Solan et al. 2004a, O'Reilly et al. 2006, Queirós et al. 2013, Queirós et al. 2015). *Amphiura filiformis* is primarily a suspension feeder that remains buried below the sediment surface and protrudes one or more arms into the water column. It actively undulates its arms and pumps its disc to achieve respiratory gas exchange, burrow ventilation and irrigation, in addition to collection and expulsion of food and waste (Vopel et al. 2003, Calder-Potts et al. 2015). *Amphiura filiformis* is also a dominant species in many coastal and shelf areas of the NE Atlantic and its effects on sediment properties may explain its structuring effect in infauna communities (Queirós et al. 2006). Negative impacts of hypoxia on the biology of *A. filiformis* have also been documented for a range of key physiological processes. Hypoxic exposure reduces *A. filiformis* disc diameter growth (Hylland et al. 1996), reduces arm regeneration rates and delays spawning (Nilsson & Sköld 1996, Nilsson 1999), reduces metabolic rates, reduces oocyte growth and delays reproductive development (Chapter 2, Calder-Potts et al. 2015). However, research that examines the links between the biological or physiological consequences of hypoxia and the subsequent impacts on organism behaviour and the repercussions for ecosystem processes are limited.

In a 'random extinction' event simulation study focused on the North Sea, the biogenic mixing depth (BMD), an indicator of bioturbation, was dependant on whether *Amphiura filiformis* was among the survivors (Solan et al. 2004a). Field data on communities exposed to fishing pressure in the Irish Sea demonstrated that community biomass and production dramatically decreased following the loss of the dominant *A. filiformis,* a species which is highly vulnerable to physical damage associated with trawling (Queirós et al. 2006). Therefore, in communities where contributions to ecosystem function are dominated by one species, stress induced loss or behavioural alterations of that dominant species can have consequences for the entire community.

In order to predict the impacts of environmental stressors, such as hypoxia, on ecosystem function in coastal environments, a better understanding is needed of the mechanisms and acclimations that functionally dominant species, such as *Amphiura filiformis,* undergo and the consequential effects on functional performance. Consequently, a 14 day mesocosm experiment was conducted that addressed the following questions: 1) Does exposure to moderate hypoxia affect *A. filiformis* behaviour, measured in terms of bioturbation activity? 2) Do any changes in *A. filiformis* behaviour affect nutrient fluxes in the sediment, as a proxy for the ability to maintain ecosystem function? 3) What role does population density play in maintaining ecosystem function? 4) If density is a significant factor, do populations with a higher density of individuals display greater resilience to hypoxic stress than populations with lower densities, possibly as a consequence of greater bioturbation activities and thus increased pore-water exchange?

Within a controlled mesocosm environment, *Amphiura filiformis* was exposed to 14 days moderate hypoxia. Bioturbation activity was measured using 2D imaging and particle tracing methods (Mahaut & Graf 1987, Gilbert et al. 2003, Solan et al. 2004b). Tracer data were then used to quantify three different parameters: maximum bioturbation depth, percentage of the sediment surface reworked, and bioturbation activity as determined using a process based random-walk model. Nutrient flux data were used as an 'experimental proxy' for ecosystem function to give an indication of how hypoxia affects the functionality of *A. filiformis*.

3.2. MATERIALS AND METHODS

The data presented here were generated from the same mesocosm experiment documented in Chapter 2. For details on sediment collection refer to Sect. 2.2.1, for details on brittlestar collection refer to Sect. 2.2.2, for details on experimental design and set up refer to Sect. 2.2.3, and for details on seawater manipulations refer to Sect. 2.2.4 and Table 2.1.

3.2.1. Acquisition of bioturbation data

3.2.1.1. Image preparation

Bioturbation data for all aquaria held within the mesocosm were acquired using a luminophore tracer technique (Mahaut & Graf 1987) and 2-D imaging under U.V. light to monitor the movement of luminophores over time. The luminophores (supplier, Partrac ltd., Glasgow, U.K.) used were chosen to match the sediment granulometry of the collection site (Cawsand, U.K.) and had a median grain size of 60 µm. The luminophore particles are naturally occurring quartz material coated with a fluorescent dye. Luminophores (0.2 g *per* cm^2 =

20 g *per* aquaria) were added to the experimental aquaria 5 d in advance of their allocated sampling day (T0, T6, T10 or T14) resulting in a staggered addition of luminophores across the experimental period. Luminophores were added to each aquarium by evenly pouring them into the overlying water. Settlement of luminophores took approximately 1 h, during which time water circulation to the aquaria was stopped (Fig. 3.1).

Each aquarium was then photographed once every 24 h (± 1 h) for a total of 6 d (n $_{images\ per\ aquarium}$ = 6) (Fig. 3.1). To this end, aquaria were individually removed from the experimental system and carefully placed at one end of a custom-made black box which housed at the other end (and at a fixed focal distance from the aquarium) a digital SLR camera (Canon EOS 1000D, 10.1 MP), and was illuminated by a 8 W ultra violet (UV) light (see Schiffers et al. 2011, supporting material, Fig. S1). A custom made frame was fixed in the camera box that held the aquaria in the exact same position each time a photograph was taken. The camera was set for an exposure of 10 s, f = 5.6, ISO = 200, was controlled remotely *via* a PC using the software GB Timelapse, (V 3.6.1). The UV light inside the photo box was necessary for luminophore excitation and produced enough light to distinguish the sediment-water profile. Images were captured in RGB format and saved using a JPEG compression (sized 3888 x 2592 pixels). After each photograph session, aquaria were returned to the experimental system and re-connected to their respective flow–through water treatment. The sixth and final photograph for each aquaria occurred on a sampling day (T0, T6, T10 or T14). At this point, designated aquaria were removed from the experiment for further analysis.

3.2.1.2. Image data extraction

Using the software, ImageJ (V. 1.4.3) all photographs were cropped to a size of 2996 x 2200 pixels, which removed the edges of the aquaria. On each image, the water-sediment interface was drawn manually. This line represented the initial reference used to calculate luminophore penetration depths. Luminophore positions in each image were quantified using custom-made, semi-automated algorithms for R 2.15.1 (R Development Core Team 2012, Queirós et al. 2015) and Image J (V. 1.4.3) modified from Queirós (2010). The algorithm acts as an automated standardised method for image segmentation (threshold analysis), which accounts for potential changes in the apparent brightness of luminophore pixels as particle mixing occurs during the aquarium incubations. In summary, each image was transformed to a binary matrix, where luminophore pixels were assigned the value of 1 and sediment pixels a value of 0. Image data were automatically compiled as a count of luminophores *per* pixel layer (i.e. depth) within each image, with sediment depth calculated relative to the linearised sediment-water interface. Luminophores *per* pixel layer were then summed creating a row total, which was used to re-construct vertical profiles of luminophores within the sediment from each photograph, in addition to profile sequences for the set of six images.

3.2.1.3. Quantifying bioturbation

The luminophore tracer profiles extracted from each image were used to estimate three aspects of bioturbation. Firstly, maximum luminophore penetration depth (MPD) was used as a proxy for maximum bioturbation depth, and estimated by determining the deepest image pixel row containing at least five luminophore pixels. Five luminophore pixels approximately equated to 330

μm, which represented one – two individual luminophore particles. Secondly, bioturbation activity was estimated by calculating the proportion of sediment surface reworked (SSR), measured as 100% minus the percentage of tracer left in the surficial layers (the first cm of sediment) at the end of each time point (Maire et al. 2006). Lastly, the open-source, bioturbation process based random-walk model detailed in Schiffers et al. (2011) was applied to the tracer profiles. The core of the model represents a random walk approach for active particle movement (local transport) and a discrete and stochastic version of an advection model which accounts for non-local transport (Schiffers et al. 2011). Model rules and parameterisation are detailed in Schiffers et al. (2011). The model output includes three parameters, two of which ('*distance*' and '*activity*') are important descriptors of bioturbation. '*Distance*' is the average distance a simulated tracer particle travels within one time step, and '*activity*' is the probability of displacement for each simulated tracer particle within one time step. The third parameter, '*tracerdiff*', sets the probability of a particle being displaced according to density differences between luminophores and natural sediment particles.
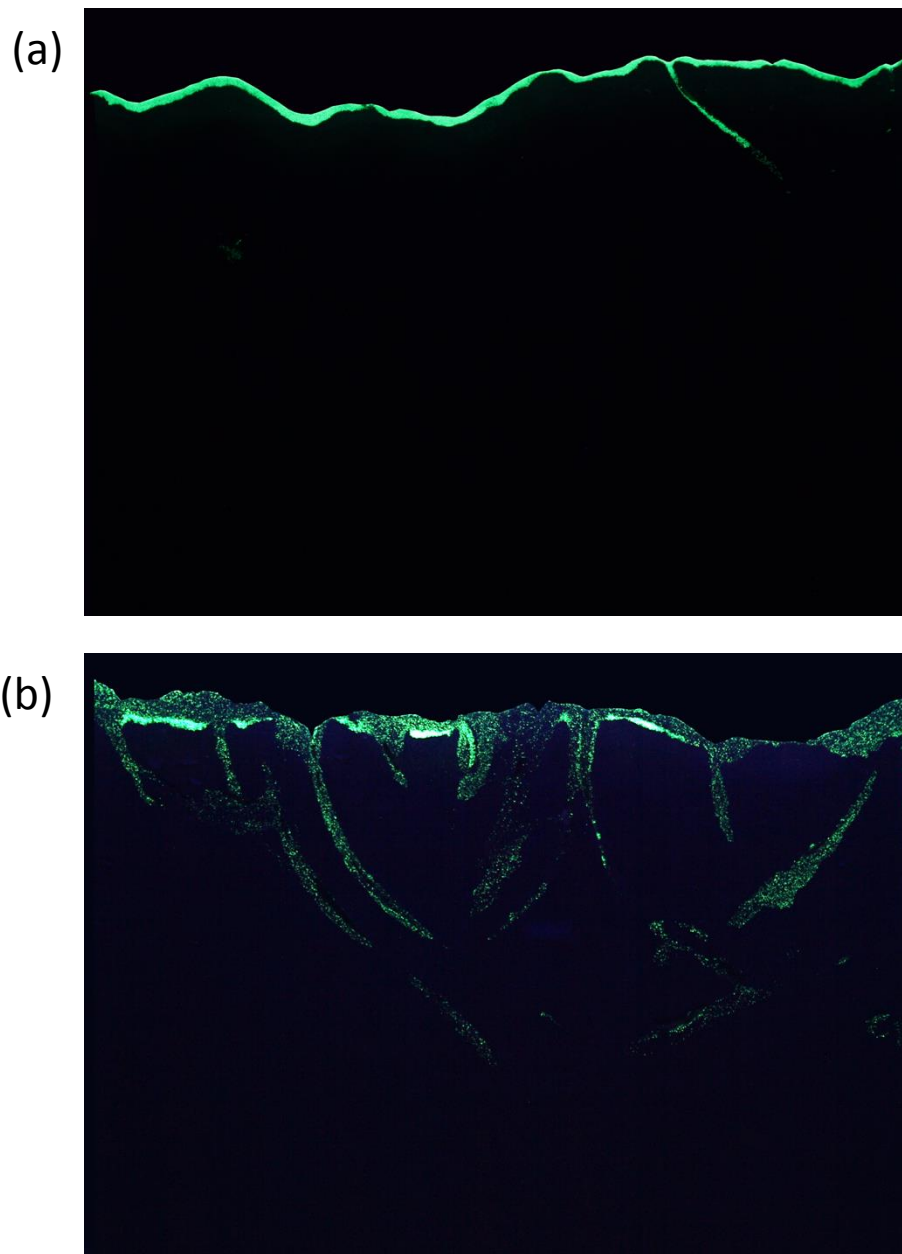
Figure 3.1. Example of luminophore mixing over a six day period within an aquaria containing 21 individuals. Image (a) represents luminophore mixing 1 hour after addition, image (b) represents luminophore mixing six days after addition.

3.2.2. Nutrient analysis

Nutrient samples were taken from each aquarium at time points T6, T10 and T14. Water overlying the sediment (1 ± 0.5 cm) and water from the inflow pipe connected to the header tanks were sampled separately, in triplicate, on each sampling day. Each 50 mL sample was filtered through a 47 mm ø GF/F filter and stored in an acid washed Nalgene bottle. Samples were stored and frozen at -20 °C until analysed using a segmented flow nutrient auto-analyser (AAIII, SEAL Analytical, Fareham, U.K.). Standard methods were used to detect ammonium, nitrate, nitrite, silicate and phosphate concentrations (Brewer & Riley 1965, Grasshoff 1976, Mantoura & Woodward 1983, Kirkwood 1989). Nutrient fluxes were calculated using Eq. (3.1) from Widdicombe and Needham (2007). Fluxes across the sediment-water interface provide an estimation of the net change of nutrient x within the experimental aquaria and give an indication of the alterations in biogeochemical cycling caused by a reduction in dissolved oxygen concentrations and also by changes in infaunal activities and abundance.

$$F_x = \frac{(C_i - C_o) \times Q}{A}$$
(3.1)

Where F_x is the flux of nutrient x (μmol m^{-2} h^{-1}), C_i is the concentration of nutrient x in the inflow water (μM), C_o is the concentration of nutrient x in the aquaria water (μM), Q is the rate of water flow through the aquaria (L h^{-1}) and A is the sediment area within the aquaria (m^2). A positive flux value indicates nutrient x is being taken up by the sediment (influx) and a negative value

indicates nutrient x is being released from the sediment (efflux) into the overlying water.

3.3. STATISTICAL ANALYSES

Statistical analyses were carried out using the software package MINITAB 17 (ver. 17.1.0). The Shapiro-Wilk test for normality and Levene's test for homogeneity of variance were completed on each parameter measured. Where necessary, either a square root or Log_{10} +1 transformation was applied. Ammonium flux data were the exception and could only be normalised using a 'sine' transformation. Each parameter was analysed using a general linear model analysis of variance (ANOVA), with 'water treatment' (normoxic or hypoxic), 'brittlestar density' (0, 5, 9, 13, 17 and 21 indiv. *per* aquaria), and 'experimental time' (0, 6, 10 and 14 d) as the factors. Prior to analyses of nutrient flux data within the experimental aquaria, nutrient measurements originating from the header tanks were tested for 'tank effects'. Header tank nutrient data could not be normalised using any transformation and were analysed using the non-parametric Mann-Whitney U rank sum test.

The treatments containing no *Amphiura filiformis* (i.e. a brittlestar density of zero) were excluded from analyses on maximum luminophore depths (MLD) and % of surface sediment reworked (% SSR) because, as expected, luminophores were not disturbed or bioturbated within these treatments. By excluding the zero density treatment, MLD and % SSR relationships with brittlestar density are not artificially strengthened or skewed due to the addition of a zero activity data point due to no brittlestars being present. The zero brittlestar density treatments were included in the nutrient flux analyses

because they provide insight into background nutrient cycling rates in the absence of *A. filiformis*.

3.4. RESULTS

3.4.1. Bioturbation activity

3.4.1.1. Maximum luminophore depths (MLD)

The average maximum luminophore depth (MLD) measured across all aquaria (excluding the 0 density treatment) was 7.99 ± 0.57 cm (mean ± 95% CI). There were no significant effects of the experimental parameters on MLD (Table 3.2 a).

Table 3.1. General linear model ANOVA for maximum luminophore depths (MLD) (a); percentage of sediment surface reworked (% SSR) (b); the parameter '*distance*' (cm) calculated from the process-based modelling of bioturbation (c). Degrees of freedom (DF); sum of squares (SS); mean squares (MS); F-value (F); and probability value (*p*). *Significant *p*-values (to 95 % significance level).

Source	DF	SS	MS	F	*p*
(a) MLD					
Water treatment	1	0.00	0.00	0.00	0.998
Density	4	14.36	3.59	1.37	0.277
Time	3	11.74	3.91	1.49	0.245
Water treatment * Density	4	19.98	5.00	1.90	0.145
Residual	22	57.71	2.62		
Total	34	110.38			
(b) % SSR					
Water treatment	1	16.8	16.83	0.16	0.692

Density	4	7578.8	1894.71	18.09	<0.001*
Time	3	1228.8	409.59	3.91	0.022*
Water treatment * Density	4	1347.7	336.93	3.22	0.032*
Residual	22	2304.5	104.75		
Total	34	13034			

(c) '*distance*' (cm)

Water treatment	1	0.0000010	0.0000010	0.33	0.572
Density	4	0.000028	0.0000070	2.48	0.076
Time	3	0.00013	0.000043	15.08	<0.001*
Water treatment * Density	4	0.000026	0.0000060	2.25	0.098
Residual	21	0.000060	0.0000030		
Total	33	0.00025			

3.4.1.2. Percentage of sediment surface reworked (% SSR)

In both the normoxic and hypoxic water treatments, the percentage of sediment surface reworked (% SSR) was significantly greater as brittlestar density increased (Fig. 3.2, Table 3.1 b). There was also a significant effect of 'experimental time' whereby on average, in both water treatments, less sediment surface was reworked the longer the brittlestars remained in the experimental system (Table 3.1 b). For example, the average % SSR across both water treatments and all density treatments at T0 was 41.67 %, which decreased to 30.56 % at T6, 28.02 % at T10 and 22.49 % at T14. There is the possibility that this was due to the animals taking time to acclimate to the conditions within the mesocosm (cf. Chapter 2).

In addition, there was a significant interaction effect between 'water treatment' and 'brittlestar density' (Table 3.1 b). This indicates that there are significant effects of each of the factors, but the effect of water treatment was modified

through the levels of brittlestar density. For example, the largest differences in % SSR between the normoxic and hypoxic aquaria occur in the highest brittlestar density treatment (21 indiv. *per* aquaria) at T6 and T14. At T6 within the normoxic aquaria % SSR = 54.80 %, whilst in the hypoxic aquaria % SSR = 29.68 %. At T14 within the normoxic aquaria % SSR = 64.16 %, whilst in the hypoxic aquaria % SSR = 26.10 %. There were no significant effects of 'water treatment' in isolation and no interaction effects between 'water treatment' and 'time' (Table 3.1 b).
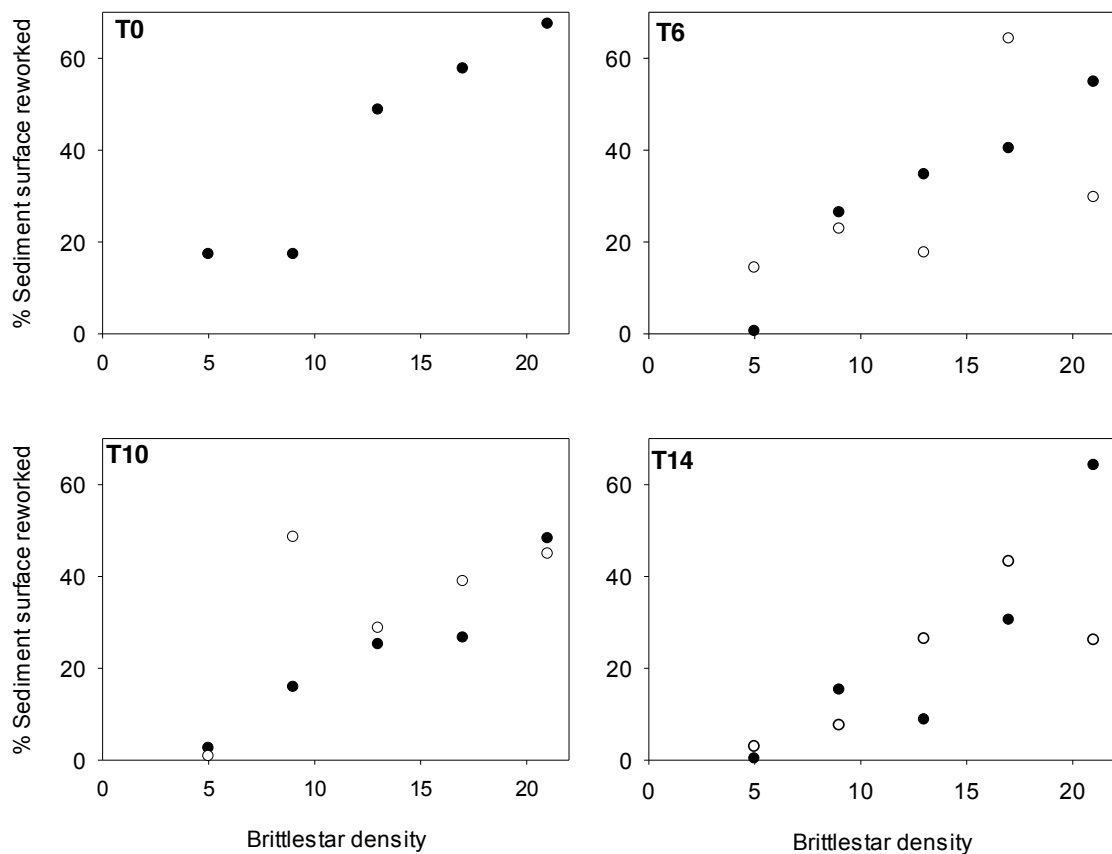
Figure 3.2. Percentage of sediment surface reworked (% SSR) (top 1 cm only) against brittlestar density at time points T0, T6, T10 and T14. Points represent individual aquaria, ● = normoxia, ○ = hypoxia.

3.4.1.3. Process-based modelling of bioturbation

Investigation of sums of squares plots indicated strong correlation in model error associated with the investigated ranges of values for the parameters '*activity*' and '*distance*'. For this reason, the two-parameter optimisation model was used by fixing one of the parameters ('*activity*'), as recommend by the model authors (Schiffers et al. 2011). To reduce computing time, model simulations were conducted on a grid containing 297 sediment layers (1 layer = 1 pixel row) equating to a depth of 2 cm, as *per* Schiffers et al. (2011). Analyses revealed that 'experimental time' had a significant effect on the parameter '*distance*' (cm) calculated from the process-based model, but 'water treatment', 'brittlestar density', and the interaction between 'water treatment' and 'brittlestar density' had no effect (Fig. 3.3, Table 3.1 c). This time effect was due to higher average values calculated at T0, with a decrease in '*distance*' (cm) as the experiment progressed, ('*distance*' (cm) at T0 = 0.0194 ± 0.0114; T6 = 0.0141 ± 0.00352; T10 = 0.0128 ± 0.00264; T14 = 0.0146 ± 0.00265; values are means ± 95 % CI). However, caution should be taken when interpreting these results, with reasons highlighted in the discussion section.
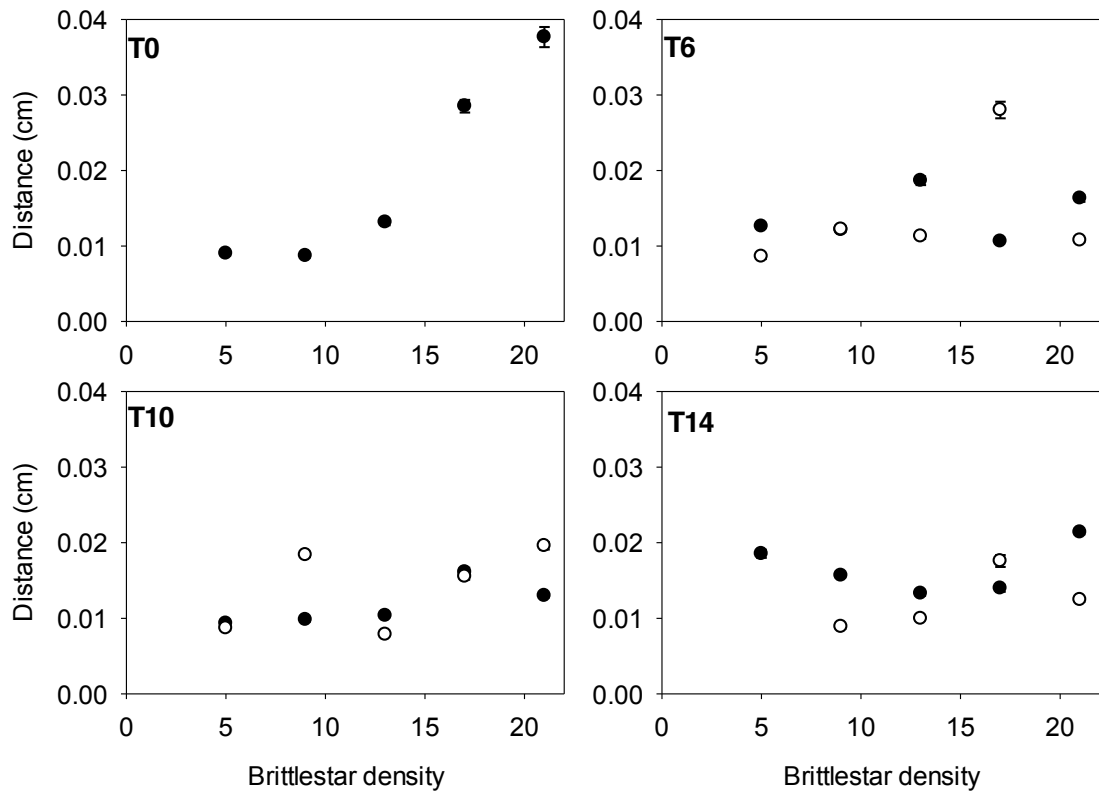
Figure 3.3. The average '*distance*' (cm) a simulated tracer particle travels within one-time step, against brittlestar density at time points T0, T6, T10 and T14. Data expressed as means for each aquaria, ± 95 % confidence intervals based on 10 model iterations, ● = normoxia, ○ = hypoxia.

3.4.2. Nutrients

3.4.2.1. Header tank effects

Analyses of nutrient measurements from the header tanks revealed that there were significant differences in nitrate and phosphate concentrations between the normoxic and hypoxic header tanks, despite the tanks receiving seawater from the same source (Table 3.2). Consequently, header tank nitrate and phosphate data were examined in more detail.

Table 3.2. Mann-Whitney U rank sum test on header tank nutrient concentrations (µM). N = 54; t-value (t); Mann-Whitney U statistic (MWU); probability value (p). * Significant p-values (to 95 % significance level)

Source	t	MWU	p
Nitrite	3037.00	1203.00	0.204
Nitrate	3510.00	730.00	<0.001*
Ammonia	2599.00	1324.00	0.868
Silicate	2837.00	1406.50	0.881
Phosphate	1448.00	70.00	<0.001*

3.4.2.2. Header tank nitrate

The differences in nitrate concentrations between the two header tanks started at T10 and increased with experimental time, with the largest differences occurring at T14. Nitrate concentration within the normoxic header tank at T10 was 6.46 µM (± 0.080) and the corresponding hypoxic nitrate concentration was 5.91 µM (± 0.059), a decrease of 8.5 % (Mann-Whitney U statistic = 1.00, t = 494.00, n = 18, p = < 0.001). At T14 nitrate concentration within the normoxic header tank had increased to 7.14 µM (± 0.12), whilst nitrate concentrations in the hypoxic header was 4.71 µM (± 0.24), a decrease of 34.0 % compared to the normoxic tank (Mann-Whitney U statistic = 0.00, t = 153.00, n = 18.00, p = < 0.001).

3.4.2.3. Header tank phosphate

Phosphate concentrations between the normoxic and hypoxic header tanks remained closely matched until T14. At T14, phosphate concentration in the normoxic header tank was 0.27 µM (± 0.015), whilst concentrations in the

hypoxic header tank were significantly lower at 0.17 µM (± 0.032), a decrease of 37.0 % (Mann-Whitney U statistic = 35.00, t = 460.00, n = 18, p = <0.001).

3.4.2.4. NO_x fluxes in experimental aquaria

To investigate the effects of hypoxia, brittlestar density and time within the experimental aquaria, combined nitrate and nitrite measurements (hereafter known as NO_x) were examined. During the experiment NO_x predominantly fluxed into the sediment in both the normoxic and hypoxic aquaria (Fig. 3.4 a - c). Analyses revealed that 'water treatment', 'brittlestar density' and 'experimental time' had no significant effects on NO_x flux, however there was a significant interaction effect between 'water treatment' and 'experimental time' (Table 3.3 a). This is due to the slight increase in NO_x flux into the sediment within the normoxic aquaria after 14 d experimental exposure (Fig. 3.4 c).

3.4.2.5. Ammonium fluxes in experimental aquaria

In aquaria containing no brittlestars there were minimal amounts of ammonium fluxing into or out of the sediment but ammonium consistently fluxed out of the sediment in aquaria that contained brittlestars, irrespective of water treatment (Fig. 3.4 d - f). Analyses revealed that 'water treatment', 'brittlestar density', 'experimental time' and their interactions did not significantly affect ammonium flux (Table 3.3 b). However, Figure 3.4 (f) shows that ammonium efflux at T14 has increased in the aquaria exposed to hypoxic seawater that contain brittlestar densities of 13, 17 and 21 indiv. *per* aquaria. A subsequent GLM ANOVA was conducted on T14 ammonium flux data from the high brittlestar density treatments (13, 17 and 21 indiv. *per* aquaria). Results revealed that at T14, within the high brittlestar density treatments, ammonium efflux was

significantly greater within the hypoxic aquaria compared to the normoxic aquaria (Fig. 3.4 f, Table 3.3 c).
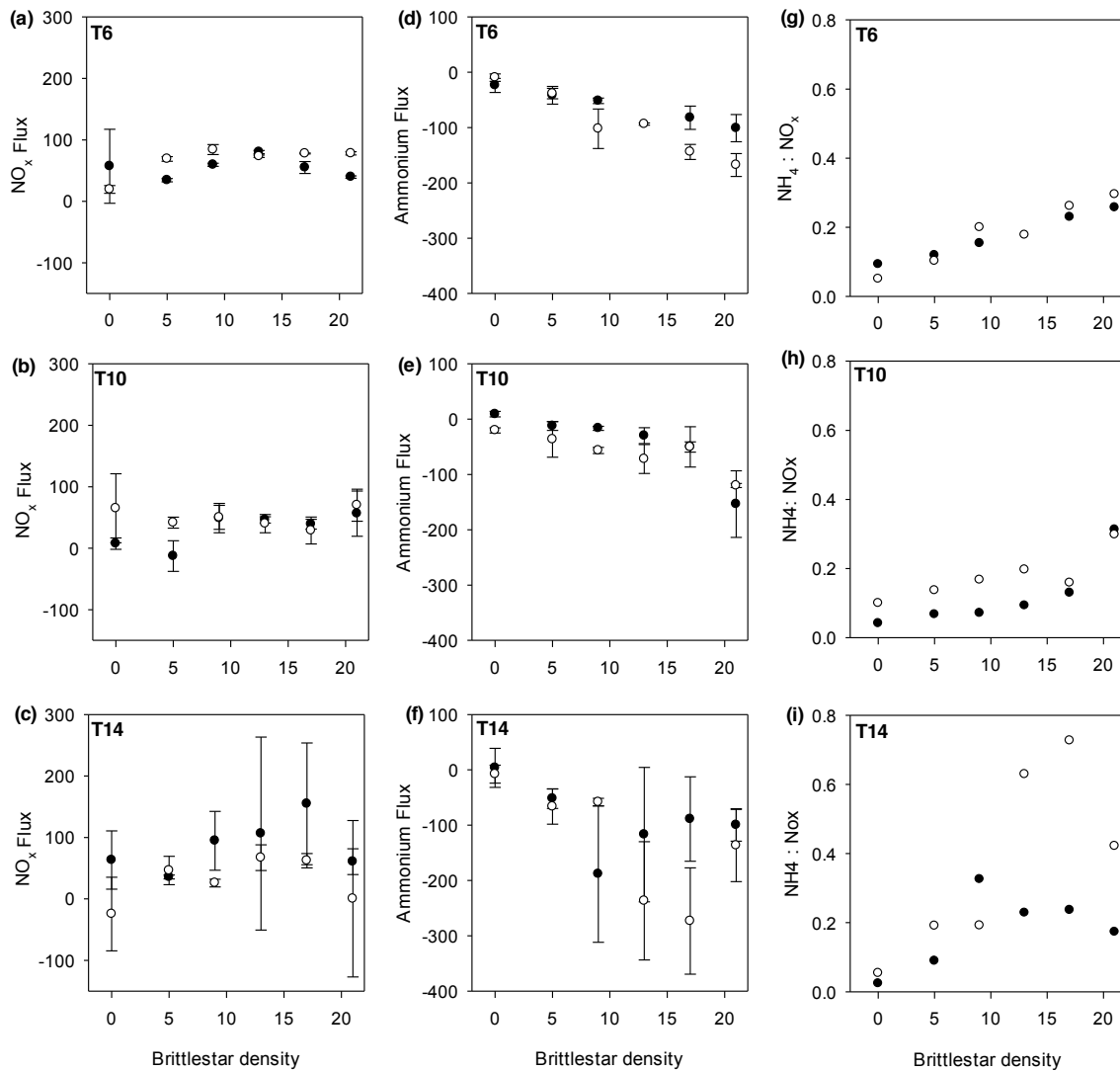
Figure 3.4. NO_x flux ($\mu mol\ m^{-2}\ h^{-1}$) (a - c); ammonium (NH_4^+) flux ($\mu mol\ m^{-2}\ h^{-1}$) (d – f); and $NH_4^+ : NO_x$ ratios (g – i), in experimental aquaria at time points T6, T10 and T14. Data for NO_x and ammonium fluxes are means ± 95 % confidence intervals, $NH_4^+ : NO_x$ ratio data calculated from mean concentrations. For NO_x and ammonium flux, positive results represent nutrient influx, whilst negative results represent nutrient efflux, ● = normoxia, ○ = hypoxia.

Table 3.3. General linear model ANOVA for NO_x flux (a); ammonium (NH_4^+) flux (complete data set) (b); ammonium flux at T14 within the high brittlestar density treatments (13, 17, and 21 indiv. *per* aquaria) (c); NH_4^+ : NO_x ratios (d); phosphate (PO_4^{3-}) flux (e); silicate (SiO_4^{4-}) flux (complete data set) (f); and silicate flux at T14 within the high brittlestar density treatments (13, 17, and 21 indiv. *per* aquaria) (g). Degrees of freedom (DF); sum of squares (SS); mean squares (MS); F-value (F); probability value (*p*). *Significant *p*-values (to 95 % significance level)

Source of variation	DF	SS	MS	F	*p*
(a) NO_x flux					
Water treatment	1	640.30	640.30	1.00	0.329
Density	5	8160.30	1632.10	2.55	0.061
Time	2	2956.30	1478.10	2.31	0.125
Water treatment * Density	5	3617.20	723.40	1.13	0.377
Water treatment * Time	2	10386.10	5193.10	8.11	0.003*
Residual	20	12805.90	640.30		
Total	35	38566.10			
(b) Ammonium flux					
Water treatment	1	0.12	0.12	0.23	0.640
Density	5	3.16	0.63	1.14	0.372
Time	2	0.15	0.07	0.13	0.875
Water treatment * Density	5	2.53	0.51	0.92	0.490
Water treatment * Time	2	0.82	0.41	0.74	0.489
Residual	19	10.49	0.55		
Total	34	17.16			
(c) Ammonium flux (T14, high density treatments)					
Water treatment	1	58240.00	58240.00	9.18	0.009*
Density	2	14844.00	7422.00	1.17	0.339
Residual	14	88774.00	6341.00		
Total	17	161859.00			

Table 3.3. Continued

Source of variation	DF	SS	MS	F	p
(d) NH_4^+ : NO_x ratios					
Water treatment	1	0.06	0.06	5.97	0.024*
Density	5	0.39	0.08	7.53	<0.001*
Time	2	0.08	0.04	3.87	0.039*
Water treatment * Density	5	0.02	0.00	0.38	0.859
Water treatment * Time	2	0.04	0.02	1.87	0.182
Residual	19	0.20	0.01		
Total	34	0.80			
(e) Phosphate flux					
Water treatment	1	16.75	16.75	0.73	0.402
Density	5	74.66	14.93	0.65	0.663
Time	2	39.76	19.88	0.87	0.434
Water treatment * Density	5	50.79	10.16	0.44	0.812
Water treatment * Time	2	0.13	0.06	0.00	0.997
Residual	20	457.30	22.87		
Total	35	639.40			
(f) Silicate flux					
Water treatment	1	427.60	427.60	0.61	0.445
Density	5	17675.20	3535.00	5.02	0.004*
Time	2	3106.10	1553.00	2.21	0.136
Water treatment * Density	5	1288.40	257.70	0.37	0.866
Water treatment * Time	2	10017.10	5008.60	7.11	0.005*
Residual	20	14082.80	704.10		
Total	35	46597.10			
(g) Silicate flux (T14, high density treatments)					
Water treatment	1	41393.90	413 93.90	17.30	0.001*
Density	2	146.80	73.40	0.03	0.970
Residual	14	33507.40	2393.40		
Total	17	75048.10			

3.4.2.6. Ratios of NH_4^+ : NO_x

Concentrations (µM) of ammonium (NH_4^+) and NO_x in the experimental aquaria have been presented as ratios [NH_4^+ : NO_x] with the aim of emphasising sedimentary processes such as nitrification and denitrification, that may drive changes in nutrient concentrations (Fig. 3.4 g - i). Analyses revealed that 'water treatment', 'brittlestar density' and 'experimental time' had a significant effect on the NH_4^+ : NO_x ratios, with the normoxic aquaria displaying lower NH_4^+ : NO_x ratios compared to the hypoxic aquaria, with differences increasing over the experimental time period (Table 3.3 d). At T6, normoxic NH_4^+ : NO_x ratios were an average of 6.07 % lower than the hypoxic aquaria ratios. At T10, this difference increased to 32.38 % and at T14, the normoxic NH_4^+ : NO_x ratios were an average of 51.35 % lower than the hypoxic aquaria. At T6 and T10, NH_4^+ : NO_x ratios increase steadily with brittlestar density (Fig. 3.4 g, h). At T14, the normoxic NH_4^+ : NO_x ratios peak at brittlestar density 9, and slightly decrease and plateau at the higher brittlestar density treatments (Fig. 3.4 i). Within the hypoxic treatment at T14, NH_4^+ : NO_x ratios remain similar to normoxic levels but only in the low density treatments (0 – 9 indiv. *per* aquaria). In the high density treatments (13 – 21 indiv. *per* aquaria) NH_4^+ : NO_x ratios increase (Fig. 3.4 i). The interactions between 'water treatment' and 'brittlestar density' and 'water treatment' and 'experimental time' had no significant effect on NH_4^+ : NO_x ratio data (Table 3.3 d).

3.4.2.7. Phosphate flux

Phosphate (PO_4^{3-}) primarily fluxed into the sediment throughout the experimental period, but there was variability within the results with some data points indicating phosphate efflux (Fig. 3.5 a - c). Analyses revealed no

significant effects of any experimental parameter on phosphate flux data (Table 3.3 e).

3.4.2.8. Silicate flux

At T6, silicate (SiO_4^{4-}) consistently fluxed out of the sediment in brittlestar density treatments of nine indiv. *per* aquaria or above, irrespective of water treatment. After T6, variability in the silicate flux results increases, resulting in some data points representing silicate efflux and others representing silicate influx (Fig. 3.5 d - f). 'Brittlestar density' significantly affected silicate flux, with the higher density treatments increasing the efflux of silicate out of the sediment. There was also an interaction effect between 'water treatment' and 'experimental time', whereby, similarly to ammonium, silicate efflux within hypoxia at T14, in the high brittlestar density treatments increased (Table 3.4 f). Further analyses focusing on silicate flux at T14 within the high brittlestar treatments (13, 17, and 21 indiv. *per* aquaria), revealed that silicate efflux was significantly greater in aquaria exposed to hypoxia compared to the corresponding normoxic treatment (Fig. 3.5 f, Table 3.3 g).
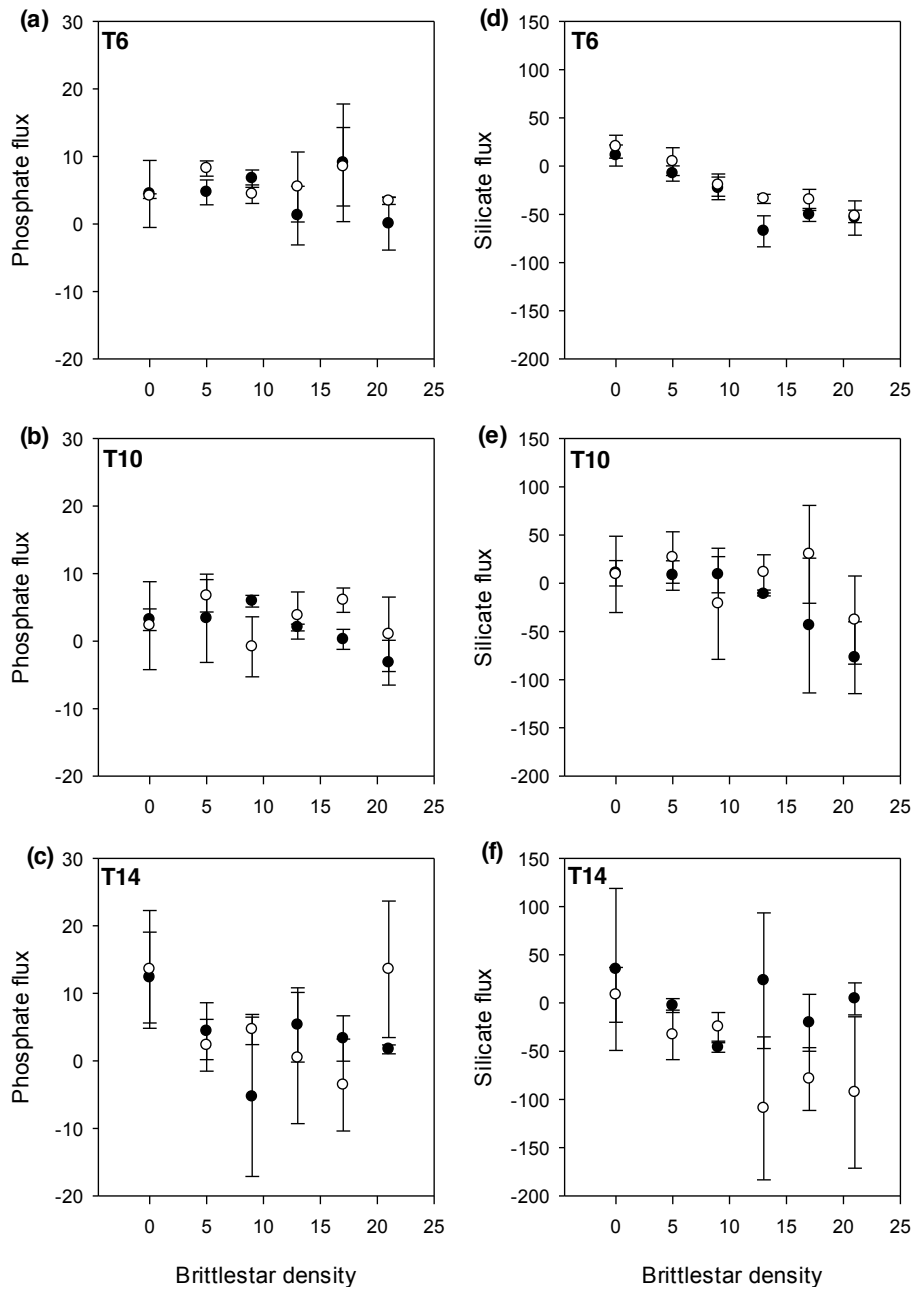
Figure 3.5. Phosphate (PO_4^{3-}) flux (µmol m^{-2} h^{-1}) (a – c); and Silicate (SiO_4^{4-}) flux (µmol m^{-2} h^{-1}) (d – f), in experimental aquaria at time points T6, T10 and T14. Data are means ± 95 % confidence intervals. Positive results represent nutrient influx, whilst negative results represent nutrient efflux, ● = normoxia, ○ = hypoxia.

3.5. DISCUSSION

Exposure of *Amphiura filiformis* to moderate hypoxia for 14 days resulted in reduced brittlestar activity (in terms of sediment surface bioturbation), increased ammonium (NH_4^+) and silicate (SiO_4^{4-}) fluxes and a disruption to NH^{4+}: NO_x ratios when brittlestar densities were high (1300, 1700, and 2100 indiv. m^{-2}). It is possible that the impact of moderate hypoxia on *A. filiformis* was so small, it only became measurable at high densities, or there was an interaction between high density aggregations and low dissolved O_2 that exacerbated the effects of hypoxia, or both. It is difficult to identify which scenario initiated the observed alterations in *A. filiformis* function, but these changes in brittlestar activity and behaviour may represent a vulnerability of this species that has not been captured before.

Chapter 2 and Calder-Potts et al. (2015) document that hypoxia (> 14 d) caused reduced respiration rates and hindered female oocyte growth and development, but brittlestar density had no effect on the physiological parameters measured. It was concluded that during hypoxia, *Amphiura filiformis* may strategically allocate its energy into locomotory arm movements to increase burrow irrigation rates and prevent the build-up of toxins. This conclusion supports the results presented here, with brittlestars in the high density and long incubation treatments potentially increasing burrow irrigation rates, explaining in part, the rise in ammonium and silicate efflux and alterations in sediment surface bioturbation patterns.

3.5.1. Bioturbation of *Amphiura filiformis* under normoxic conditions

Under normoxic conditions, the percentage of sediment surface reworked (% SSR) increased with brittlestar density. Within the normoxic treatment, this relationship between brittlestar density and % SSR appears robust, where at the highest brittlestar density, the highest % SSR was detected, and at the lowest brittlestar density, the lowest % SSR was detected. In normoxic conditions, brittlestars appeared to continue with routine burrow maintenance, with visible excavated mounds, feeding arms protruding, and all individuals buried within the sediment. Therefore, it is reasonable to assume that each individual (which were similar in size) may have equally contributed to surface sediment bioturbation activities, producing the observed relationship with density. Previous measurements of % SSR by *Amphiura filiformis* from the same location, measured at natural field densities, (214.28 indiv. *per* m^{-2}) ranged between 1 – 27 % (Queirós et al. 2015). This is comparable to the % SSR measurements for our lowest experimental densities of 500 – 1300 indiv. m^{-2}, with a mean of 5 - 27 % SSR respectively. However, due to higher brittlestar densities, these current results represent less sediment surface mixing per individual. Furthermore, experimental time significantly affected the % SSR, with decreased surface mixing, in both water treatments, as the experiment progressed. Although a five day settling period was allocated to allow for burrow creation and acclimation, bioturbation activities may have initially been higher at the start of the experiment if acclimation to experimental conditions took longer than five days. This conclusion is supported by the study reported in Chapter 2, where it was suggested that brittlestars kept within normoxic seawater increased their oxygen uptake rates over the 14 day period as a result of acclimation to experimental conditions. In the context of oxygen

uptake rates, exposure to hypoxia hindered this acclimation process. The effect of experimental time and reduced surface bioturbation rates per individual compared to previous measurements may be due to natural variability, differences in experimental set-ups, i.e. feeding regime, and adjustment to the different density treatments used in this experiment, which were higher than natural field densities.

In laboratory experiments it has previously been observed that once *Amphiura filiformis* buries itself, it can remain within the burrow cavity for weeks or even months, if conditions are favourable (Woodley 1975). Other experiments have shown that *A. filiformis* can exhibit density-dependent migration, moving within and on the sediment to less populated areas, given the space to do so (Rosenberg et al. 1997). It has also been described from observations in a natural population that *A. filiformis* disc chamber placement can occur in alternating patterns of 1 shallow, 1 deep ranging from depths of 2.0 cm to 6.5 cm deep (O'Reilly et al. 2006). Clearly it is difficult to pinpoint the exact effects of being confined within experimental aquaria, but it is likely that experimental procedures limit migratory movements within the sediment and may affect optimal dispersal patterns. However, despite this, it would be expected that bioturbation activities for burrow maintenance and feeding activities would be maintained. During this experiment, food supply was not limited, water velocity within aquaria was low, and brittlestars were contained within a confined space. Therefore, it is also plausible that maximum luminophore depths remained constant because brittlestars remained within their original burrow structures throughout the experiment, sensing conspecifics nearby, reducing migratory movements within, but not necessarily on the sediment.

3.5.2. Bioturbation of *Amphiura filiformis* under hypoxic conditions

When exposed to hypoxia, the relationship between % SSR and brittlestar density appears to be less predictable compared to the reworking rates in the normoxic aquaria. After six days exposure, the highest rates of % SSR did not occur within the highest brittlestar density treatment and after ten days of hypoxic exposure the highest rates of % SSR were observed in aquaria containing the second lowest density treatment (900 indiv. m^{-2}). Within the highest brittlestar density treatments, after the longest incubation, % SSR was 38.06 % less in the hypoxic treatment compared to the equivalent density in normoxic conditions.

Within the hypoxic treatment, brittlestars did remain buried within the sediment for the majority of the experiment, but occasionally sightings of individuals on the sediment surface were observed. Although the experiment was not monitored during darkness hours, it is possible that brittlestars within the hypoxic treatment left their burrows and spent time on the sediment surface in search of more favourable conditions, as has been observed in other fauna experiencing hypoxia (Sturdivant et al. 2012). This possible exploration at the sediment surface, in addition to increased bioirrigation rates may have moved and mixed the sediment in a different way to brittlestars within the normoxic treatment. This small shift in behaviour from routine burrow maintenance, as observed in the normoxic aquaria, to potentially, extended periods of burrow irrigation and surface exploration due to hypoxic exposure, may represent the initial 'fine-scale' effects of hypoxia on bioturbation activity and could be a possible explanation for the differences in surface sediment bioturbation patterns. These subtle behavioural changes may eventually affect the

persistence of *Amphiura filiformis'* bioturbation traces and ultimately its ecological performance.

There was no evidence to suggest that moderate hypoxia affected the burrow depths of *Amphiura filiformis*. If brittlestar (or disc chamber) placement did move closer to the sediment surface during hypoxic exposure, this was not detected with the luminophore imaging techniques used here, most likely as a result of some tracer particles being left in the deeper sediment where the original burrow structures were formed prior to experimentation. Sturdivant et al. (2012) found that during *in situ* experiments within the Rappahannock River, Virginia, severe hypoxia as low as 0.1 mg L^{-1} reduced burrow length, burrow production and burrow depth of macrofaunal communities. Although some brittlestars were occasionally spotted on the sediment surface for brief periods of time, it is possible that the hypoxic treatment level used here was not severe enough to reduce burrow depths.

Results from the process-based bioturbation model are discussed in greater detail within the section 'experimental limitations'.

3.5.3. Cycling of NO_x and NH_4^+ during normoxia

The majority of recycled N released from the sediments to the overlying water is in the form of ammonium (NH_4^+), which is generally regenerated from the decomposition and deamination of organic matter, and then passes from the sediments to the overlying water *via* diffusion or advection (bio-irrigation), where it can be assimilated by phytoplankton (Kemp et al. 1990). Before it escapes the sediments and when oxygen is present, a portion of this ammonium is oxidised

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to nitrate (NO_3^-), a process known as nitrification and can then, in turn, be used by denitrifying bacteria (Kemp et al. 1990).

In our experiment NO_x fluxed into the sediment, whilst ammonium consistently fluxed out of the sediment. This suggests that rates of within sediment nitrification were not sufficient to supply all the necessary nitrite (NO_2^-) and nitrate required for other processes such as denitrification and anammox. Previous studies using *Amphiura filiformis* and sediment from nearby sites within Plymouth Sound have documented the same basic sediment nutrient sink / source properties as this study, with the sediment acting as a source of ammonium and a sink for NO_x in control conditions (Wood et al. 2009, Murray et al. 2013). When comparing the $NH_4 : NO_x$ ratios it would appear that this undersupply was worse at higher brittlestar densities implying that *A. filiformis* was increasing the production of ammonium more than it stimulated ammonium oxidation processes.

Overall, formal statistical analysis suggested that under normoxic conditions, 'brittlestar density' had no significant effect on NO_x or ammonium fluxes. Other mesocosm studies using similar sediment type and densities of *Amphiura filiformis,* also found that brittlestar density had no significant effects on NO_x or ammonium fluxes when in control conditions (Wood et al. 2009, Murray et al. 2013). It is reasonable to expect ammonium efflux to increase with brittlestar density as excretion products rise and bacterial mineralisation of organic matter could intensify as burrow structures increase in numbers and surface area (Papaspyrou et al. 2005). Bacterial abundance and activity can be 10-fold higher in burrow walls compared to the surrounding environment, aiding

sedimentary processes such as nitrification and denitrification (Papaspyrou et al. 2005, Laverock et al. 2010).

In this experiment, under normoxic conditions, the fluxes of NO_x and ammonium, as well as the $NH_4^+ : NO_x$ ratio, remained relatively stable, with variability in the data only increasing at the end of the longest incubations. It is generally accepted that the most important role of bioturbation in stimulating remineralisation reactions is the introduction of oxygen into subsurface sediments (Kristensen & Kostka 2005). Consequently, it is possible that *Amphiura filiformis* is stimulating NH_4^+ oxidation processes, such as nitrification and denitrification, but, as previously discussed, not initially at a rate sufficient to totally keep pace with the increase in ammonium. This leads to a slight rise in both ammonium flux and the $NH_4^+ : NO_x$ ratio when brittlestar density increased.

After 14 days, the $NH^{4+} : NO_x$ ratios were not following a linear trend with *Amphiura filiformis* density and were noticeably lower at high brittlestar densities. This suggests that the discrepancy between ammonium production and ammonium oxidation is falling. One explanation might be because the current experiment used sieved homogenised sediment and that this will have disrupted established microbial communities. It is possible that, after 14 days experimental exposure, the natural burrow wall microflora are finally re-establishing to near natural conditions and starting to stimulate ammonium oxidation processes in the *A. filiformis* burrows. It would therefore be preferential for future such studies with *A. filiformis,* to allow a longer period of establishment within the mesocosm before exposure experiments are conducted. Furthermore, by only measuring net fluxes it is highly likely that

large changes in both those processes that produce and remove ammonium and NO_x, were detected, causing no net effect on fluxes to be observed. In future studies, more targeted sampling of specific N cycling processes, coupled with microbial functional group analysis, would be of great value.

3.5.4. Cycling of NO_x and NH_4^+ during hypoxia

Within the longest incubations, when brittlestar densities were high (1300 – 2100 indiv. m^{-2}), ammonium efflux in hypoxia was significantly greater than the control. This supports previous work by Villnäs et al. (2012) where an increase in the duration of hypoxic exposure significantly increased the efflux of ammonium. Whilst it is difficult to separate out how hypoxia, bioturbation, excretion and bacterial remineralization are independently affecting ammonium fluxes, results from Villnäs et al. (2012) highlight how benthic abundance and biomass were of importance. Therefore, in this experiment it is probable that ammonium from excretion processes and through increased bio-irrigation of burrow structures, enhanced the advection of ammonium into the overlying water. Additionally, it is likely that those microbial processes responsible for ammonium removal (i.e. nitrification) cannot keep pace with processes of hypoxic ammonium generation, especially at high brittlestar density treatments, and thereby cannot maintain the balance seen under normoxic conditions. Therefore, moderate hypoxia may have indirect consequences to sedimentary microbial processes and nutrient cycling through changes in organism behaviour that affect the system's ability to remove excess ammonium.

3.5.5. Cycling of phosphate and silicate during normoxia

In oxygenated conditions, and oxidised areas, such as burrow walls, phosphate (PO_4^{3-}) sorption onto insoluble iron-manganese compounds can readily occur, resulting in phosphate influx into the sediment. The capacity of this process is determined by the supply of Fe III in the sediment, with macrofaunal activities increasing the amount of oxidised surface area available for phosphate accumulation (Karlson et al. 2007). During this experiment, phosphate primarily fluxed into the sediment, with experimental parameters having no effect. Previous laboratory experiments using *Amphiura filiformis* and sediment from Plymouth Sound have reported contradictory results. Wood et al (2008) found brittlestar density significantly increased sediment uptake of phosphate, whilst Murray et al (2013) found no significant effects on phosphate flux when *A. filiformis* was present compared to aquaria with no macrofauna. It is likely that, given the high degree of variability within the phosphate flux results, statistically significant outcomes were unlikely.

Silicate (SiO_4^{4-}) fluxes are thought to be a balance between oxic precipitations into the sediment and excretion of silicate rich waste from infauna and diatom decomposition. Infaunal bioturbation activities contribute to nutrient fluxes through promotion of an oxidised environment within the sediment adjacent to burrows within which, compound oxidation may occur. In this experiment, the majority of aquaria exhibited silicate efflux, representing silicate regeneration into the water column, but at T10 some measurements indicated influx of silicate, possibly explained by microalgal uptake or adsorption processes at the sediment-water interface (Bartoli et al. 2009). In this experiment brittlestar density significantly increased silicate efflux, possibly due to increased

mobilisation of silicate from porewaters (Bartoli et al. 2009). Previous mesocosm experiments failed to detect a significant effect of *Amphiura filiformis* on silicate flux (Wood et al. 2009). However, discrepancies between experiments is possible due to variability in the amount of organic matter and the degradation of benthic diatoms within sediments (Villnäs et al. 2012).

3.5.6. Cycling of phosphate and silicate during hypoxia

In hypoxic conditions iron-bound phosphate released into the pore-water as Fe(III) is reduced to Fe(II), causing efflux of phosphate (Belias et al. 2007). However, in this experiment, phosphate generally fluxed into the sediment, with 'water treatment' having no effect. During the experiment, oxygen was limited but not unavailable (i.e. anoxic). It may be reasonable to assume that with some oxygen still present in the hypoxic treatment, phosphate adsorption onto ferric iron still occurred. However, Villnäs et al. (2012) did not observe an increase in phosphate efflux from sediments exposed to hypoxia, and concluded that it was likely to be due to the low content of phosphate in the sediment. This may be true for this experiment, and could mask any potential effects of hypoxia and brittlestars. Unfortunately, no analyses for dissolved and particulate phosphate in the sediments used here were carried out.

Similarly to the case with ammonium, prolonged exposure to hypoxia, in addition to high brittlestar densities (1300 – 2100 indiv. m^{-2}), resulted in increased silicate efflux compared to the normoxic aquaria. In support of these results, previous studies have also documented a rise in silicate efflux during prolonged hypoxia (Villnäs et al. 2012). It is likely that a combination of bioturbation and bioirrigation activities, degradation of benthic diatoms, and

release of silicate from surfaces of hydrated oxides of iron due to reduced oxic precipitation into the sediments, contributed to the silicate efflux results observed here (Villnäs et al. 2012).

3.5.7. Experimental limitations

3.5.7.1. Changes in header tank nutrient concentrations

Reductions of nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations occurred within the hypoxic header tank after 10 d (NO_3^-) and 14 d (PO_4^{3-}) experimental exposure, despite both header tanks receiving filtered seawater from the same source. Unfortunately, at the time of experimentation samples to test for microbial growth within the header tanks and aquaria were not taken. However, despite the reduction in nitrate levels within the hypoxic header tank, the hypoxic NO_x flux and NH_4^+ : NO_x ratio values can be evaluated with confidence for several reasons: (1) There is no difference in NO_x fluxes between the hypoxic and normoxic experimental aquaria at T10 (Fig. 3.5 b), indicating that the sedimentary processes occurring within the experimental aquaria are not significantly affected by the differences in header tank concentrations; (2) The differences in NO_x fluxes at T14 (Fig. 3.5 c) are caused by an increase in NO_x flux in the normoxic aquaria, not a reduction in NO_x within the hypoxic aquaria compared to previous time points; (3) the similarity in NH_4^+ : NO_x ratios from T6 to T10 in both water treatments, indicate that the processes occurring within each experimental aquarium are comparable and similar, despite the reduction in nitrate concentrations in the hypoxic header tank at T10. The reduction in phosphate concentrations within the hypoxic header tank at T14 did not cause any significant effects to the aquaria flux results, and the high levels of

variability in phosphate flux data occurred within both the normoxic and hypoxic treatments.

3.5.7.2. Process-based modelling of bioturbation

It is beyond the scope of this chapter and thesis to evaluate the open source simulation model for sediment bioturbation from Schiffers et al. (2011), but feedback regarding model use, output accuracy and limitations are beneficial to researchers in this field. Although there was a significant effect of 'experimental time' on the estimated parameter '*distance*' (cm), which may correspond to the experimental time effects observed in the % SSR data (discussed earlier), these results demonstrate that the simulation results obtained here have severely under-represented actual particle movement and conclude that the experimental design has pushed the simulation model to its operational limits. We believe this to be true for a number of reasons: (1) the simulated tracer profile graphs produced during the model run show limited tracer particle movement compared to the actual data, even after many interactions to push parameter space to deeper mixing through the model user routines; (2) only 6 photos *per* aquaria were captured, resulting in 6 time points for parameter estimation. In previous applications, the number of data points has been significantly higher; (3) images for each aquarium were taken 24 hours apart, which compared to previous applications of the model, is a much longer time-step; (4) focusing on the top 2 cm of sediment did not exclude a large part of burrow dynamics and bioturbation activity, because in all aquaria, over 80 % of luminophores remained in the top 2 cm of the sediment, strengthening our argument that the experimental set up was the limiting factor; (5) When comparing the model output ('*distance*' (cm)) to previously published data, the

results obtained in this experiment are considerably different. For example: at T0, density 5 (equivalent of 500 indiv. m^{-2}), distance (cm h^{-1}) = 0.000379. For density 21 (equivalent of 2100 indiv. m^{-2}), distance (cm h^{-1}) = 0.00157. Results from Queirós et al. (2015) for *Amphiura filiformis*, at a density of 208 indiv. m^{-2} were 0.014 cm h^{-1} (for ease of comparison, the results have been standardised to distance travelled in one hour, not per time-step). However, many reasons could cause discrepancies between the model outputs from different experiments such as type of food, rate of feeding and water flow within the aquaria, so although no direct comparison can be made, our results are still below previous values.

3.5.8. Ecological effects and conclusions

Moderate hypoxia may not cause an immediate loss in biodiversity and species richness compared to severe hypoxic and anoxic events, but it may initiate alterations in organism physiology and behaviour that have the potential to impede ecosystem function. We have demonstrated how population density plays an important role in determining the negative impacts of hypoxia, as dense patches of *Amphiura filiformis* may actually exhibit larger changes in behaviour and shifts in ecosystem function, compared to sparse patches, as competition for oxygen and resources heighten, and oxygen diffusion into the sediment reduces. Furthermore, the duration of a hypoxic event will also be important in determining the individual and community effects, as different species have varying thresholds and sensitivities to decreased oxygen concentrations. In the current study, and previous work (Chapter 2 and Calder-Potts et al. 2015) *A. filiformis* exhibited an initial tolerance to hypoxia, with significant effects only occurring after 14 days exposure. Furthermore, the

results presented in Chapter 2, were consistent with the view that *A. filiformis* is an 'oxyconformer', reducing its metabolic rate with declining pO_2 (partial pressure of O_2). However, when oxygen is still available, 'oxyconformers' can be behavioural 'oxyregulators', attempting to maintain oxygen levels in their burrows or body fluids constant through compensatory adjustments in ventilatory efforts, such as burrow irrigation (Pörtner 2010). This concept supports our conclusions that after prolonged hypoxic conditions, *A. filiformis* may have increased burrow irrigation rates, in an attempt to maintain oxygen levels within the burrow, in addition to avoiding toxin build-up. This subtle change in brittlestar behaviour altered sediment surface bioturbation patterns, and increased the efflux of ammonium and silicate, possibly reducing nitrification rates. In areas where persistent hypoxia and reduced oxygen diffusion into the sediments occur, inhibition of nitrification, and the subsequent decrease in denitrification, could result in a build-up of nitrogen and further unpredictable eutrophication phenomena (Huesemann et al. 2002), which would inhibit an area's ability to recover and rehabilitate, further causing a loss of biodiversity and functionality.

CHAPTER 4: IMPACT OF HYPOXIA ON BENTHIC COMMUNITY STRUCTURE AND DIVERSITY

Assessing the sensitivity and community response (in terms of structure and diversity) of a typical U.K. benthic community to moderate hypoxic stress.

4.1. INTRODUCTION

Coastal ecosystems are characteristically complex, both ecologically and physically, and are among the most productive and diverse areas on Earth (Mann 1988, Struyf et al. 2004, Meadows et al. 2012). They encompass a broad range of habitat types and sustain a unique wealth of Biodiversity, providing resources and services that are vital for human health and survival (Burke et al. 2001). Consequently, healthy and productive marine ecosystems are a unique life support system for marine life and humans alike, where any degradation in environmental status is recognised as having far-reaching effects (IPCC 2007, Diaz & Rosenberg 2008). Consequently, there is considerable interest in understanding how Biodiversity loss might alter ecological processes vital to the functioning of ecosystems (Cardinale et al. 2000). Of particular concern and of global importance is the increasing frequency and intensity of the depletion of oxygen in the bottom waters of coastal systems, referred to as hypoxia, and its effects on the infaunal benthic community (Vaquer-Sunyer & Duarte 2008, Meire et al. 2013). Changes in climate and increased nutrient loadings in coastal areas are two aspects of global change that are predicted to profoundly impact the manifestation of coastal hypoxia (Meire et al. 2012) presumably with consequences for infaunal community and diversity dynamics.

Marine soft sediment habitats contribute to intense nutrient cycling, primary production and carbon fixation, with benthic infauna playing an important role in mediating these processes (Canfield & Farquhar 2009, Boyle et al. 2014). Carbon and nitrogen cycling, oxygen, pH and redox gradients, sediment porosity and permeability, as well as microbial activity are all influenced by the diversity, abundance and functional traits of benthic infauna (Herbert 1999,

Laverock et al. 2010, Bertics et al. 2013). Understanding organismal and community responses to hypoxia is, therefore, an important 'first-step' in our knowledge of how these changes may alter the ecological processes that drive ecosystem function. However, organism response to hypoxia is species-specific and dependent on the duration and severity of the event. Furthermore, community response to hypoxia is not a simple 'summation' of isolated effects expressed at the individual level.

Individual responses (physiological, behavioural and ecological) are connected through interactions with other species at the same or adjacent trophic levels (Walther 2010). For example, behavioural responses to hypoxia include shallowing of burrow depths, elongated siphons, and altered locomotion, which may alter predator-prey relationships and food-finding behaviour (Boyd et al. 2002, Sturdivant et al. 2012). Subsequently, behavioural changes as a result of hypoxia can have direct and indirect consequences for individual fitness with cascading responses across several levels of biological organisation, including effects to community composition and ecosystem function (Solan et al. 2004a, Zhang et al. 2010, Riedel et al. 2014). Therefore, the community and ecosystem level response to hypoxia will depend on complex reactions linked to what species are lost, how community degradation patterns occur, the functional traits that are impaired, and how these changes impact species interactions, behaviours and functionality. Furthermore, there is evidence that species loss or extinction due to disturbance is a non-random process, which is in-part, determined by life-history traits such as rarity, body size and sensitivity, in addition to species interactions and behaviour as mentioned above (Solan et al. 2004a).

Ultimately, the benthic response to hypoxia is complex and nested within many interacting factors. Therefore, predicting how a community will respond to disturbance such as hypoxia, using successional models and ecological theory is limited due to numerous site-specific parameters that will influence community response and recovery. Consequently, both field and laboratory experiments on specific communities are required to investigate how these communities respond to disturbance and to determine how resilient they are.

Station L4 is one of the main sites of the Western Channel Observatory (WCO), located approximately 10 km southwest of Plymouth, U.K., and is part of a long-standing oceanographic time-series and marine Biodiversity reference site (http://www.westernchannelobservatory.org.uk). Although the WCO represents one of the best studied marine regions in Europe, with a rich dataset derived from both environmental and biological samples, no information specific to this area currently documents L4 benthic community degradation patterns in response to hypoxia.

Station L4 is classified as a coastal site, often characterised by summer thermal stratification and nutrient depletion, but intense summer precipitation can increase riverine inputs into the system, resulting in pulses of increased nitrate concentrations and surface water freshening (Smyth et al. 2010). The L4 site does not currently experience regular severe hypoxic events, preventing established resilience to hypoxia within the benthic community, but fluctuations in oxygen concentrations do occur, often following the phytoplankton spring bloom (Zhang et al. 2015). In 2013, the year this investigation was conducted, levels of dissolved oxygen (DO) throughout the water column at L4 varied from

5.99 mg O_2 L^{-1} to 10.58 mg O_2 L^{-1} measured from January through to December as part of the Western Channel Observatory monitoring program. The lowest observed DO levels occurred during July and August and correspond to the occurrence of the summer thermocline, but were not considered hypoxic. In 2012, an unusually large and long-lived spring bloom occurred which was approximately 3x greater and 50 % longer than any previous year (Zhang et al. 2015). Despite this increase in organic matter reaching the benthos and although fluctuations in DO were observed, hypoxia was not detected and DO levels did not fall below 6.02 mg O_2 L^{-1}.

However, future predictions for the severity and occurrence of marine hypoxia are expected worsen, in both coastal regions and the open ocean (Diaz & Rosenberg 2011), with higher global temperatures expected to directly lower oxygen concentrations and enhance stratification (Gnanadesikan et al. 2011). Given its coastal location, summer stratification regimes and potential influences from riverine inputs, Station L4 may be vulnerable to future changes in DO associated with global change and anthropogenic activities. Whilst severe hypoxia (< 2.0 mg O_2 L^{-1}) can cause mortality, the non-lethal impacts of reduced DO to levels above this limit on nearshore communities is not well understood, despite the importance of these habitats for numerous species and humans alike (Froehlich et al. 2015).

Consequently, the aim of this chapter is to document how the benthic assemblage present at L4 responds to changes in DO. The work is conducted within the laboratory but results are also interpreted in light of field samples collected concurrently at L4. These field samples will experience the effects of

the spring bloom, and the changing patterns of oxygenation overlying the station *in situ* during the same time period.

The community's sensitivity to hypoxia will be assessed using replicate intact sediment cores containing the natural benthic community from site L4 translocated to a laboratory mesocosm facility. At the same time key aspects of the ecology of the assemblage, together with the measurement of some physio-chemical characteristics of the overlying water mass will be monitored for comparison. A null hypothesis was proposed, namely that exposure to hypoxia for 3 and 6 weeks will have no effect on the community structure, diversity and biomass of the L4 benthic macrofaunal community in the laboratory. In doing so, this is the first study of its kind to experimentally examine the effects of prolonged and moderate hypoxia on the L4 benthic community and will provide a benchmark of community resilience for the future, should Station L4 become affected by more serious hypoxic events.

4.2. MATERIALS AND METHODS

4.2.1. Sediment and fauna collection

On the 24th and 25th June 2013 sediment cores containing *in-situ* macro and meio-fauna communities were collected from Station L4 (50° 13.30'N, 4° 11.40'W) a mixed shell gravel site, ~ 10 km from Plymouth, U.K., using a 0.1 m^2 US-NL box-corer. The overlying water captured with each core was carefully drained, and sediment was immediately sub-sampled into clear acrylic aquaria (0.2 m x 0.2 m x 0.4 m) using a square 0.04 m^2 steel corer that tightly fitted into the aquaria tanks, preserving the sediment structure. Each aquarium was then gently filled with fresh unfiltered seawater (T = 12.32 °C, S = 35.02), loosely

covered with a black PVC tarpaulin, and placed in large containers surrounded by fresh locally collected seawater to keep them at ambient seawater temperature during transport back to Plymouth Marine Laboratory (PML) which took approximately 1 h. On arrival to PML, temperature of the overlaying water was checked and was typically within 1 °C of local seawater temperatures. Each aquarium contained sediment to a depth of 17.75 ± 1.25 cm, overlaid by approximately 8.90 L of seawater. Only one sub-core was taken per 0.1 m^2 box-core, with a total of 30 aquaria filled and transported back to PML over the two day sampling period. Of the 15 cores collected on the 24th June, ten were randomly placed within the experimental set up and the remaining five were prepared for immediate community analysis. These five samples will hereafter be known as 'initial fields' and represent the natural community structure at L4 at the time our experimental cores were collected. All 15 cores collected on the 25th June were randomly placed within the experimental system upon arrival to the mesocosm facility.
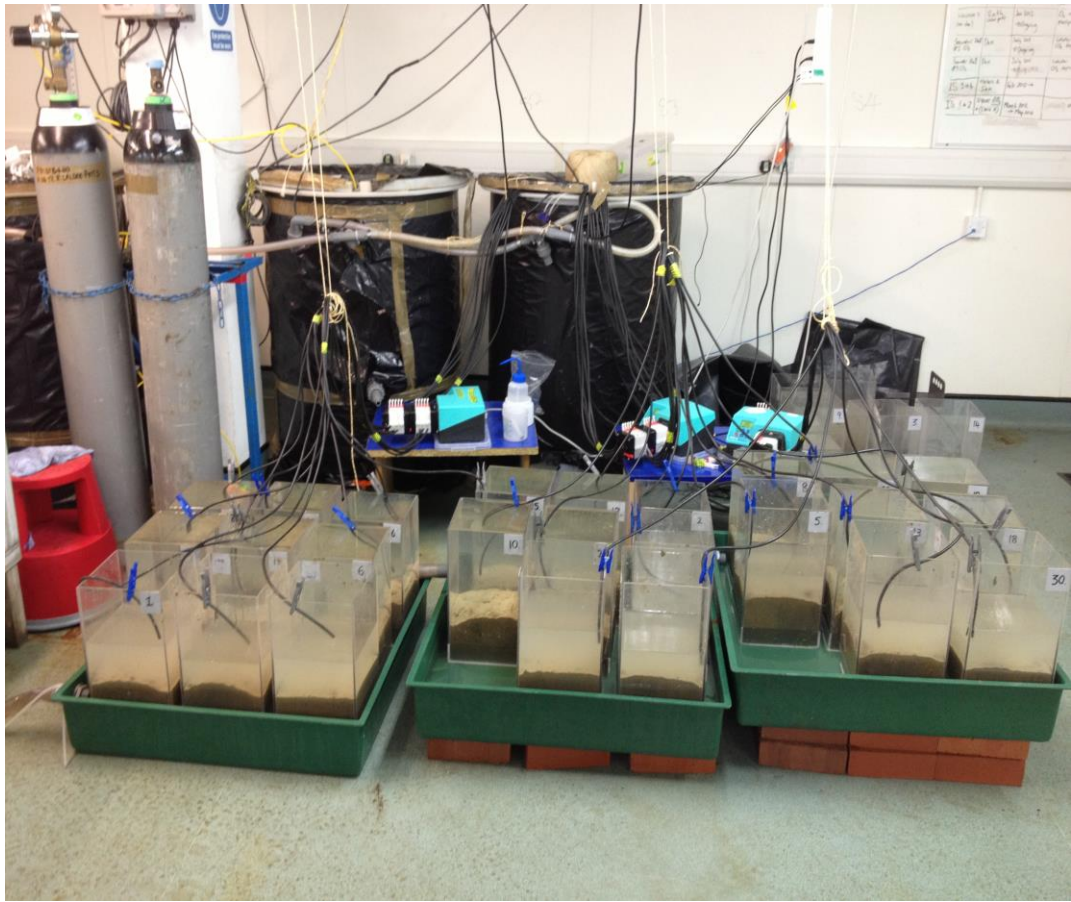
Figure 4.1. Image of the experimental set up, with header tanks, peristaltic pumps and experimental aquaria.

4.2.2. Experimental design and holding conditions

The mesocosm experiment consisted of 25 aquaria, all of which underwent a settling time of 6 – 7 d (depending on collection date) prior to experimental exposures (Fig. 4.1). On arrival at the mesocosm facility aquaria were connected to a flow-through seawater system that delivered aerated, twice filtered (10 µm and 1 µm diam. Hydrex carbon block filters) seawater from a 450 L header tank (DO = 7.32 ± 0.061 mg O_2 L^{-1}, S = 35.15 ± 0.046, T = 13.80 ± 0.10 °C, pH = 8.07 ± 0.012, means ± 95 % C.I.) *via* a peristaltic pump (323E, Watson Marlow, Falmouth, U.K.) at a rate of 18 ± 0.5 mL min^{-1}. Aquaria water temperature was typically within 1 °C of the mean field temperatures during

June, July and August. After the 6 – 7 d acclimation period, five aquaria were randomly selected and removed from the experimental set up for analysis. These samples are hereafter referred to as 'T0' samples. At the same time, 10 of the remaining 20 aquaria within the experimental set up were randomly selected and their inlet pipes were switched to the hypoxic header tank (2.61 ± 0.039 mg O_2 L^{-1}), this marked the beginning of the experimental exposures. At time point 'T3' (3 wks. experimental exposure), ten aquaria (five from each water treatment) were randomly selected and removed for analysis, these samples will henceforth be known as 'T3 normoxic' and 'T3 hypoxic'. The remaining aquaria were removed from the experiment at time point 'T6' (6 wks. experimental exposure), henceforth known as 'T6 normoxic' and 'T6 hypoxic'.

On the days prior to the experimental time points T3 and T6, five additional cores were collected from the same field location (Station L4), using identical methods as previously described. These samples underwent immediate analysis upon their arrival to the PML mesocosm facility and are hereafter referred to as 'T3 fields' and 'T6 fields'. Field samples were collected during the experimental timeframe to allow comparisons between the natural field community and the aquaria exposed to normoxic seawater, giving indication of any potential 'experimental effects' and also to assess if there was any natural variations in community structure whilst the experiment was in progress.

4.2.3. Seawater manipulations

Within the mesocosm, hypoxic and normoxic seawaters were held in two separate header tanks (vol. = 450 L each). To produce hypoxic seawater, DO was modified using a computerised control system (Walchem Webmaster

Series, Holliston, MA, U.S.A), which regulated the addition of oxygen-free nitrogen gas. Each header tank was connected to its respective experimental aquaria, *via* a peristaltic pump, which supplied a constant supply of seawater. Full details of this system are documented in Sect. 2.2.4 and in Calder-Potts et al. (2015). The seawater within both header tanks and the experimental aquaria were monitored daily throughout the experiment for DO, temperature, salinity and pH (Table 4.1) using a Multiprobe (556MPS, YSI, Yellow Springs, OH, U.S.A.).

Table 4.1. Summary of seawater treatment conditions throughout the experiment. Values are means ± 95 % confidence intervals and the range of values is given in brackets (min – max). L4 field data is taken from the Western Channel Observatory (http://www.westernchannelobservatory.org.uk), measured between June, July and August 2013, at a depth of 50 – 53.5 m. Dissolved oxygen (DO) measurements are corrected for atmospheric pressure and temperature.

	Normoxic header	Normoxic aquaria	Hypoxic header	Hypoxic aquaria	L4 field conditions
DO (mg L^{-1})	7.06 ± 0.16 (6.37 – 7.71)	7.32 ± 0.061 (6.46 – 8.18)	2.61 ± 0.039 (2.44 – 2.75)	3.09 ± 0.03 (2.37 – 3.41)	7.67 ± 0.11 (6.87 – 10.83)
DO (%)	85.93 ± 2.01 (77.10 – 93.74)	88.52 ± 0.74 (78.08 – 98.92)	31.50 ± 0.47 (29.45 – 33.33)	37.45 ± 0.39 (28.69 – 41.29)	-
Temperature (°C)	13.95 ± 0.15 (13.60 – 14.74)	13.80 ± 0.10 (12.90 – 15.22)	14.29 ± 0.21 (13.50 – 14.99)	13.69 ± 0.091 (12.80 – 15.30)	12.87 ± 0.18 (10.30 – 15.89)
Salinity (ppt)	35.26 ± 0.18 (34.20 – 35.70)	35.15 ± 0.046 (34.66 – 35.83)	35.02 ± 0.17 (34.30 – 35.19)	35.16 ± 0.049 (34.65 – 35.68)	35.18 ± 0.047 (35.12 – 35.25)
pH (NBS)	8.11 ± 0.039 (7.98 – 8.18)	8.07 ± 0.012 (7.91 – 8.23)	8.13 ± 0.030 (7.99 – 8.18)	8.07 ± 0.012 (7.91 – 8.22)	-

4.2.4. Feeding regime

Water in each experimental aquarium was supplied with a daily dilution of a marine microalgae concentrate mix (1800 Instant Algae® Marine Microalgae Concentrated Shellfish Diet, Reed Mariculture, Campbell, CA, U.S.A.), a mixture of *Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii* and *Tetraselmis* sp. (cell count 2 billion mL^{-1}, 8 % dry-mass). Dilutions were prepared on a daily basis to match 20 % of macrofauna dry-mass to surface area (0.04 m^2), an estimate based on L4 community biomass data for the corresponding time of year between 2008 – 2013, (Dashfield & McNeill 2014), and as *per* Queirós et al. (2015) and Ricciardi & Bourget (1998).

Feeding began at the start of the mesocosm settling time and was repeated daily for the duration of the experiment. Algae was delivered *via* syringe and released into the water approximately 2 cm above the sediment surface and evenly dispersed. Water inlet pipes to each aquarium were fixed at 1.5 ± 0.5 cm above the sediment surface and water flow was ceased for 1 h after the addition of algae.

4.2.5. Macrofauna sampling

On the designated sampling days (T0, T3 or T6) sediment and fauna within the aquariums was carefully extracted and sieved (0.5 mm) in filtered seawater (10 μm diam. Hydrex filters) and placed into individual 10 L storage buckets. Sediment and fauna were placed in 10 % formaldehyde solution and stored until further analysis. Upon removal from storage, samples were thoroughly rinsed in tap water over a 0.5 mm sieve to remove the formaldehyde and the entire sample was elutriated a minimum of 5 times over a 0.5 mm sieve. The

remaining residue was checked for uncaptured macrofauna. If macrofauna was still present in the residue, further elutriations were completed. Extracted macrofauna were stored in 75 % IMS, and were counted and identified to the lowest taxonomic level possible using compound and dissection microscopes (Leica Wild M3Z, (x 65 – 400 magnification), Wetzlar, Germany, and Nikon Optihot-2, (x 100 – 1000 magnification) Tokyo, Japan). Blotted wet mass' for each taxon were recorded to five decimal places using a precision balance (Sartorius R200-D, Göttingen, Germany). Field samples underwent the exact same macrofauna extraction and identification methods upon immediate arrival to the PML mesocosm facilities.

4.2.6. Environmental variables at L4 during 2013

To support interpretation of the macrofaunal community response over time and to provide an environmental context, a number of core environmental parameters from the L4 Western Channel Observatory (WCO) datasets were examined. These data are publically available from the WCO website (http://www.westernchannelobservatory.org.uk) which also contains details of how the data were generated. The parameters of temperature (°C), salinity (psu), oxygen (mg L^{-1}) and chlorophyll a (µg L^{-1}) were plotted using Ocean Data Viewer (ODV) and illustrates each parameter for the year of 2013 through the water column (0 - 52 m).

4.3. STATISTICAL ANALYSES

4.3.1. Analyses of diversity indices

A diversity index is a mathematical measure that represents how many different types of species there are in a given community, simultaneously including

evenness components, and either reflects a dominance or an information statistic index. There are many different diversity indices available, e.g. Shannon's or Simpson's index, as examples. However, the simplest definition of biodiversity is the number of species, or 'species richness' (S) found in an area. Species richness is a simple measure, and assumes all taxa are the same, it is therefore used frequently in the context of biological conservation and ecology (Hengeveld 1996). Consequently, the number of species (S), number of individuals (NI) and Pielou's species evenness (J') were calculated for each community sample using the software PRIMER 6 (ver. 6.1.18). Blotted wet biomass and average body size (calculated using abundance and biomass data) for each community was also analysed. Each parameter underwent tests for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) using the software MINITAB 17 (ver. 17.1.0) and when appropriate, suitable transformations were applied. Using MINTAB, Kruskal-Wallis tests (when data could not be normalised) and one-way ANOVA tests were used to examine the effect of sampling time on the diversity indices for the field samples, and a two-way ANOVA was used to investigate differences between treatment groups (i.e. field, normoxic and hypoxic samples) and the effects of time (T0, T3, T6).

4.3.2. Analyses of community structure

Multivariate analyses were conducted using PRIMER 6 and PERMANOVA + (ver. 6.1.18). Bray-Curtis similarity matrices were constructed for untransformed abundance data and presence / absence transformed data. Analyses using the untransformed data identify changes in the relative abundance of the dominant taxa, whilst the presence / absence transformation down weighs the influence of

very abundant species, and upgrades the influence of rare species, effectively giving equal weight to all species. Permutational multivariate analyses of variance (PERMANOVA) was used to assess differences in community structure and non-metric multi-dimensional scaling (MDS) ordination plots were constructed to help visualise the relative distances apart of samples based on the rank similarity matrix.

4.3.3. Exploratory Analysis

Abundance-Biomass Comparison (ABC) curves were created for the initial field samples and T0 normoxic samples using PRIMER, as a means to assess the levels of biological stress as a result of transplantation into the mesocosm. Species are ranked in order of importance in terms of abundance or biomass on the *x*-axis (logarithmic scale) and percentage dominance on the *y*-axis (cumulative scale) (Clarke & Warwick 2001). In an undisturbed / unstressed marine benthic community, the biomass curve is elevated above the species abundance curve as interspecific competition will lead to *k*-selected species being dominant (biomass is dominated by one or a few large species). When disturbance occurs, smaller *r*-selected (opportunistic) species become dominant in terms of numbers, but do not dominate the biomass because they are small-bodied. This causes a reversal in the ABC curves, with the abundance curve being elevated above the biomass curve (Clarke & Warwick 2001). The *W*-statistic created from the ABC curves is a measure of the difference between the abundance and biomass lines, and ranges from -1 to 1 (Clarke 1990). The closer the *W*-statistic is to -1, the more disturbed the system. Within MINITAB, a t-test was used to assess the differences in the *W*-statistic between the initial field and T0 normoxic samples.

Further exploratory analysis used the SIMPER procedure in PRIMER 6 to assess the dissimilarities between communities from different treatments. It indicates which specific species were most responsible for the dissimilarity between two specific groups.

The average taxonomic distinctness (Δ^+) was calculated for each sample using PRIMER 6 as a supplementary investigation into community diversity. In addition to species abundances, the taxonomic distance through the classification tree between every pair of individuals is incorporated. Taxonomic distinctness is a measure of the degree to which individuals in an assemblage are related to each other (Clarke & Warwick 2001). Average taxonomic distinctness values are displayed in a 95 % probability funnel plot, whereby the funnel is drawn based on species frequency measures from the field samples only and represents the likelihood of communities falling within this expected spread of Δ^+ based on the number of species within each community sample.

As a final measure to investigate the levels of biological stress within each community, a Biotic Coefficient (BC) was calculated for each sample as *per* Borja et al. (2000). Each species was assigned to one of five ecological groups according to their sensitivity to an increasing stress gradient (increasing organic matter enrichment) and the BC calculated based on the percentages of abundance of each ecological group within each sample. 'Group 1' contains very sensitive species which are present under unpolluted conditions, 'group 2' contains species indifferent to enrichment, present in low densities, 'group 3' contains species tolerant to excess organic enrichment, 'group 4' contains

second-order opportunistic species and 'group 5' contains first-order opportunistic species which proliferate in reduced sediments. Where information on specific species was not available, a 'not assigned' status was given.

4.4. RESULTS

The field and experimental communities examined contained fauna from a large number of major taxa including Annelida, Mollusca, Echinodermata, Cnidaria, Sipuncula, Chaetognatha, Nematoda, Nemertea and Phoronida. In total, 281 macrofaunal species categories were identified to the lowest taxonomic category possible, consisting mainly of annelids (150 species categories), arthropods (65 species categories), molluscs (39 species categories) and echinoderms (9 species categories). The Nemertea and Nematoda (identified to phyla only) were also included in the community analyses for all samples.

4.4.1. Environmental conditions at L4 during 2013

The temporal changes in temperature, salinity, oxygen and chlorophyll *a* at L4 during 2013 are shown in Figure 4.2. There was evidence of strong thermal stratification during the summer months of July and August, with the water column becoming less stratified and a more uniform temperature from mid-September onwards (Fig. 4.2 a). At depth (> 40 m) the salinity at L4 generally remained stable from May to October, but there was evidence of reduced surface water salinity, mainly during the cooler months, which is indicative of periods of wet and windy weather (Fig. 4.2 b). During May and June the highest levels of dissolved oxygen were recorded, which corresponds to the highest concentrations of chlorophyll *a*, representing the spring bloom (Fig. 4.1 c, d). From mid-July to September there was evidence of slightly reduced dissolved

oxygen levels at depths that occurred below the thermocline (Fig. 4.2 a, c). Chlorophyll *a* concentrations peaked between May and June, and were detected throughout the water column, but especially at depth (30 – 50 m). This indicates that the organic material associated with the spring bloom was rapidly mixed within the water column reaching the benthos, providing a valuable food source for benthic fauna. In July and August, at depth (40 – 52 m), chlorophyll *a* concentrations rapidly decreased, possibly indicating swift remineralisation of the spring bloom organic matter, and a reduced food supply for benthic organisms from this time onwards.

Field samples were collected from the L4 site on the 24th June, 25th July and the 22nd August 2013. Consequently, the 'initial field' (24th June) samples were collected prior to the July-August formation of the thermocline, and at the tail-end of the May-June spring bloom, when oxygen concentrations were approximately 8.0 mg O_2 L^{-1}. The 'T3 field' (25th July) samples were collected when the thermocline was strong, and chlorophyll *a* concentrations were low, with oxygen concentrations approximately 7.8 mg O_2 L^{-1}. The 'T6 field' (22nd August) samples were also collected during the summer thermocline, when oxygen (6.91 mg O_2 L^{-1}) and chlorophyll *a* concentrations were at their lowest. Community samples collected for use in the mesocosm were taken from L4 on the 24th and 25th June 2013. As a result, all mesocosm samples were collected just before the July – August thermocline formed, and at the tail-end of the May-June spring bloom, a period when food supply to the benthos was at its maximum.
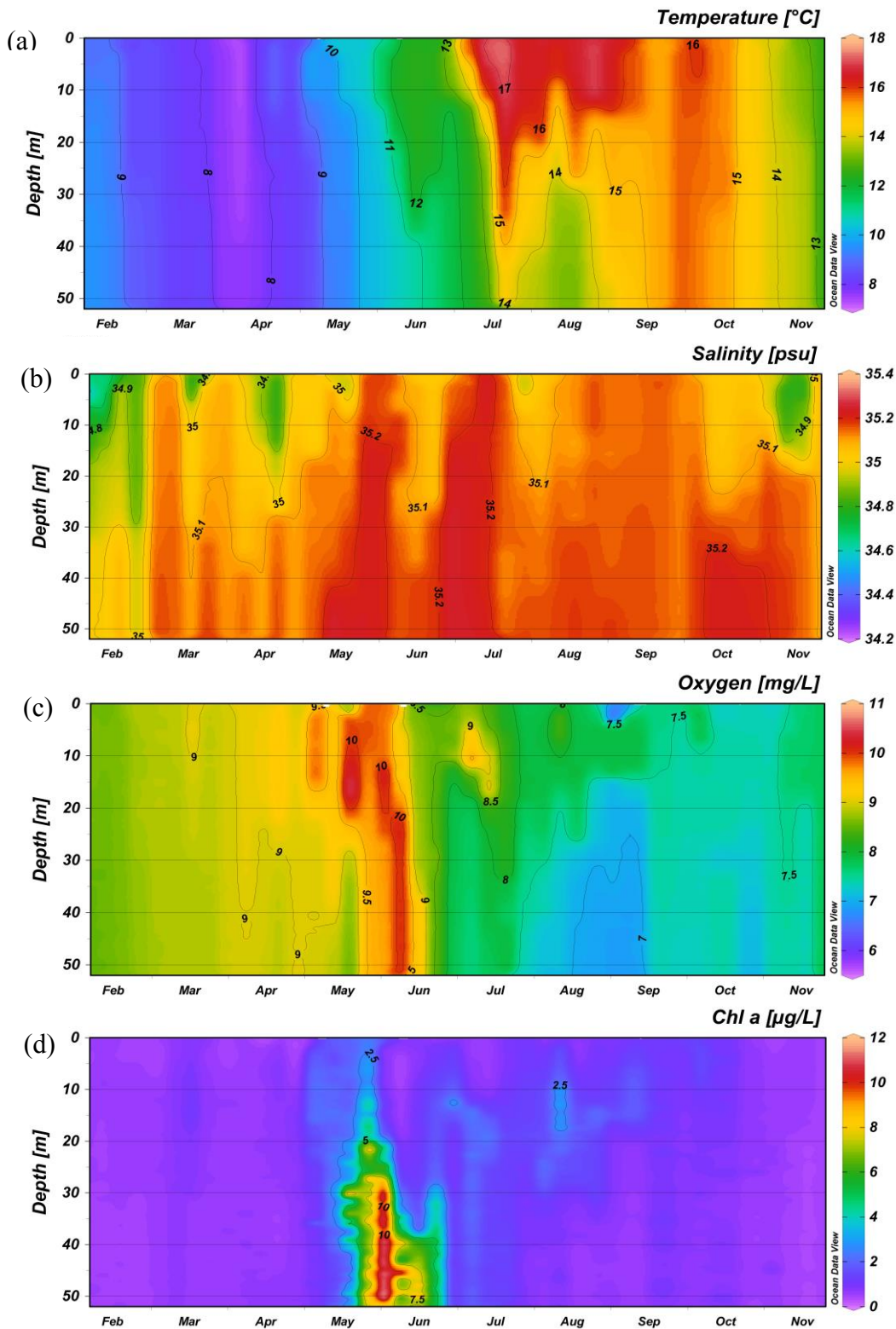
Figure 4.2. ODV plot showing key water column environmental variables at L4 during 2013: Temperature (a); salinity (b); oxygen concentration (c); and chlorophyll *a* concentration (d). Data obtained from the Western Channel Observatory (http://www.westernchannelobservatory.org.uk).

4.4.2. L4 field samples

One-way ANOVA and Kruskal-Wallis tests (when data could not be normalised) both showed there were no significant effects of sampling time ('initial fields', 'T3 fields' and 'T6 fields') on the total number of species (DF = 2, n = 15, H = 0.74, p = 0.692), number of individuals ($F_{(DF = 14)}$ = 0.70, p = 0.518), species evenness ($F_{(DF = 14)}$ = 0.27, p = 0.765), community biomass ($F_{(DF = 14)}$ = 0.50, p = 0.621) and average body size (DF = 2, n = 15, H = 1.52, p = 0.468) in the communities sampled directly from the field (Fig. 4.3). However, PERMANOVA analyses revealed that the species abundance community structure data and the transformed presence / absence community structure data from the L4 field samples changed significantly with sampling time (Table 4.2). In support of these results the multi-dimensional scaling (MDS) plots (Fig. 4.4) show groupings of the samples collected at different time points. Samples collected at the end of the experiment ('T6 fields') show a much tighter clustering, indicating greater similarity and less variability, compared to the samples collected at the beginning of the experiment ('initial fields') that are not tightly grouped, indicating greater variability.
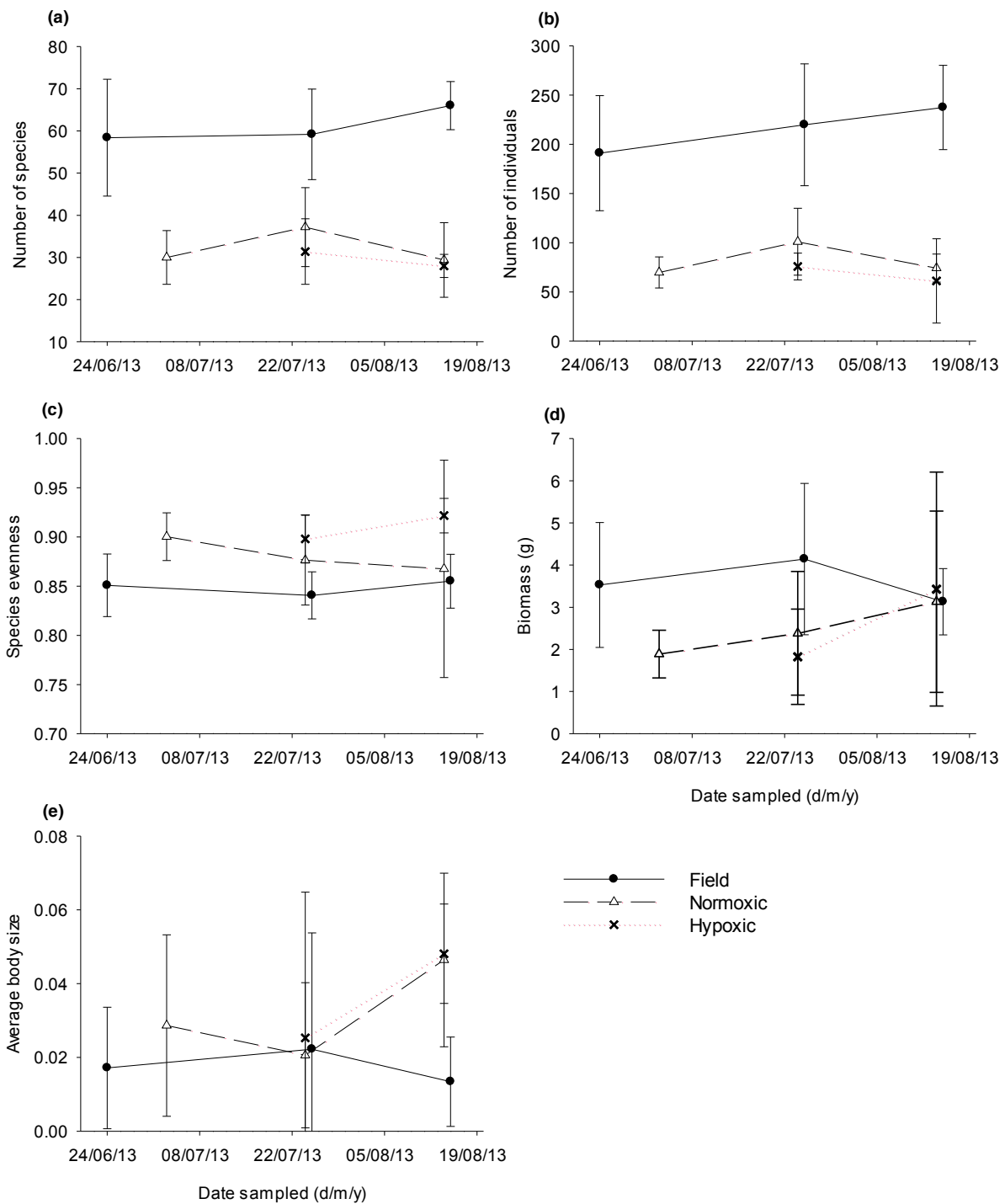
Figure 4.3. The number of species (a); number of individuals (b); species evenness (c); biomass (d); and average body size (e), for communities in the initial fields, T3 and T6 fields, T0, T3 and T6 normoxic and T3 and T6 hypoxic aquaria. Data points are mean values for communities exposed to the same experimental conditions ± 95 % confidence intervals.

Table 4.2. Permutational multivariate analyses of variance of the effects of one fixed factor, sampling time (three levels: 'field initials', 'T3 fields' and 'T6 fields') on the untransformed (a); and the transformed presence / absence (b), community structure data. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), the pseudo F-value (Pseudo-F), the permutational probability value (p(perm)) and the number of permutations of residuals carried out (Unique perms). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	Pseudo-F	p(perm)	Unique perms
(a) Species abundance community structure data						
Time	2	7015.50	3507.70	2.47	0.001*	992
Residual	12	17025.00	1418.70			
Total	14	24040.00				
(b) Presence / absence community structure data						
Time	2	4642.30	2321.20	2.00	0.002*	993
Residual	12	12.00	13910.00	1159.10		
Total	14	14.00	18552.00			
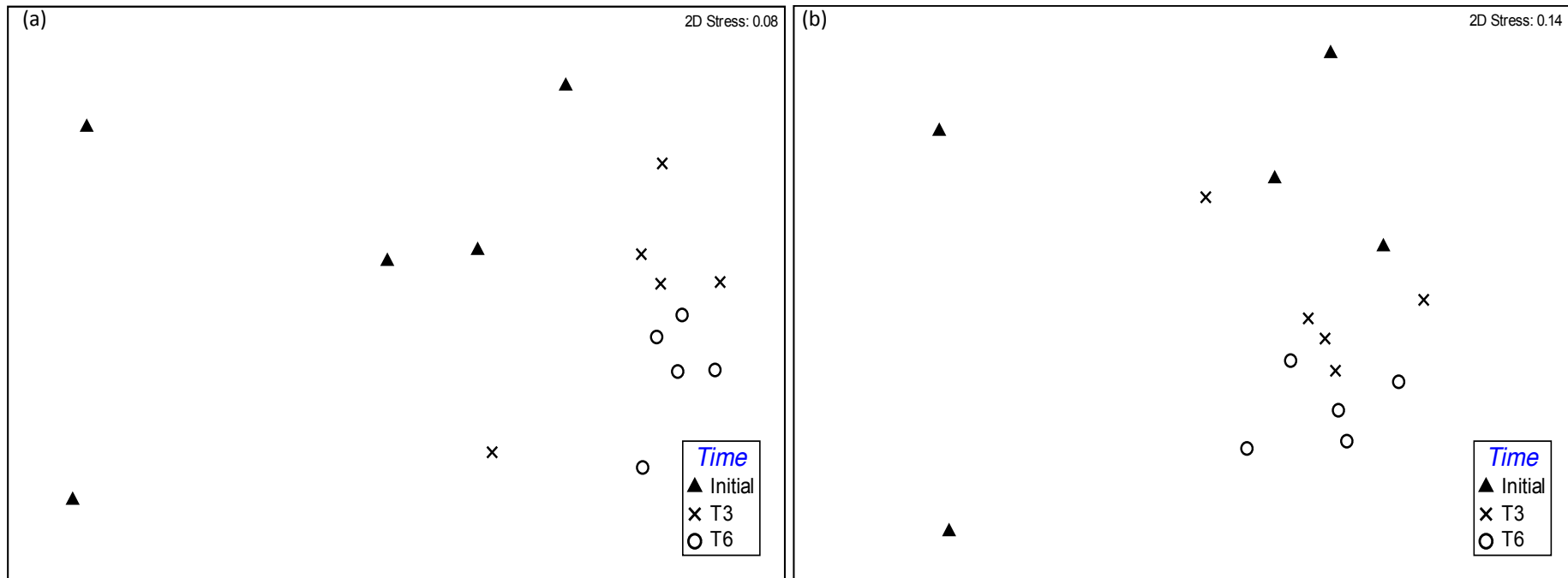
Figure 4.4. Non-metric multi-dimensional scaling (MDS) ordination plots for the Bray-Curtis similarity untransformed species abundance community structure data (a); and the transformed presence / absence community structure data (b), for samples collected from the field (site L4) at different time points (Initial, T3, T6).

4.4.3. Mesocosm effects

Both the number of species and the number of individuals were significantly and consistently higher in the field samples compared to the normoxic experimental samples with sampling time having no effect on each diversity parameter (Fig. 4.3 a, b, Table 4.3 a, b, c). This is seen in Figure 4.3 a and b, where the reduction in the number of species and individuals within the mesocosm aquaria occurred during the first 7 days of being transferred to the mesocosm, with subsequent measures of species numbers and individuals remaining relatively constant. There were no significant differences in species evenness, biomass and average body size between the field samples and normoxic experimental samples (Fig. 4.3 c, d, e, Table 4.3 c, d, e).

There was an average decrease of 47.38 % in the number of species found within the normoxic experimental samples, and a 62.21 % decrease in the number of individuals, compared to the field samples respectively. These reductions in species numbers and individuals within the mesocosm aquaria may be a factor of being confined within a system that is closed to immigration or emigration (excluding death and consumption) whereas the field samples would be open to immigration or recolonization possible. The discrepancy between these data raises questions relating to the experimental effects of transfer to mesocosm conditions. Consequently, the differences between the 'initial fields' and 'T0 normoxic' samples were examined to determine the effects of bringing natural community samples into the laboratory, and to separate out the effects of transplantation into the mesocosm and the effects of hypoxia *per se*.

Table 4.3. Two-way ANOVA (or Kruskal-Wallis test) between the field samples and normoxic experimental samples at all time points for the total number of species (a); number of individuals (b); species evenness (c); biomass (d); and average body size (e). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F) (or H-value (H) for Krusskal-Wallis test), and probability value (p). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	F / H	p
(a) Number of species					
Field *vs.* Normoxic	1	6307.50	6307.50	53.15	<0.001*
Time	2	95.00	47.50	0.40	0.675
Field *vs.* Normoxic * Time	2	267.80	133.90	1.13	0.340
Residual	24	2848.00	118.67		
Total	29	9518.30			
(b) Number of individuals					
Field *vs.* Normoxic	1	1.32	1.32	54.83	<0.001*
Time	2	0.057	0.028	1.17	0.327
Field *vs.* Normoxic * Time	2	0.030	0.015	0.62	0.546
Residual	24	0.58	0.024		
Total	29	1.99			
(c) Species evenness					
Field *vs.* Normoxic	1	0.0080	0.0080	2.14	0.157
Time	2	0.0017	0.00084	0.22	0.801
Field *vs.* Normoxic * Time	2	0.0018	0.00087	0.23	0.793
Residual	24	0.090	0.0037		
Total	29	0.10			

Table 4.3. Continued

Source	DF	SS	MS	F / H	*p*
(d) Biomass					
Field *vs.* Normoxic	1	9.64	9.64	3.39	0.078
Time	2	1.67	0.83	0.29	0.749
Field *vs.* Normoxic * Time	2	4.82	2.41	0.85	0.441
Residual	24	68.37	2.848		
Total	29	84.50			
(e) Average body size					
Field *vs.* Normoxic	1			3.41	0.065
Time	2			0.66	0.719

4.4.4. Transplantation effects into the mesocosm

As previously highlighted the major differences between the field and normoxic samples occurred during the first seven days after transplantation into the mesocosm. One-way ANOVA analyses revealed that the 'initial field' samples had a significantly higher number of species ($F_{(DF = 9)}$ = 13.3, *p* = 0.007), a greater number of individuals ($F_{(DF = 9)}$ = 15.14, *p* = 0.005) and a lower species evenness ($F_{(DF = 9)}$ = 5.87, *p* = 0.042) when compared to the 'T0 normoxic' community samples (Fig. 4.5).
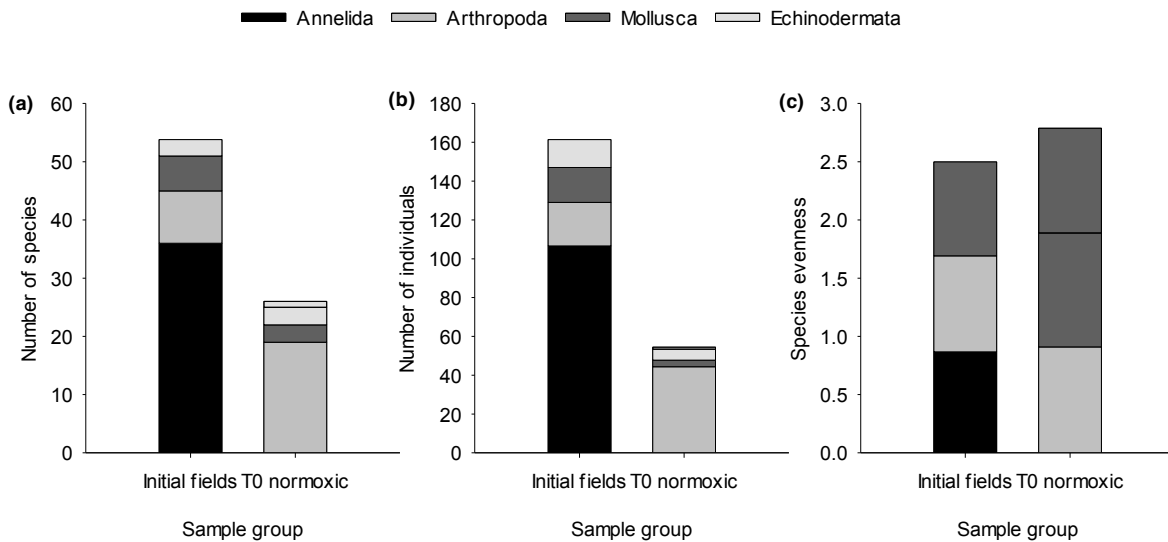
Figure 4.5. Number of species (a); number of individuals (b); and species evenness (c), for the 'initial field' samples and 'T0 normoxic' samples. Bar's split into contributions from the 4 main phyla; Annelida, Arthropoda, Mollusca and Echinodermata.

Furthermore, PERMANOVA analysis revealed that there was a significant difference in community structure between the 'initial field' and 'T0 normoxic' samples in both the untransformed community abundance data (Pseudo-F = 2.19, p(perm) = 0.028, unique permutations = 126) and the transformed presence / absence abundance data (Pseudo-F = 2.13, p(perm) = 0.016, unique permutations = 126). These differences in community structure are represented in Figure 4.6, where the T0 normoxic samples appear to be within a more distinct group compared to the initial field samples.
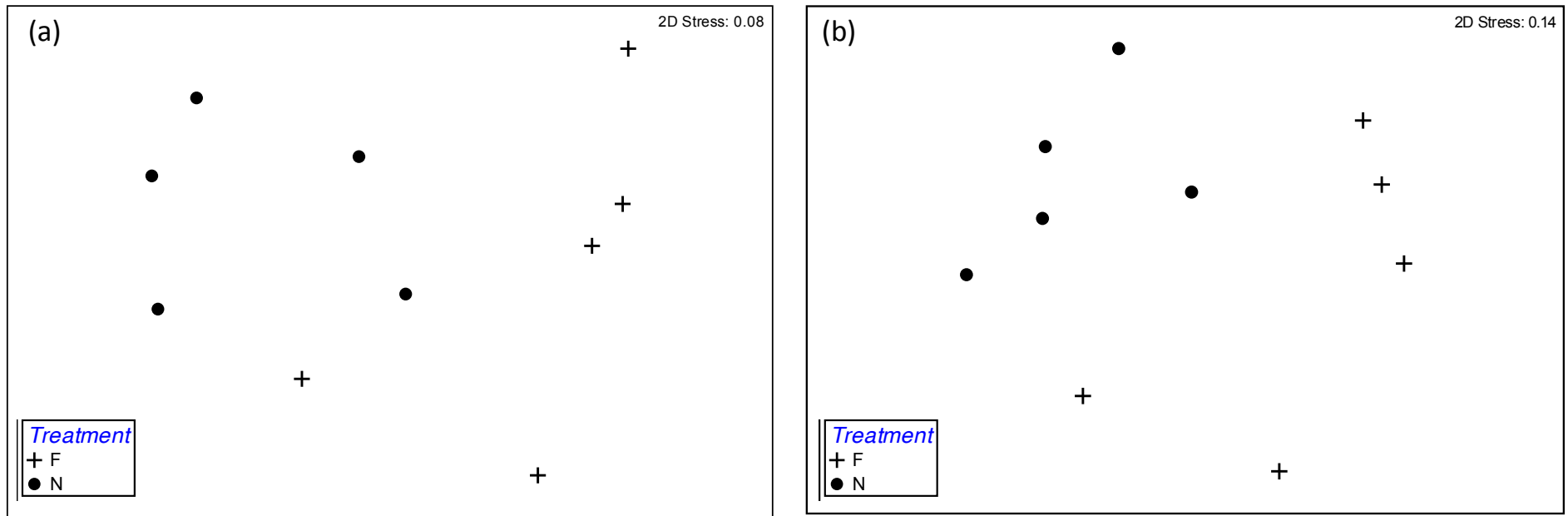
Figure 4.6. Non-metric multi-dimensional scaling (MDS) ordination plots for the Bray-Curtis similarity for the untransformed species abundance community data (a); and for the presence / absence transformed species abundance data (b), for 'initial field' samples and 'T0 normoxic' samples (+ = initial fields (F), ● = T0 normoxic samples (N)).

Differences in the four largest contributing phyla were also examined (Fig. 4.5, Table 4.4). The initial field samples contained a significantly greater number of annelid, arthropod and mollusc species compared to the T0 normoxic samples, in addition to a greater number of individual annelids, arthropods and echinoderms (Table 4.4). Additionally the species evenness within the phylum 'Annelida' was significantly lower in the initial field samples compared to the T0 normoxic samples.

Table 4.4. One-way ANOVA (or Kruskal-Wallis test) between the 'initial field' samples and 'T0 normoxic' experimental samples for the 4 main phyla groups, Annelida (a); Arthropoda (b); Mollusca (c); and Echinodermata (d), for the total number of species (i); number of individuals (ii); and species evenness (iii). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F) (or H-value (H) for Krusskal-Wallis test), and probability value (*p*). * Significant *p* values (to 95 % significance level).

Source	DF	SS	MS	F / H-value	*p*-value
(a) Annelida					
(i) Number of species					
Field *vs.* Normoxic	1	792.1	792.1	18.38	0.003*
Residual	8	344.8	43.1		
Total	9	1136.9			
(ii) Number of individuals					
Field *vs.* Normoxic	1	9672.0	9672.1	10.49	0.012*
Residual	8	7378.0	922.3		
Total	9	17051.0			
(iii) Species evenness					
Field *vs.* Normoxic	1	0.00453	0.00453	9.31	0.016*
Residual	8	0.00389	0.00048		
Total	9	0.00842			
(b) Arthropoda					
(i) Number of species					
Field *vs.* Normoxic	1	90.0	90.0	6.92	0.030*
Residual	8	104.0	13.0		
Total	9	194.0			
(ii) Number of individuals					
Field *vs.* Normoxic	1	1.294	1.29395	13.79	0.006*

Table 4.4. Continued

Source	DF	Adj SS	Adj MS	F / H-value	p-value
Residual	8	0.7505	0.09381		
Total	9	2.0445			
(c) Mollusca					
(i) Number of species					
Field *vs.* Normoxic	1	0.13626	0.13626	11.12	0.010*
Residual	8	0.09803	0.01225		
Total	9	0.2343			
(ii) Number of individuals					
Field *vs.* Normoxic	1	0.3434	0.34339	4.68	0.062
Residual	8	0.587	0.07338		
Total	9	0.9304			
(iii) Species evenness					
	1			0.17	0.676
(d) Echinodermata					
(i) Number of species					
Field *vs.* Normoxic	1	0.1954	0.19542	2.6	0.146
Residual	8	0.6017	0.07521		
Total	9	0.7971			
(ii) Number of individuals					
Field *vs.* Normoxic	1	1.062	1.0616	6.14	0.038*
Residual	8	1.384	0.173		
Total	9	2.445			

Individual ABC curves were created for each aquarium within the 'initial field' and 'T0 normoxic' samples, and were combined to create a mean ABC curve for each treatment group (Fig. 4.7). For each community analysed, biomass curves were consistently above the abundance curves with the W-statistic ranging between 0.311 – 0.577. The 'initial field' samples had a W-statistic of 0.387 ± 0.041 (mean ± 95 % CI), whilst the 'T0 normoxic' samples W-statistic = 0.508 ± 0.051 (mean ± 95 % CI). A t-test revealed that the 'initial field' samples had a significantly lower W-statistic compared to the 'T0 normoxic' samples (n = 5, DF = 8, t = 3.59, p = 0.004), meaning that the 'initial field' samples were dominated by a higher number of individuals with a smaller biomass, compared to the 'T0 normoxic' samples.

Although formal statistical analyses did not show any significant difference in body size between the 'initial fields' and 'T0 normoxic' groups (one-way ANOVA, $F_{(DF = 9)}$ = 3.44, p = 0.101), average body size was smaller within the 'initial field' samples (0.0172 ± 0.0047, mean ± 95 % CI) compared to the 'T0 normoxic' samples (0.0286 ± 0.012, mean ± 95 % CI), complementing the results observed from the W-statistic calculated from the ABC curves.
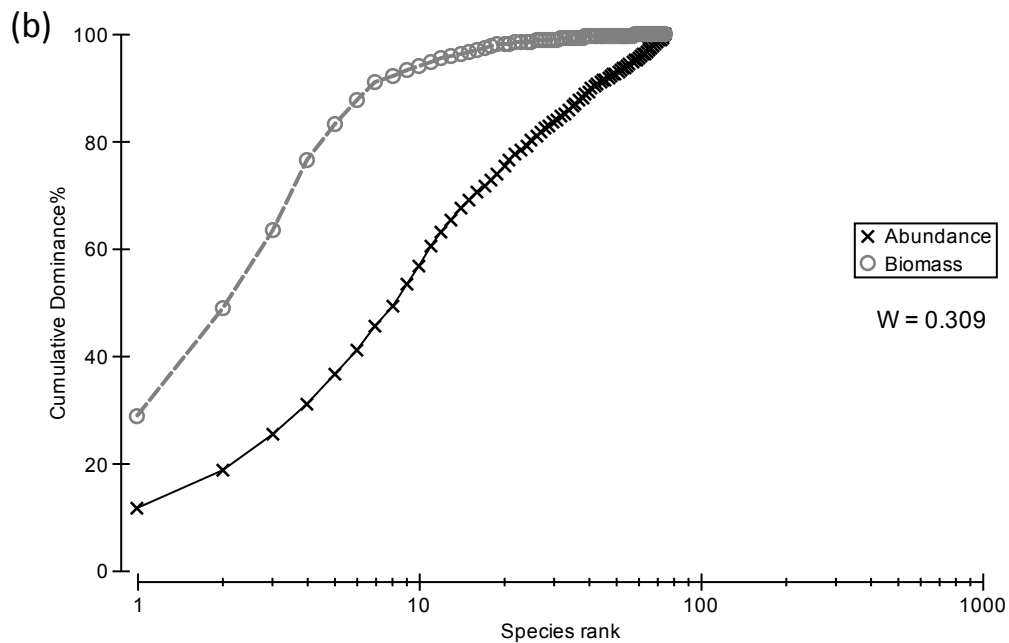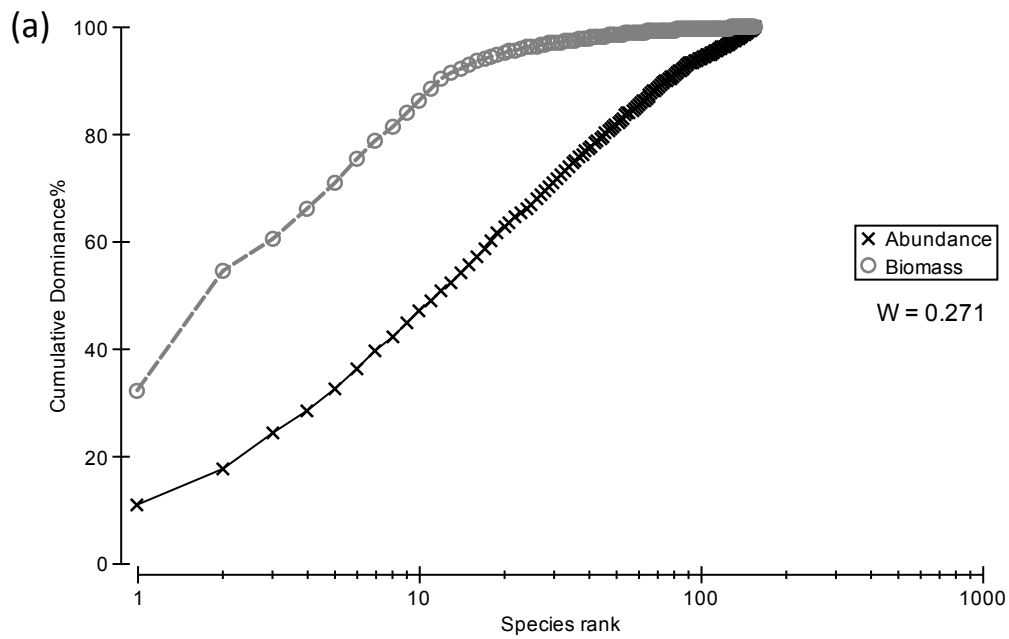
Figure 4.7. Mean ABC curves comparing 'initial field' samples (a); and 'T0 normoxic' samples (b). The *W*-statistic displayed is the difference between the biomass and abundance lines for the specific graphs and not the average *W*-statistic calculated for the treatment groups.

4.4.5. SIMPER analysis comparing field and normoxic samples

SIMPER analysis revealed that the average dissimilarity between all samples collected from the field and all normoxic experimental samples was 70.09. This is comprised of 11.81 % from the annelid *Poecilochaetus serpens*, 5.20 % from the nematodes, 3.82 % from the annelid *Lumbrineris cingulata*, 3.70 % from *Echinocardium cordatum* juv., 3.67 % from the copepods, 3.57 % from the amphipod *Ampelisca tenuicornis*, 2.40 % from the annelid *Parexogone (= Exogone) hebes*, 1.93 % from the annelid *Peresiella clymenoides* and a further 93 different taxa each with a diminishing contribution towards dissimilarity until the tests cut off point of 90 % was reached.

The majority of these species mentioned above contribute to the dissimilarity figure through a reduction in abundance within the normoxic aquaria. For example, *Poecilochaetus serpens* decreased by 82.67 % in the normoxic aquaria compared to the field samples, *Lumbrineris cingulata* decreased by 49.70 %, *Echinocardium cordatum* juv. decreased by 90.97 %, Copepoda was not recorded within any normoxic sample, *Parexogone hebes* decreased by 32.25 % and *Peresiella clymenoides* decreased by 43.92 %. On average the numbers of nematodes were similar within each aquarium, but abundance figures show a decrease in nematode numbers in the field samples, and an increase in nematode abundance in the normoxic samples over time. The amphipod *Ampelisca tenuicornis* had an average of 6.86 indiv. *per* field sample, with every sample, bar one, containing this species. Within the field samples, numbers of individuals ranged from 1 to 16. In the normoxic aquaria, only 9 out of 15 aquaria contained *A. tenuicornis,* ranging from 0 to 3 individuals. However, at 21 d one normoxic sample contained 48 individuals, which when

included in analyses causes an average of a 2.04 % increase in *A. tenuicornis* numbers within the normoxic samples over the experimental period. If this anomaly is removed, the mean number of individuals per normoxic aquaria becomes two, which equates to an average decrease of 70.83 % in the number of *A. tenuicornis* individuals.

4.4.6. Species absent from mesocosm communities

Some species were completely absent from the normoxic mesocosm samples, which may be due to species sensitivity and/ or their rarity. Table 4.5 lists the species categories that were not recorded within any of the normoxic aquaria, but were present within the field samples. Species marked with an asterisk (*) were recorded within the hypoxic experimental aquaria, albeit in low abundance. Excluding the Copepoda and Echinoida juv., the species that were completely absent in the normoxic aquaria, generally had low abundance values within the field samples (between 0.3 – 1.6 indiv. *per* aquaria). Consequently, each species category recorded during the entire experimental period was assigned an abundance rank, calculated from the total abundance counts for the duration of the experimental period. Species categories that had the same total abundance shared a rank value, meaning that of the 281 species categories identified, ranks were assigned from 1 – 60 (1 = most abundant, (i.e. *Poecilochaetus serpens* with a total abundance count of 513), 60 = least abundant, (i.e. one observation during the experimental period)).

The Copepoda and Echinoida juv., were assigned an abundance rank of 12 and 16 respectively, meaning they were within the top 27 % most abundant species recorded during the experimental period, mainly within the field samples. The

total loss of Echinoida juv. in both normoxic and hypoxic aquaria and the near total loss of Copepoda (one recorded in the hypoxic aquaria) may mean that these two species categories are highly sensitive to disturbance, movement and general mesocosm conditions. The other species listed in Table 4.5 that were absent from the normoxic aquaria, were assigned an abundance rank from 37 to 56, representing the last 61.6 % - 93.3 % of species recorded in all samples. These species are typically rarer (less abundant) in the field samples, and are subsequently absent within the normoxic samples, possibly due to their reduced abundance (rarity).

Table 4.5. Species absent from the normoxic aquaria for the duration of the experimental period. (* = species present within some hypoxic aquaria, ** = assigned ecological group taken from Borja et al. (2000) as a measure of sensitivity, 1 = species very sensitive, present under unpolluted conditions, to 5 = tolerant species that can proliferate in reduced sediments, N.A. = not assigned.

Species category	Major group	Total abundance in hypoxic aquaria (*)	Total abundance in field samples	Average abundance per field aquaria	% contribution to the SIMPER dissimilarity figure between field and normoxic samples	Calculated abundance rank	Assigned ecological group (**)
COPEPODA*	Crustacean	1	111	7.40	3.67	12	N.A
ECHINOIDA juv.	Echinoderm		62	4.13	1.74	16	1
Caprella linearis	Crustacean		23	1.53	0.81	39	N.A
Diastylis laevis	Crustacean		25	1.67	0.74	37	1
Aoridae Sp.	Crustacean		17	1.13	0.50	44	1
Atherospio guillei	Annelid		14	0.93	0.46	47	4
Phyllodoce longipes	Annelid		10	0.67	0.30	51	2
Westwoodilla caecula	Crustacean		10	0.67	0.28	51	N.A
Ebalia granulosa*	Crustacean	4	9	0.60	0.28	48	N.A
Aricidea catherinae*	Annelid	3	8	0.53	0.23	50	1
Pleocyemata zoea	Crustacean		7	0.47	0.21	54	N.A
Leptocheirus pectinatus	Crustacean		6	0.40	0.21	55	3
Malmgrenia indet.*	Annelid	1	6	0.40	0.19	54	2
Phaxas pellucidus (large)	Mollusc		5	0.33	0.19	56	1
Magelona filiformis*	Annelid	1	6	0.40	0.17	54	1

4.4.7. Taxonomic distinctness

Figure 4.8 displays the average taxonomic distinctness (Δ^+) for each community sample. Nearly all of the samples analysed (bar 3) fall within the 95 % probability funnel, meaning that Δ^+ is comparable across most samples, with little impact on taxonomic diversity. Figure 4.8 helps to confirm that specific groups of species have not been lost, i.e. taxonomic diversity is not affected, but the mesocosm samples (normoxic and hypoxic) have a clear generic loss in the number of species they contain.
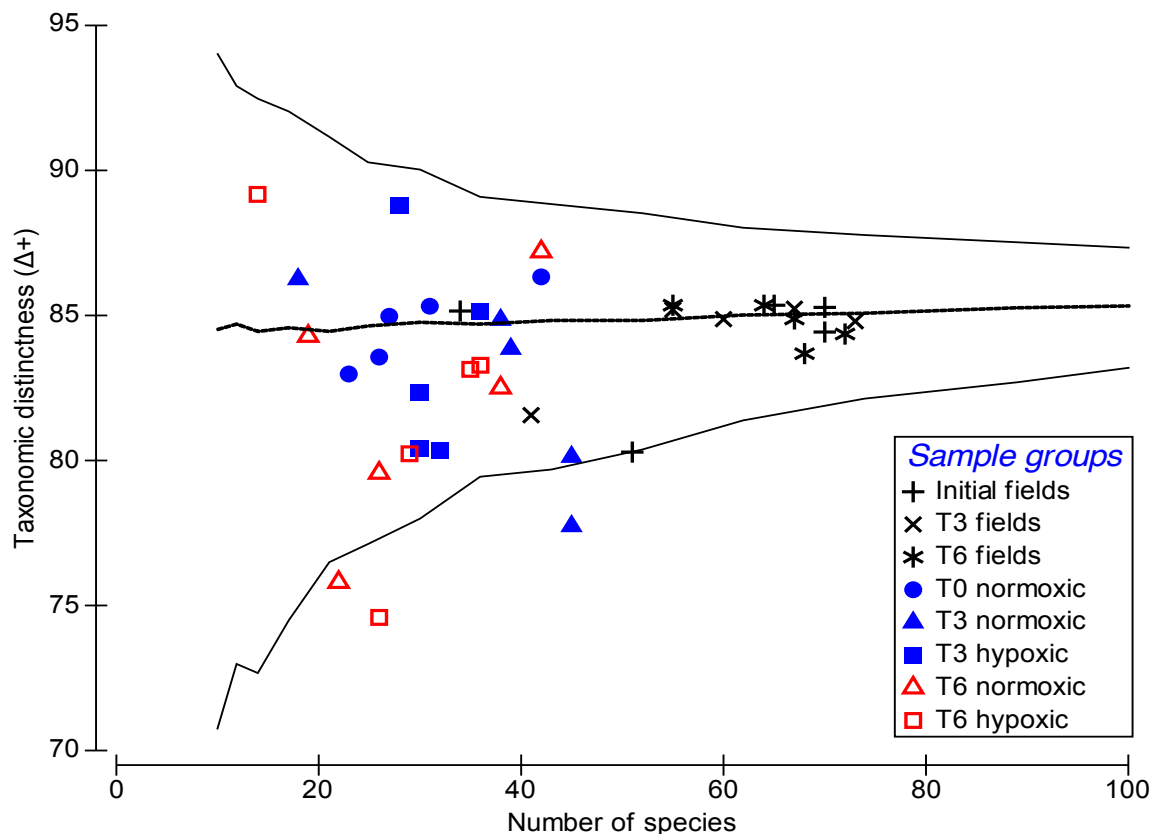
Figure 4.8. Average taxonomic distinctness (Δ^+) for each sample group (n = 5) plotted against the number of species within each community. The 95 % probability funnel (or confidence limit) is drawn based on species frequency measures from the field samples only and represents the likelihood of random subsets of communities falling within the expected spread of Δ^+ based on the field community samples.

4.4.8. The effect of hypoxia

Two-way ANOVAs revealed there were no significant differences in the number of species, number of individuals, species evenness and community biomass between samples in the normoxic and hypoxic water treatments at both time points (T3 and T6) (Fig 4.3, Table 4.6). PERMANOVA analyses revealed there was no significant effect of hypoxia or experimental time on the untransformed community abundance data and the transformed presence / absence community abundance data (Table 4.7 a, b).

Table 4.6. Two-way ANOVA between normoxic and hypoxic experimental samples at time points T3 and T6 for the total number of species, (a); number of individuals (b); species evenness (c); community biomass (d). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F) and probability value (p). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	F	p
(a) Number of species					
Water treatment	1	64.80	64.80	0.85	0.370
Time	1	156.80	156.80	2.06	0.170
Water treatment * Time	1	24.20	24.20	0.32	0.581
Residual	16	1217.20	76.08		
Total	19	1463.00			
(b) Number of individuals					
Water treatment	1	1824.1	1824.1	2.72	0.119
Time	1	2142.5	2142.5	3.20	0.093
Water treatment * Time	1	186.1	186.1	0.28	0.606
Residual	16	10726.4	670.4		
Total	19	14879.0			
(c) Species evenness					
Water treatment	1	0.000418	0.000418	1.45	0.245
Time	1	0.000009	0.000009	0.030	0.865
Water treatment * Time	1	0.000084	0.000084	0.29	0.597
Residual	16	0.00460	0.000287		
Total	19	0.00512			
(d) Community biomass					
Water treatment	1	0.083	0.083	0.02	0.901
Time	1	6.92	6.92	1.35	0.263
Water treatment * Time	1	0.91	0.92	0.18	0.678
Residual	16	82.09	5.13		
Total	19	90.01			

Table 4.7. Permutational multivariate analyses of variance of the effects of two fixed factors, water treatment (two levels: normoxic and hypoxic) and experimental time (two levels: T3 and T6) on the untransformed (a); and transformed presence / absence (b), community data. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), the pseudo F-value (Pseudo-F), the permutational probability value (p(perm)) and the number of permutations of residuals carried out (Unique perms). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	Pseudo-F	p (perm)	Unique perms
(a) Species abundance community structure data						
Water treatment	1	2512.3	2512.3	1.316	0.098	998
Time	1	1821.9	1821.9	0.95436	0.535	998
Water treatment * Time	1	1893.2	1893.2	0.99169	0.469	998
Residual	20	38180	1909			
Total	24	46599				
(b) Presence / absence community structure data						
Water treatment	1	1464.1	1464.1	0.95249	0.550	997
Time	1	2041.1	2041.1	1.3279	0.130	997
Water treatment * Time	1	1383.9	1383.9	0.90029	0.605	997
Residual	20	30742	1537.1			
Total	24	38254				

4.4.9. Assessing the ecological quality of communities

The mean Biotic Coefficients calculated for each treatment group are displayed in Figure 4.9 and Table 4.8. Figure 4.9 shows that the T3 and T6 field samples have a lower BC compared to all other sample groups, with the T3 field samples having a mean BC of 0.971, falling within the 'impoverished' category for benthic community health and the 'unpolluted' site classification according to

Borja et al. (2000). T6 field samples were also close to this category, but with a mean BC of 1.247, fell within the 'unbalanced' category for community health and the 'slightly polluted' site classification. The initial field samples have a higher mean BC value that is comparable to BC values calculated for the mesocosm samples.

PERMANOVA analyses examining the BC scores for each sample within T3 and T6, revealed that the T3 and T6 field samples have a significantly lower BC score compared to the T3 and T6 mesocosm samples but there is no difference in BC scores between the hypoxic and normoxic samples (Table 4.9). The Initial field samples and consequently the T0 normoxic samples, were excluded due to the potential influence of the spring bloom on the initial field samples.
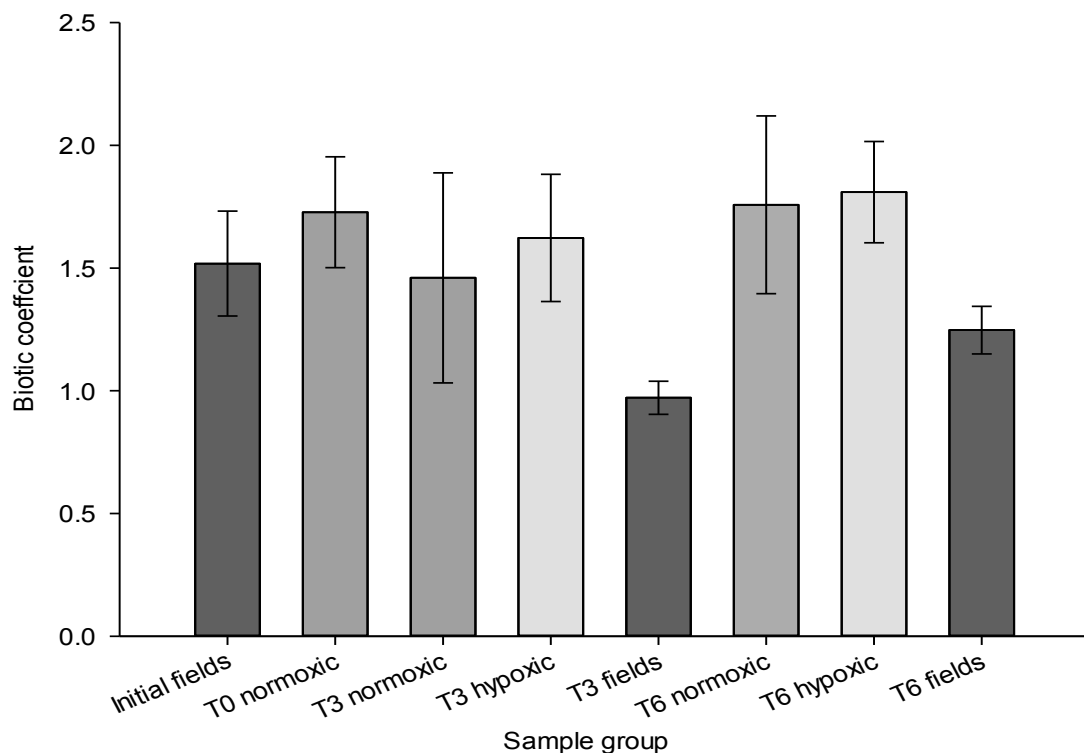
Figure 4.9. Biotic Coefficient (BC) scores for each sample group (means ± 95 % confidence intervals) calculated as *per* Borja et al. (2000).

Table 4.8. Left-hand side: Mean Biotic Coefficients calculated for all sample groups, with their assigned dominating ecological group and health status based on BC scores. Right-hand side: Biotic Coefficient scale and corresponding benthic community health information adapted from Borja et al. (2000) and Grall & Glémarec (1997).

Sample	Mean Biotic Coefficient	Dominating ecological group	Assigned benthic community health status	Biotic coefficient scale	Benthic community health
Initial fields	1.518	III	Unbalanced	0.0 < BC ≤ 0.2	Normal
T0 normoxic	1.727	III	Unbalanced	0.2 < BC ≤ 1.2	Impoverished
T3 normoxic	1.460	III	Unbalanced	1.2 < BC ≤ 3.3	Unbalanced
T3 hypoxic	1.623	III	Unbalanced	3.3 < BC ≤ 4.3	Transitional to pollution
T3 fields	0.971	II	Impoverished	4.5 < BC ≤ 5.0	Polluted
T6 normoxic	1.758	III	Unbalanced	5.0 < BC ≤ 5.5	Transitional to heavy pollution
T6 hypoxic	1.810	III	Unbalanced	5.5 < BC ≤ 6.0	Heavy polluted
T6 fields	1.247	III	Unbalanced	Azoic	Azoic

Table 4.9. Permutational multivariate analyses of variance of the effects of two fixed factors, water treatment (three levels: fields, normoxic and hypoxic) and experimental time (two levels: T3 and T6) on the Biotic Coefficient scores for each community sample. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), the pseudo F-value (Pseudo-F), the permutational probability value (p(perm)) and the number of permutations of residuals carried out (Unique perms). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	Pseudo-F	p(perm)	Unique perms
(a) Main PERMANOVA						
Treatment	2	2.098	1.049	11.033	0.002*	998
Time	1	67.982	67.982	714.900	0.001*	999
Treatment * Time	2	0.017	0.009	0.091	0.976	999
Residual	24	2.282	0.095			
Total	29	72.379				

(b) Pairwise tests			
Groups	t	p(perm)	Unique perms
normoxic, hypoxic	0.64629	0.662	993
normoxic , fields	3.4163	0.002*	993
hypoxic, fields	6.7641	0.001*	997

4.5. DISCUSSION

Moderate (3.09 ± 0.03 mg L^{-1}) hypoxic exposure for three and six weeks had no effects on the L4 benthic invertebrate community in terms of structure and diversity. However, during the course of the experiment, natural field samples were collected which offered an enhanced insight into L4 community dynamics and the limitations of the mesocosm experiment.

Rather than a 'hypoxic' effect on the benthic community, a significant 'translocation' or 'mesocosm' effect occurred. However, it was not possible to determine how much the 'translocation / mesocosm' effect masked any potential hypoxic effects. In the current context, these findings indicate that the L4 benthic community appears tolerant of moderate hypoxia, but the investigation also raises questions about the efficacy of transplanting complex community systems into 'simpler' mesocosm environments, without having concomitant field sampling campaigns to compare with. It also emphasises the influence and strong driving dynamics of pelagic systems on benthic communities and accentuates the need for *in situ* hypoxia experiments to be used more widely.

4.5.1. Field samples

Field sample community structure changed significantly with sampling time, which is likely to reflect natural variability due to the time they were collected from the field in relation to the May-June spring bloom. Phytoplankton production constitutes the main source of organic inputs into coastal areas that drive benthic faunal changes, and often occur seasonally, typically as spring and autumn blooms (Pearson & Rosenberg 1978, Smyth et al. 2010). The initial

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field samples were collected at the tail-end of the May-June spring bloom when deposition of phytoplankton was high and therefore during a time when the benthos was not food-limited. Zhang et al. (2015) found that macro-benthic abundance rapidly increased as a result of the L4 spring bloom, with the initial response being an increase in the abundance of small body-sized organisms. Although no difference in biomass or average body size was detected between the initial field samples, T3 and T6 field samples, the variability observed in community structure over the time period of the experiment is not surprising and is likely to be related to the complex natural cycles and natural variability occurring in a dynamic coastal system.

4.5.2. Translocation / mesocosm effects

Mesocosms are useful tools for the establishment of causal relationships, since they allow controlled experimentation with replicated experimental units to randomly disperse treatments and controls (Kraufvelin 1998). However, mesocosms typically lack the complexity of whole ecosystems, so that such features such as complex sediment-water exchanges, inclusion of the activities of numerous organisms and appropriate spatial and temporal scales to assess responses are often not included (Schindler 1998). The results from this experiment indicate that it was the translocation of community samples into the mesocosm that caused the subsequent loss in species and individuals within all mesocosm community samples.

Mesocosm experimental samples were collected from Station L4 on 24th and 25th June, and translocated directly into the mesocosm experimental set up within 2 hours of collection. All aquaria were then allowed 7 - 8 days (depending

on collection date) to acclimatise before official experimental treatments began and the T0 normoxic aquaria were removed and sampled. It was during this first week that the reduction in species and individuals occurred in both the normoxic and hypoxic aquaria. Numbers of species and individuals remained relatively constant thereafter, demonstrating that no further degradation in the community occurred after this initial period. This indicates that the loss of species and individuals is likely to be due to the translocation process and the change in conditions once within the mesocosm. Although measures were taken to replicate natural conditions as much as possible unfortunately every mesocosm investigation can suffer from its intermediate position of being caught between realism and control (Kraufvelin 1998). However, with the addition of concomitant sampling of the natural field community, the significant effects of the mesocosm experiment have become apparent and can be discussed in greater detail.

4.5.2.1. Examining abundance – biomass relationships

The abundance-biomass-comparison (ABC) curves between the initial field samples and the T0 normoxic samples revealed that the communities within the initial field samples had a lower *W*-statistic than the communities in the T0 normoxic treatment. This result means that the initial field samples had a higher number of smaller (often opportunistic) species that are dominant in terms of numbers but not biomass, because they are small bodied (Clarke & Warwick 2001). Although formal statistical analysis did not show any significant differences in body size between the initial fields and T0 normoxic treatment groups, the initial field samples did have a smaller mean body size compared to the T0 samples. However, this does not necessarily mean that the field samples were 'more disturbed or stressed' than the T0 experimental samples. Due to the

timing of the collection of the initial field samples, the increase in smaller bodied individuals is likely to be due to the final stages of the spring bloom, when there is strong connectivity between the pelagic and benthic systems at L4, as previously described by Zhang et al. (2015). Benthic macrofauna communities can change very rapidly at L4 in terms of abundance, biomass, diversity and community composition, with evidence to suggest that small-bodied deposit feeders rapidly increase during the spring bloom (Zhang et al. 2015). It is likely that the effects of the May-June 2013 spring bloom at L4 on the benthic community were still remaining whilst the initial field samples and experimental mesocosm samples were collected. Consequently, the smaller individuals within the initial field samples were likely to be a transient part of the community due to the increased food supply from the spring bloom, creating a community that is dominated by smaller bodied individuals and hence the lower *W*-statistic calculated from the ABC curves. It is probable that these smaller individuals did not survive the translocation into the mesocosm, causing the T0 normoxic samples to have an increased average body size value, and lower mean biomass, albeit not statistically different from the initial fields in formal tests.

4.5.2.2. Phyla and species lost due to translocation effects

The reduction of species numbers and individuals within the mesocosm samples was from a broad range of phyla and taxonomic groups. Within the T0 normoxic samples each of the four major phyla (Annelida, Arthropoda, Mollusca, and Echinodermata) all demonstrated a significant reduction in species numbers and numbers of individuals compared to the initial field samples. The SIMPER analysis confirmed that over 100 species contributed towards the dissimilarity between the field and normoxic samples, with the

largest contribution from the annelid *Poecilochaetus serpens* which only formed 11.81 % of the dissimilarity. This generic loss in species numbers across all four of the main phyla groups suggests that a large number of species were sensitive to the translocation effects of being removed from the field and placed within the mesocosm. This raises questions over the reliability of using natural community samples within mesocosm or laboratory experiments, without comparisons to field samples to quantify the translocation effects. However, there were no significant differences in taxonomic distinctness between any treatment and time group, indicating that the communities within the mesocosm samples were representative of L4 field communities, (i.e. taxonomic diversity was not affected, albeit a significant reduction in the number of species and individuals they contained). Again, it is probable that this difference between the initial field samples and T0 normoxic samples may have occurred due to the loss of the 'transient' smaller-bodied organisms captured within the field samples that were present due to the effects of the spring bloom at L4.

In addition to a generic decrease in species numbers and individuals, species that were completely absent from the mesocosm samples were also investigated to assess if these species were particularly sensitive. With the exception of 'Copepoda' and 'Echinoida juv.' all of the species that were completely absent in mesocosm samples were low in abundance within the field samples, indicating that species rarity played a large role in causing some species to be 'extinct' from mesocosm samples, rather than their sensitivity to stress. For species that were absent within the mesocosm samples, a sensitivity score was assigned to them as *per* Borja et al. (2000). The scores assigned ranged from 1 (very sensitive species) to 4 (second order opportunistic

species), further supporting the conclusion that species rarity, rather than species sensitivity to stress, played a dominating role in determining the species that were 'extinct' within mesocosm samples. Extinction risk is typically higher for rare species with low local abundances, because smaller populations are more vulnerable to environmental stochasticity (McKinney 1997, Solan et al. 2004a). Consequently, when community samples are brought into mesocosm experiments for investigation, it is possible that the rare, less abundant species are always lost due to the stress of translocation and change in conditions, but this loss may not have been detected if comparisons to field samples were not conducted. Alternatively, it could be argued that depending on the research questions being investigated, the 'extinction' of a few rare species within a community is acceptable to allow for the controlled study of the remaining population. For example, when studying bioturbation, small populations typically contribute less, with previous studies indicating that the extinction of rare species has little impact on the biogenic mixing depth of the sediment (Solan et al. 2004a).

Copepoda and Echinoida juv. were the only two groups that were absent from the mesocosm samples but were relativity abundant within the field samples. Consequently, the loss of these two groups was likely due to their sensitivity to transplantation and mesocosm conditions, not rarity. A sensitivity score could not be assigned to the Copepoda, but the Echinoida juv. were assigned a sensitivity score of '1' as *per* Borja et al. (2000), which represent species that are very sensitive and only present under unpolluted conditions.

4.5.3. Effects of hypoxia

Exposure to hypoxia for three and six weeks had no significant effects on any of the diversity indices measured or community structure within the mesocosm samples. Due to the significant loss of species and numbers of individuals when communities were brought into the mesocosm, the 'translocation / mesocosm' effect may have masked any potential effects of hypoxia by reducing the most sensitive species within the community. Biotic coefficients were calculated for each community sample to investigate the levels of biological stress as *per* Borja et al. (2000). Each species within the community was assigned to one of five ecological groups according to their sensitivity to an increasing stress gradient (increasing organic matter enrichment). After 3 and 6 weeks, mesocosm communities had a significantly higher biotic coefficient than the T3 and T6 field samples. This high biotic coefficient within the mesocosm communities, in both normoxic and hypoxic treatments, indicates that the species within the mesocosm communities were likely to be more tolerant to stress compared to the field samples, possibly signifying that the most sensitive species were affected during the translocation process into the mesocosm, which may have hindered the detection of any hypoxic effects. However, all communities sampled, field, normoxic and hypoxic, (except the field samples collected after 3 weeks), were dominated by species falling within the 3rd ecological group classification as detailed by Borja et al. (2000), of 'species tolerant to excess organic enrichment'. The field samples collected at T3 were dominated by species falling within the 2nd ecological group of 'species indifferent to enrichment, present in low densities'. Although the values of the Biotic Coefficients were different for field and mesocosm samples, the fact the majority of the samples were dominated by species from the same ecological

group may help explain why the taxonomic diversity between samples was not affected. Furthermore, L4 is a highly dynamic coastal site, with huge variability occurring in the duration and strength of the spring and autumn blooms. In 2006 the L4 spring bloom lasted only 14 days, whilst in 2012 the spring bloom lasted an unusual 223 days (Zhang et al. 2015). Zhang et al. (2015) presented evidence to suggest that there is likely to be extremely close coupling between phytoplankton biomass and food supply (and hence organic enrichment) reaching the benthos at site L4, so although not known to be a polluted site, the benthic community at L4 is likely to be adapted to regular organic enrichment as a result of the spring and autumn blooms, with a corresponding benthic community health status as 'unbalanced' (classified as *per* Borja et al. 2000).

Alternatively, the macrobenthic community could be relatively tolerant to moderate hypoxia for the time frame investigated here, offering explanation as to why there were no significant changes in community abundance and diversity measures over the three and six-week experimental period. In their classic review paper, Diaz and Rosenberg (1995) state that benthic macrofauna actually have a rather high tolerance to hypoxia, with most species exhibiting behavioural responses before mortality occurs (Diaz & Rosenberg 1995). However, there are numerous studies that provide evidence to suggest that exposure to hypoxia and anoxia can cause significant negative effects to benthic communities (Rainer & Fitzhardinge 1981, Llansó 1992, Diaz & Rosenberg 1995, Levin et al. 2009, Seitz et al. 2009, Kodama et al. 2012) but the severity, intensity and durations of hypoxic exposure in these studies was greater than used in the present study.

In fjords, sea-lochs or protected embayments the DO level critical to most benthic organisms appears to be around 1.4 ml L^{-1} (2.0 mg L^{-1}) (Rosenberg et al. 1992), whilst in estuaries and open coasts, the critical oxygen concentration is closer to 0.7 ml L^{-1} (< 1.0 mg L^{-1}) (Llansó 1992, Diaz & Rosenberg 1995). In the Gullmar fjord, western Sweden, DO levels declined gradually through the 1970s but remained above 2.0 ml L^{-1} (3.0 mg L^{-1}) until 1980. During this time benthic communities remained stable in abundance and biomass, but in January 1980 when DO levels dropped to below 2.0 ml L^{-1} the entire community was eliminated (Josefson & Widbom 1988). Kodama et al. (2012) examined the macrofaunal community within Tokyo Bay, Japan, from June through to September. During June and July, when DO was ~ 2.1 – 3.5 ml L^{-1} (3.0 – 5.0 mg L^{-1}), macrofauna were found throughout the bay. During August and September macrofaunal defaunation occurred when DO levels dropped to 1.1 ml l^{-1} (1.5 mg L^{-1}). A meta-analysis of numerous studies investigating the effects of hypoxia on marine invertebrates found that the mean lethal oxygen concentration (LC_{50}) for all organisms reviewed (872 published experiments on 206 species) was found to be 2.05 ± 0.09 mg O_2 L^{-1} with crustaceans exhibiting the most sensitivity and molluscs exhibiting the greatest tolerance towards hypoxia (Vaquer-Sunyer & Duarte 2008). Consequently, in the context of community structure and species abundance, the evidence suggests that benthic communities are relativity tolerant to moderate levels of hypoxia. Benthic macrofaunal response will depend on the duration, predictability and intensity of hypoxia, in addition to if H_2S is formed (Levin et al. 2009). When hypoxia is severe enough there is usually a loss of diversity in intolerant species, elevated dominance in tolerant or opportunistic species and reductions in body size (Levin et al. 2009). The level of hypoxia used in this experiment

(3.09 ± 0.03 mg O_2 L^{-1}) for three and six weeks was likely to be not severe or long enough to cause any changes in community composition, offering reasonable explanation as to why no hypoxic affect was observed.

4.5.4. Limitations of the mesocosm

Experiments serve to reconstruct partial aspects of the ecosystem and allow for inferred statements or causal relationships to be discussed (Haag & Matschonat 2001). During mesocosm experiments complexity is often increased at the expense of control, and it is down to the primary investigator to find the balance between the two. However, despite their usefulness and contribution to advancement in science, mesocosms present specific physical, chemical and biological shortcomings, demonstrating that the discrepancy between experimental set ups and the ecosystem scale is not resolved easily (Schindler 1998, Haag & Matschonat 2001).

It is generally accepted that good experimental practice provides stable settings to allow for causal inference; however within natural systems the setting is not stable. Heterogeneity, variability and instability prevail in natural conditions, making predictions of community and ecosystem behaviour from experimentally derived factors challenging (Haag & Matschonat 2001). Specifically at station L4, there is documented evidence of a strong sequence of community change through time, with communities even changing throughout the period of the spring bloom. There is also evidence of strong connectivity between the pelagic and benthic systems at this coastal site (Zhang et al. 2015). This level of natural variability and complexity cannot be recreated within the mesocosm, immediately affecting community samples once brought into experimental

conditions. However, the results presented here indicate that although there was a translocation effect to the benthic community when initially brought into the mesocosm, no further degradation of the community took place over the following three and six week sampling regime. Furthermore, the taxonomic distinctness of communities was still representative of field samples, and the assigned benthic community health status based on Biotic Coefficient scores generally fell within the 'unbalanced' category for all field and mesocosm samples (except the T3 field samples). Consequently, although it is difficult to determine how the translocation effect may have masked or hindered any potential hypoxic effects, the mesocosm experiment conducted was robust and reliable enough to conclude that the diversity and taxonomic structure of the L4 benthic community is fairly resilient to moderate hypoxic stress.

4.5.5. Conclusions

This experiment is the first of its kind to experimentally examine the effects of moderate hypoxia for 3 and 6 weeks on the L4 benthic community. In conjunction with natural field samples collected throughout the experimental period, the results presented here indicate that the L4 benthic community is fairly resilient to moderate hypoxic stress in terms of community structure and diversity, but exhibits some sensitivity when translocated from the field into mesocosm conditions.

The results presented here, support previous studies that have shown benthic community resilience to hypoxia when DO levels are considered 'moderate' (~ 3.0 mg O_2 L^{-1} or above) (Josefson & Widbom 1988, Diaz & Rosenberg 1995, Kodama et al. 2012) and highlight how influential natural cycles and pelagic

phytoplankton dynamics can be on driving benthic dynamics. Consequently, the timing of this experiment, with sample collection at the tail-end of the May – June Spring bloom possibly captured the benthic community in its most dynamic state, as opportunistic species thrived as a result of the increased food supply. This may have enhanced the translocation effects observed, and caused the significant reduction in species numbers and individuals within all mesocosm samples. Mesocosm experiments conducted in the winter may therefore offer a more stable benthic community to work with, but stressors such as hypoxia, often occur in the summer, after the spring bloom, when the community is most dynamic. Mesocosm experiments are therefore always a trade-off.

In-situ experiments would incorporate the natural conditions that have such strong influence over benthic dynamics and can capture actual behavioural responses, intra-and interspecific interactions, mortality sequences and community level processes in the natural environment (Stachowitsch et al. 2007). However, they can require costly equipment, specialist boat and field work programmes, and experience numerous environmental factors that cannot be controlled. Consequently, a combination of mesocosm experiments supported with field sampling campaigns and the development of *in situ* experiments would offer an all-round insight into the effects of hypoxia on specific communities.

As moderate hypoxia had no specific effects on community composition and diversity, sub-lethal effects such as changes in organism behaviour or physiology require investigation. Alterations in behaviour or physiology may

affect the functional role of organisms within the community when under moderate hypoxic stress, possibly influencing important ecosystem processes.

CHAPTER 5: IMPACT OF HYPOXIA ON BENTHIC COMMUNITY BIOTURBATION AND ECOSYSTEM FUNCTION

Assessing the ecological responses (in terms of behaviour and function) of a typical U.K. benthic community to moderate hypoxic stress.

5.1. INTRODUCTION

Chapter 4 examined and documented the sensitivity and community response (in terms of structure and compositional changes) of a typical U.K. benthic infaunal community when exposed to different oxygen concentrations. The study presented in Chapter 4 indicated that moderate hypoxia (3.09 ± 0.03 mg O_2 L^{-1}) did not affect the L4 benthic community in terms of species richness, community structure or other 'traditional' diversity indices during a three and six week exposure period. However, species exhibit a range of sub-lethal responses when exposed to hypoxia, depending on the severity and intensity of the event, physiological thresholds, life-history traits and community interactions (Gray et al. 2002). These changes can manifest in organism behaviour and physiology generally preceding species loss or mortality, especially when dissolved oxygen (DO) is reduced but not severely hypoxic or anoxic (Gray et al. 2002, Vaquer-Sunyer & Duarte 2008). Consequently, when assessing the negative impacts of hypoxia, especially moderate or minor alterations in DO, it is important to incorporate and investigate other community components (i.e. performance, functionality and behaviour) in addition to the more 'classic' species richness and diversity measures (Waldbusser et al. 2004). There is also a lack of information on how moderate alterations in DO may impact organisms and communities. Therefore, to further our understanding of the ecological effects of moderate hypoxia on the L4 benthic community, and in light of the conclusions of Chapter 4, it is important to consider the sub-lethal effects of hypoxia that have the potential to impact on community performance, ecosystem processes and other aspects of diversity (i.e. functionality), despite their being no change in macrofaunal abundance or diversity *per se*.

Previous research has focused on the effects of biodiversity loss on ecosystem functioning (Naeem et al. 1994, Loreau et al. 2001, Cardinale et al. 2002) with the general consensus that declines in biodiversity (species loss) have negative consequences for ecosystem functioning (Gamfeldt et al. 2015). However, when species loss is not observed, research examining important community interactions (for example, species functional diversity, behaviour and physiology) that can also affect ecosystem functioning is minimal and requires further attention (Gamfeldt et al. 2015).

Undisturbed benthic communities may contain larger or deeper burrowing species that enhance sediment oxygen penetration depths and stimulate microbial growth, diversity and activity (Pearson & Rosenberg 1978). This creates extensions of important oxic micro-niches where nitrate mineralisation can be enhanced and phosphate and silicate sorption can readily occur (Kemp et al. 1990, Karlson et al. 2007, Bartoli et al. 2009), thereby enhancing certain ecosystem processes. Results from Chapter 3 showed that moderate hypoxia (3.59 ± 0.04 mg O_2 L^{-1}) affected *Amphiura filiformis* behaviour in terms of bioturbatory activity, with measurable consequences to nutrient fluxes. Additionally, previous studies have also documented important behavioural changes within the benthic community when exposed to hypoxia, such as reduced burrow depths, abandonment of burrows and tubes, stretched out bivalve siphons, and movement away from oxygen poor areas (Diaz & Rosenberg 1995, Sturdivant et al. 2012).

These changes in organism behaviour and position within the sediment not only affect how organisms within the community interact (e.g. predator-prey

dynamics), but they also affect physical properties of the sediment (Nilsson & Rosenberg 2000, Sturdivant et al. 2012). Although it is the microbial communities within the sediment that perform the oxidation of organic compounds and the regeneration of nutrients essential for sustaining primary production (Herbert 1999), infaunal invertebrates have significant influence over many sedimentary processes (Queirós et al. 2013). The consequences of inhibiting bioturbation activities are likely to cascade to changes in a variety of physical, biological and chemical processes (Sturdivant et al. 2012) and have implications for nutrient fluxes, rates of primary production, pH and redox gradients, oxygen penetration depths and chemically important micro-niches for microbial communities (Herbert 1999, Rosenberg et al. 2001, Shull 2009). Consequently, the positive effects to ecosystem processes mediated by the presence of infauna and their biological activity can be altered under hypoxic stress, not necessarily through changes in species richness or diversity, but through alterations in organism behaviour and physiology (Diaz & Rosenberg 1995). Therefore, it is essential to understand the dynamics and functioning of species populations and how potential disruptions to behavioural processes can influence community dynamics and ecosystem functioning.

Given the existing knowledge that hypoxia can cause alterations in bioturbatory behaviour in benthic invertebrates, assessments of community bioturbation activity, especially in light of anticipated changes to coastal systems associated with anthropogenic activities, can contribute to a better understanding of how ecosystem processes are mediated by biological activity and their potential alterations in times of change (Queirós et al. 2013).

Villnäs et al. (2012) found that during an *in-situ* hypoxia experiment, benthic infauna were stressed after three days of hypoxia, with observations of bivalve siphons protruding out of the sediment, and several infaunal polychaetes appearing at the sediment surface. Specific ecosystem processes (sediment oxygen consumption and the efflux of silicate and ammonia) were affected by the duration of hypoxia, but alterations in the benthic invertebrate community explained a larger proportion of the variance observed in these processes compared to the effects of hypoxia alone. This emphasises the role that the macrofaunal benthic community plays in important processes that contribute to ecosystem function, despite the primary role of microbes in the regeneration of nutrients.

Consequently, the aims of this chapter are to further our understanding of the ecological responses of the L4 benthic community to hypoxic exposure by investigating the relationship between community bioturbation measures and potential effects to ecosystem processes. Subsequently, the following null-hypotheses were proposed: (1) exposure to moderate hypoxia for 3 and 6 weeks does not affect the burrowing depth and activity of the L4 benthic community; (2) any consequential changes to macrofaunal behavioural (bioturbatory) activities will have no effect on ecosystem processes such as nutrient fluxes and estimated secondary productivity.

5.2. MATERIALS AND METHODS

The data presented here were generated from the mesocosm experiment presented in Chapter 4. For details on sediment and fauna collection refer to Sect. 4.2.1; for details on experimental design and holding conditions refer to

Sect. 4.2.2; for details on seawater manipulations refer to Chapter 2, Sect. 2.2.4, for details on seawater chemistry for this experiment refer to Chapter 4, Table 4.1 and for details on the feeding regime refer to Sect. 4.2.4.

5.2.1. Acquisition of bioturbation data

Acquisition of bioturbation data was completed using the same luminophore tracer technique methods outlined in Sect. 3.2.4. However, there were some methodological differences and they will be referred to below.

5.2.1.1. Image preparation and data extraction

For full details on image preparation and data extraction refer to Sect. 3.2.4.1. The luminophores (supplier, Partrac ltd., Glasgow, UK) used in this experiment differed slightly from the luminophores used in Chapter 3, as they were sourced to match the sediment granulometry of the collection site, Station L4, a mixed shell gravel site. Sediment grain size fractions for the luminophores for site L4 comprised of 28 % > 1 mm, 17 % > 250 μm and 55 % < 250 μm. The same amount of luminophores were added to each aquaria *per* cm^2 (0.2 g *per* cm^2), but due to the increase in aquarium size the total amount of luminophores added per aquaria in this experiment was 80 g. The camera was set for an exposure of 10 s, f = 5.6, ISO = 400 (pixel size = 0.00061 cm^2) and was controlled remotely *via* a PC using the software GB Timelapse, (V 3.6.1). Images were captured in RGB format and saved using a JPEG compression (sized 1280 x 853 pixels). After each photograph session, aquaria were returned to the experimental system and re-connected to their respective flow–through water treatment. Each aquarium was photographed once every 24 h (± 1 h) for a total of 6 d (n $_{images\ per\ aquarium}$ = 6). The sixth and final photograph for

each aquaria occurred on a sampling day (T0, T3 or T6). At this point, designated aquaria were removed from the experiment for further analysis. Using the software ImageJ (V. 1.4.3) all photographs were cropped to a size of 809 x 719 pixels, which removed the edges of the glass aquaria.

5.2.1.2. Quantifying bioturbation

The luminophore tracer profiles extracted from each image were used to estimate two aspects of bioturbation. Firstly, maximum luminophore penetration depth (MPD) was used as a proxy for maximum bioturbation depth, and estimated by determining the deepest image pixel row containing at least five luminophore pixels. Secondly, bioturbation activity was estimated by calculating the proportion of sediment surface reworked (SSR), measured as 100% minus the percentage of tracer left in the surficial layers (the first cm of sediment) at the end of each time point (Maire et al. 2006).

5.2.2. Calculation of ecosystem function

Ecosystem functioning, may be measured in a variety of ways (Biles et al. 2002), and can be loosely defined as processes occurring in the system, (such as productivity, decomposition, nutrient cycling, transfers between trophic positions) and ecosystem stability (resistance and resilience). During this experiment measures of ecosystem function were calculated in three different ways to give an indication of how hypoxia may affect certain processes that contribute towards ecosystem function. Macrofaunal abundance and biomass data (taken from data presented in Chapter 4) were used to calculate the first two measures of ecosystem function; (i) secondary production (P) and (ii) community bioturbation potential (BP_c), a metric first described by Solan et al.

(2004a). Nutrient samples were collected from each experimental aquarium and were used to calculate nutrient fluxes occurring within each aquarium, which provided the third and final measure to predict changes in ecosystem function.

5.2.2.1. Secondary production

Secondary production (P) was calculated using abundance (A) and biomass (B) data (collected during macrofaunal analyses in Chapter 4), using the methods outlined in Warwick & Clarke (1993), equation 5.1.

$$P = (B/A)^{0.73} * A \tag{5.1}$$

The value of 0.73 is the average exponent of the regression of annual production on body-size for macro-benthic invertebrates derived by Brey (1990) and used by Warwick & Clarke (1993) and Zhang et al. (2015).

5.2.2.2. Community bioturbation potential (BP_c)

BP_c is a widely used metric to characterise the effect of the macrofaunal community on sediment mixing, and is determined using equation 5.2 (Solan et al. 2004a, supporting online material, Zhang et al. 2015). Although not a direct measure of bioturbation, it can be used as an effective proxy for some bioturbation processes, such as the distance sediment particles move (Queirós et al. 2015, Zhang et al. 2015).

$$BPc = \sum_{i=1}^{n} \sqrt{\frac{Bi}{Ai}} \cdot Ai \cdot Mi \cdot Ri \tag{5.2}$$

The metric accounts for three biological traits known to influence sediment bioturbation: (1) B_i is the total biomass of any particular taxon, whilst A_i is the total number of individuals for that taxon, consequently B_i / A_i equates to the mean body size for that taxon; (2) propensity to move through the sediment matrix (mobility, M_i) and (3) the method of reworking sediments (reworking mode, R_i).

Mobility, M_i, was scored on a categorical scale that reflects increasing activity of the species (1 = fixed in a tube, 2 = limited movement, sessile, but not in a tube, 3 = slow movement through the sediment, 4 = free movement through the sediment *via* a burrow system) (Solan et al. 2004a, supporting online material). Reworking mode, R_i, was also scored on a categorical scale to reflect the increasing impacts on sediment turnover (1 = epifauna that bioturbate at the sediment-water interface, 2 = surficial modifiers, whose activities are limited to <1 – 2 cm of the sediment profile, 3 = head-down / head-up feeders that actively transport sediment to/from the sediment surface, 4 = biodiffusers, whose activity result in a constant and random diffusive transport of particles over short distances, 5 = regenerators that excavate holes, transferring sediment at depth to the surface (Solan et al. 2004a, supporting online material). Values for M_i and R_i for each species were taken from Queirós et al. (2013).

5.2.2.3. Nutrient analysis

Nutrient samples were taken from each experimental aquarium at time points T3 and T6 using methods detailed in Sect. 3.2.5. Fluxes across the sediment-water interface provide an estimation of the net change of nutrient x within the experimental aquaria and give an indication of the alterations in biogeochemical

cycling caused by a reduction in dissolved oxygen concentrations and also by changes in community bioturbation activity. A positive flux value indicates nutrient x is being taken up by the sediment (influx) and a negative value indicates nutrient x is being released from the sediment (efflux) into the overlying water.

5.3. STATISTICAL ANALYSES

Statistical analyses were carried out using the software package SigmaPlot 13 (ver. 13.0.0.83). The Shapiro-Wilk test for normality and the Brown-Forsythe test for homogeneity of variance were completed on each parameter measured. Where necessary, either square root or Log_{10} +1 transformations were applied. Two-way ANOVAs were used to detect differences between 'water treatment' groups (i.e. normoxic and hypoxic) and 'experimental time' groups (i.e. T3 and T6). When available, data from T0 (pre-exposure) is included in the figures, but excluded from ANOVA tests, as in the formal tests it prevented interaction analyses between 'water treatment' and 'experimental time' being completed. The hypotheses being tested are about the effects of hypoxia over 3 and 6 weeks, so although it was useful to have the initial pre-exposure data (T0) it is not always necessary to include in the formal statistical analyses unless specifically demanded by the specific questions being addressed (Somerfield, 2016, pers. comms.).

When significant differences between 'water treatment' or 'experimental time' groups were detected, *post-hoc* pairwise comparisons (Holm-Sidak method) were completed to highlight where the significant differences occurred. Only one set of data, the NH_4^+ : NO_x ratios could not be normalised by any

transformation, so were analysed using permutational analysis of variance (PERMANOVA) procedures using the software PRIMER 6 (ver. 6.1.18). To assess the relationship between nutrient fluxes and bioturbation measures, Pearson correlation analyses were conducted using the software SigmaPlot 13.

5.4. RESULTS

5.4.1. Maximum luminophore depths (MLD)

The average maximum luminophore depth (MLD) measured across all experimental aquaria was 2.96 ± 0.90 cm (mean ± 95% CI). Analyses revealed no significant effects of water treatment (normoxic or hypoxic) or experimental time (T3, T6) on MLD (Fig. 5.1, Table 5.1).
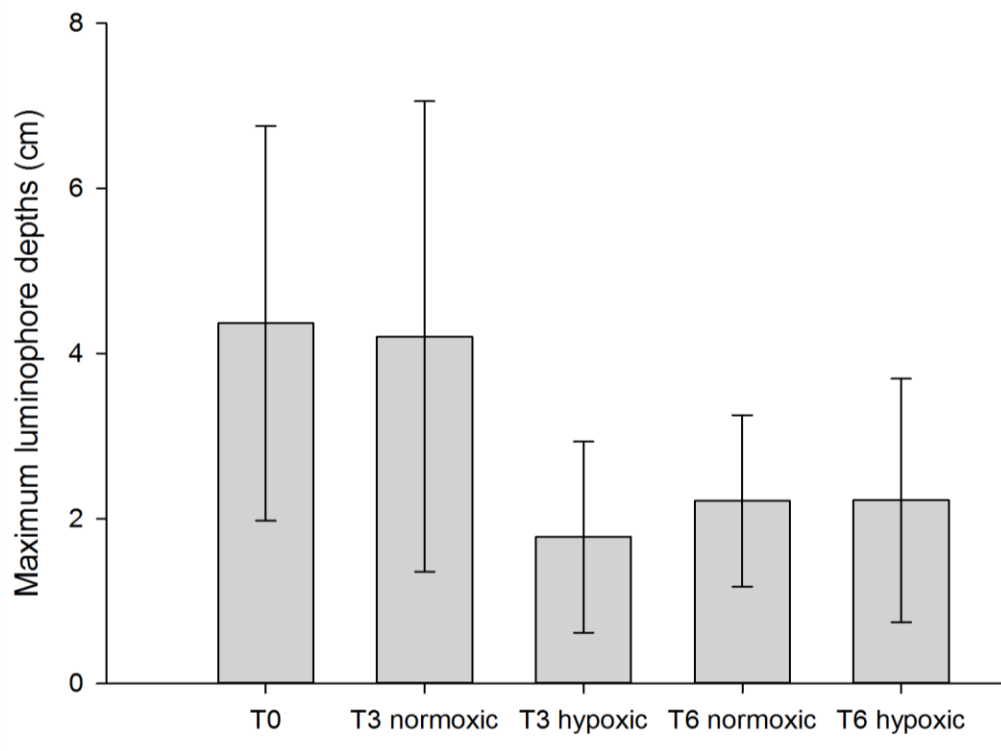
Figure 5.1. Maximum luminophore depths (MLD) (cm) across treatment and time groups. Data are means ± 95 % confidence intervals.

Table 5.1. Two-way ANOVA on maximum luminophore depth (MLD) (cm) data for experimental samples at time points T3, and T6, and water treatments normoxic and hypoxic. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	F	p
Water treatment	1	0.16	0.16	1.38	0.258
Time	1	0.013	0.13	0.12	0.739
Water treatment * Time	1	0.088	0.088	0.76	0.392
Residual	16	1.82	0.11		
Total	19	2.07	0.11		

5.4.2. Percentage of sediment surface reworked (% SSR)

Analyses revealed no significant effects of water treatment (normoxic or hypoxic) or experimental time (T3 or T6) on the percentage of sediment surface reworked (% SSR) (Fig. 5.2, Table 5.2). High levels of variability occurred within the amounts of sediment surface reworked, with values ranging from 0 – 9.77 %.
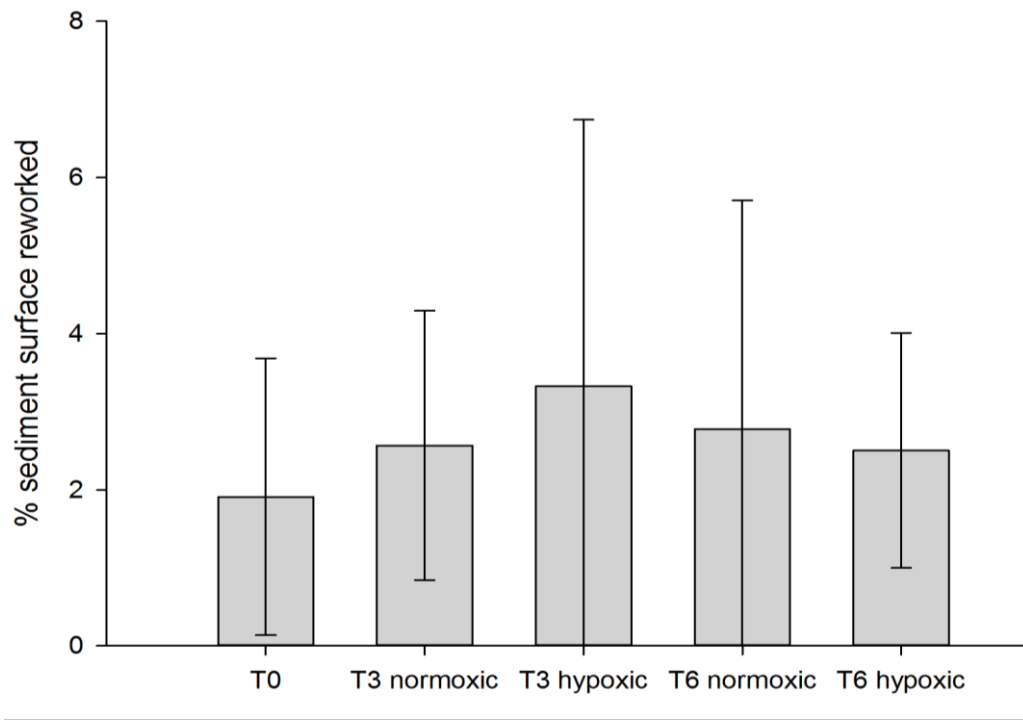
Figure 5.2. Percentage of sediment surface reworked (% SSR) across water treatment and time groups. Values presented as means ± 95 % confidence intervals.

Table 5.2. Two-way ANOVA on percentage of sediment surface reworked (% SSR) data for experimental samples at time points T3 and T6, and water treatments normoxic and hypoxic. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source	DF	SS	MS	F	p
Water treatment	1	0.020	0.020	0.049	0.827
Time	1	0.0017	0.0017	0.0041	0.949
Water treatment * Time	1	0.055	0.055	0.14	0.718
Residual	16	6.46	0.40		
Total	19	6.54	0.34		

5.4.3. Measurements of ecosystem function

5.4.3.1. Secondary productivity

Secondary Productivity estimates could be calculated for field samples in addition to mesocosm experimental samples due to the abundance and biomass data collected in Chapter 4. A two-way ANOVA between field and normoxic samples revealed there was a significant difference in secondary production estimates between these two sample groups, but no effect of time (Fig. 5.3, Table 5.3). On average the field samples had higher values for estimates of secondary productivity compared to the normoxic experimental samples. *Post-hoc* pairwise comparisons did not reveal any significant differences between individual treatment or time groups, only a significant difference of means between all field samples and all normoxic samples (field *vs.* normoxic: Diff. of means = 0.59, t = 2.20, *p* = 0.038). Further analyses examining the effect of hypoxic exposure revealed no significant differences of estimated secondary productivity between experimental samples (normoxic or hypoxic) or experimental time (T3 or T6) (Fig. 5.3, Table 5.4).

Table 5.3. Two-way ANOVA on macrofaunal secondary productivity values for field samples (initial fields, T3 fields, T6 fields) and experimental samples (T0 normoxic, T3 normoxic and T6 normoxic). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (*p*). * Significant *p* values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	*p*
Time	2	0.35	0.18	0.33	0.720
Field *vs*. Normoxic	1	2.57	2.57	4.82	0.038*
Field *vs*. Normoxic * Time	2	0.42	0.21	0.40	0.677
Residual	24	12.78	0.53		
Total	29	16.12	0.56		
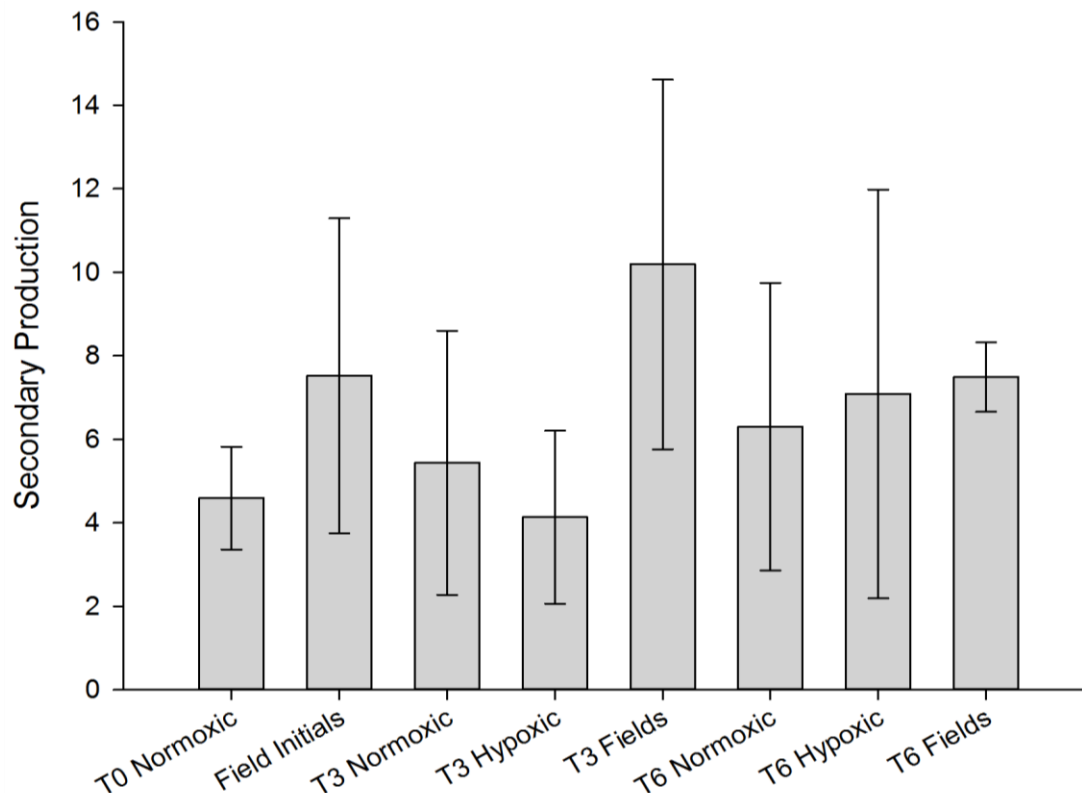
Figure 5.3. Changes in macrofaunal secondary production as calculated by Warwick & Clarke (1993) within L4 field samples and mesocosm experimental samples. Values presented as means ± 95 % confidence intervals.

Table 5.4. Two-way ANOVA on estimates of secondary productivity values for experimental samples at time points T3, and T6, and water treatments normoxic and hypoxic. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
Time	1	18.20	18.20	1.12	0.306
Water treatment	1	0.33	0.33	0.020	0.889
Water treatment * Time	1	5.46	5.46	0.34	0.571
Residual	16	260.86	16.30		
Total	19	284.85	14.99		

5.4.3.2. Community bioturbation potential (BP_c)

Community bioturbation potential (BP_c) was calculated for field samples in addition to mesocosm experimental samples. A two-way ANOVA between field and normoxic samples revealed there was a significant difference in BP_c, with field samples having higher values for BP_c compared to the normoxic experimental samples. There was no effect of experimental time (Fig. 5.4, Table 5.5). *Post-hoc* pairwise comparisons revealed that there were significant differences in BP_c between the field and normoxic samples within each individual time group (T0: Diff. of means = 1.72, t = 2.13, p = 0.043; T3: Diff. of means = 2.03, t = 2.52, p = 0.019; T6: Diff. of means = 3.24, t = 4.01, $p <$ 0.001). Further analyses examining the effect of hypoxic exposure revealed no significant differences of BP_c between experimental samples (normoxic or hypoxic) or experimental time (T3 or T6) (Fig. 5.4, Table 5.6).

Table 5.5. Two-way ANOVA on community bioturbation potential (BP_c) values for field samples (initial fields, T3 fields, T6 fields) and normoxic experimental samples (T0 normoxic, T3 normoxic, T6 normoxic). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (*p*). * Significant *p* values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	*p*
Time	2	1.43	0.72	0.44	0.649
Field *vs.* Normoxic	1	40.66	40.66	25.00	< 0.001*
Field *vs.* Normoxic * Time	2	3.20	1.60	0.99	0.388
Residual	24	39.03	1.63		
Total	29	84.32	2.91		
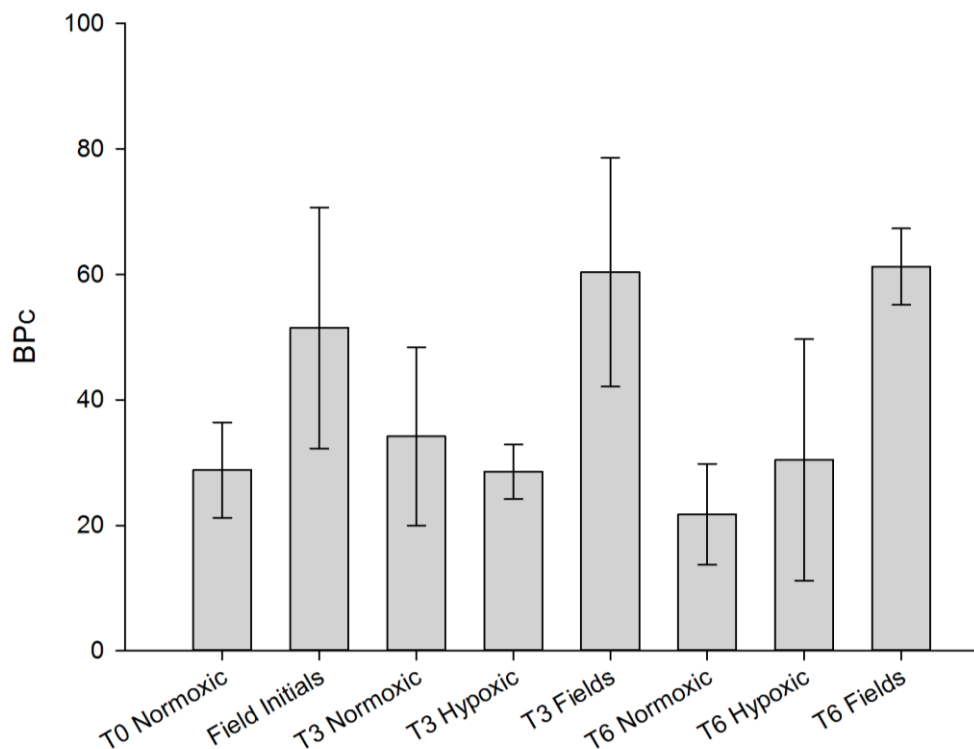
Figure 5.4. Changes in the community bioturbation potential (BP_c) within L4 field samples and mesocosm experimental samples. Values presented as means ± 95 % confidence intervals.

Table 5.6. Two-way ANOVA on community bioturbation potential (BP_c) values for experimental samples at time points T3, and T6, and water treatments normoxic and hypoxic. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
Time	1	1.78	1.78	0.81	0.382
Water treatment	1	0.11	0.11	0.050	0.826
Water treatment * Time	1	1.03	1.03	0.47	0.505
Residual	16	35.21	2.20		
Total	19	38.12	2.01		

5.4.3.3. Functional group contributions to BP_c

Contributions to total BP_c values were split up into functional groups (assigned biological trait groups used to calculate BP_c, detailed in Sect. 5.2.6.2) to assess if any particular group were most responsible for the changes in BP_c between the field and mesocosm samples, and also to assess if hypoxia had any effect on any specific functional groups. Investigating the proportions of functional groups revealed that the majority of bioturbation processes within all field and experimental samples was provided by biodiffusers ($Ri = 4$), which display slow movement through the sediment ($Mi = 3$) (Fig. 5.5, a, b). This same contribution to bioturbation for the L4 community has also been documented in Zhang et al. (2015).
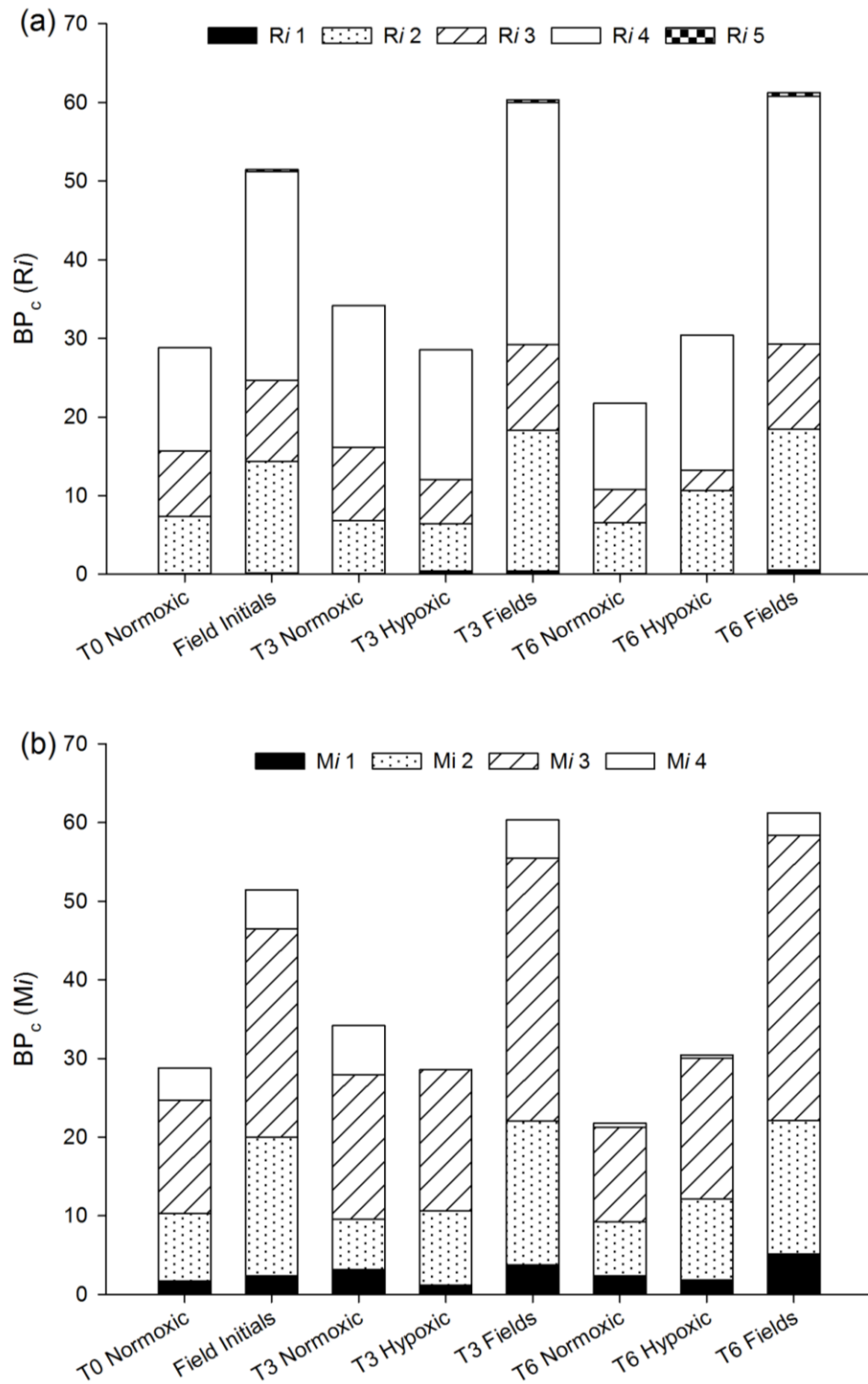
Figure 5.5. Relative contributions of each of the reworking (R_i) (a); and mobility (M_i) (b), categories to total BP_c for L4 field samples and mesocosm experimental samples. Values presented as means.

5.4.3.3.1. BP_c contributions split by sediment reworking mode (R_i)

Two-way ANOVAs between field and normoxic samples across all time points (T0, T3, T6) revealed a significant reduction in contributions to BP_c within sediment reworking (R_i) categories 2 (surficial modifiers) and 4 (biodiffusers) within the normoxic experimental samples compared to the field samples (Fig. 5.5 a, Table 5.7). No significant differences occurred in contributions to BP_c from sediment reworking (R_i) category 3 (upwards and downwards conveyors) (Table 5.7). No test could be completed for R_i categories 1 (epifauna) and 5 (regenerators) due to the high number of absent species within these R_i categories in a large number of the community samples.

Post-hoc pairwise comparisons revealed that at each individual time point (T0, T3 and T6) contributions to BP_c within R_i category 2 was consistently higher within the field samples compared to the normoxic experimental samples (T0: Diff. of means = 6.82, t = 2.13, p = 0.043; T3: Diff. of means = 11.05, t = 3.46, p = 0.002; T6: Diff. of means = 11.45, t = 3.58, p = 0.001). Within R_i category 4, post-hoc pairwise comparisons revealed that only at time points T0 and T6 were contributions to BP_c greater within the field samples compared to the normoxic experimental samples (T0: Diff. of means = 13.39, t = 2.13, p = 0.044; T3: Diff. of means = 12.79, t = 2.04, p = 0.053; T6: Diff. of means = 20.54, t = 3.27, p = 0.003).

Further analyses examining the effect of hypoxic exposure on BP_c contributions from the R_i categories revealed that water treatment (normoxic or hypoxic) and experimental time (T3 or T6) caused no significant differences in BP_c between R_i categories 2, 3, and 4 (Fig. 5.5 a, Table 5.8).

Table 5.7. Two-way ANOVA on contributions to BP_c grouped by R_i (modes of sediment reworking) for BP_c (R_i 2) (a); BP_c (R_i 3) (b); and BP_c (R_i 4) (c), on values for field samples (initial fields, T3 fields, T6 fields) and normoxic experimental samples (T0 normoxic, T3 normoxic and T6 normoxic). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
(a) BP_c (R_i 2)					
Time	2	15.25	7.63	0.30	0.744
Field *vs.* Normoxic	1	716.34	716.34	28.07	< 0.001*
Field *vs.* Normoxic * Time	2	32.94	16.47	0.65	0.533
Residual	24	612.58	25.52		
Total	29	1377.11	47.49		
(b) BP_c (R_i 3)					
Time	2	34.08	17.04	0.34	0.719
Field *vs.* Normoxic	1	85.55	85.55	1.68	0.207
Field *vs.* Normoxic * Time	2	38.17	19.08	0.38	0.691
Residual	24	1221.76	50.91		
Total	29	1379.55	47.57		
(c) BP_c (R_i 4)					
Time	2	108.65	54.33	0.55	0.584
Field *vs.* Normoxic	1	1818.20	1818.20	18.44	< 0.001*
Field *vs.* Normoxic * Time	2	92.93	46.47	0.47	0.630
Residual	24	2366.74	98.61		
Total	29	4386.53	151.26		

Table 5.8. Two-way ANOVAs on contributions to BP_c grouped by R_i (modes of sediment reworking) for BP_c (R_i 2) (a); BP_c (R_i 3) (b); and BP_c (R_i 4) (c), for experimental samples (normoxic and hypoxic) at time points T3 and T6. Due to missing data points from lack of species within some R_i categories, interaction tests between water treatment and time could not be completed. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
(a) BP_c (R_i 2)					
Time	2	24.84	12.42	0.70	0.507
Water treatment	1	13.67	13.67	0.77	0.389
Residual	21	371.56	17.69		
Total	24	408.43	17.02		
(b) BP_c (R_i 3)					
Time	2	89.26	44.63	1.37	0.277
Water treatment	1	36.96	36.96	1.13	0.299
Residual	21	685.44	32.64		
Total	24	836.45	34.85		
(c) BP_c (R_i 4)					
Time	2	56.17	28.09	0.29	0.752
Water treatment	1	29.04	29.04	0.30	0.590
Residual	21	2038.18	97.06		
Total	24	2143.16	89.30		

5.4.3.3.2. BP_c contributions split by macrofaunal propensity to move through the sediment (M_i)

Two-way ANOVAs between field and normoxic samples across all time points (T0, T3, T6) revealed a significant reduction in contributions to BP_c within macrofaunal mobility (M_i) categories 2 (limited movement) and 3 (slow, free movement through the sediment matrix) within the normoxic experimental samples compared to the field samples (Fig. 5.5 b, Table 5.9 b, c). No significant differences occurred in contributions to BP_c from mobility (M_i) category 1 (organisms that live in fixed tubes) (Table 5.9 a). No test could be completed for M_i category 4 (free movement *via* a burrow system) due to the high number of absent species within this M_i category in a large number of the community samples.

Post-hoc pairwise comparisons revealed that at each individual time point (T0, T3 and T6) contributions to BP_c within M_i category 2 were consistently higher within the field samples compared to the normoxic experimental samples (T0: Diff. of means = 9.12, t = 2.38, *p* = 0.025; T3: Diff. of means = 11.88, t = 3.11, *p* = 0.005; T6: Diff. of means = 10.13, t = 2.64, *p* = 0.014). Within M_i category 3, post-hoc pairwise comparisons revealed that only at time points T3 and T6 were contributions to BP_c greater within the field samples compared to the normoxic experimental samples (T0: Diff. of means = 12.05, t = 2.00, *p* = 0.057; T3: Diff. of means = 15.12, t = 2.51, *p* = 0.019; T6: Diff. of means = 24.22, t = 4.02, *p* < 0.001).

Further analyses examining the effect of hypoxic exposure on BP_c contributions from the M_i categories revealed that water treatment (normoxic or hypoxic) and

experimental time (T3 or T6) caused no significant differences in BP_c between

M_i categories 1, 2 and 3 (Fig. 5.5 b, Table 5.10).

Table 5.9. Two-way ANOVA on contributions to BP_c grouped by M_i (macrofaunal mobility) categories for BP_c (M_i 1) (a); BP_c (M_i 2) (b); and BP_c (M_i 3) (c), on values for field samples (initial fields, T3 fields, T6 fields) and normoxic experimental samples (T0 normoxic, T3 normoxic and T6 normoxic). Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
(a) BP_c (M_i 1)					
Time	2	16.68	8.34	2.69	0.089
Field *vs.* Normoxic	1	13.17	13.17	4.24	0.050
Field *vs.* Normoxic * Time	2	7.87	3.93	1.27	0.299
Residual	24	74.52	3.10		
Total	29	112.25	3.87		
(b) BP_c (M_i 2)					
Time	2	6.97	3.48	0.095	0.910
Field *vs.* Normoxic	1	807.93	807.93	22.06	< 0.001*
Field *vs.* Normoxic * Time	2	9.77	4.88	0.13	0.876
Residual	24	878.76	36.61		
Total	29	1703.44	58.74		
(c) BP_c (M_i 3)					
Time	2	156.52	78.26	0.86	0.435
Field *vs.* Normoxic	1	2201.82	2201.82	24.27	< 0.001*
Field *vs.* Normoxic * Time	2	200.30	100.15	1.10	0.348
Residual	24	2176.89	90.70		
Total	29	4735.54	163.29		

Table 5.10. Two-way ANOVA on contributions to BP_c grouped by M_i (macrofaunal mobility) categories for BP_c (M_i 1) (a); BP_c (M_i 2) (b); and BP_c (M_i 3) (c), for experimental samples (normoxic and hypoxic) at time points T3 and T6. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-value (F), and probability value (p). * Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
(a) BP_c (M_i 1)					
Time	1	0.042	0.042	0.015	0.905
Water treatment	1	7.79	7.79	2.72	0.119
Water treatment * Time	1	2.77	2.77	0.97	0.341
Residual	16	45.93	2.87		
Total	19	56.54	2.98		
(b) BP_c (M_i 2)					
Time	1	2.22	2.22	0.085	0.774
Water treatment	1	51.86	51.86	1.99	0.178
Water treatment * Time	1	0.13	0.13	0.0049	0.945
Residual	16	418.06	26.13		
Total	19	472.27	24.86		
(c) BP_c (M_i 3)					
Time	1	50.56	50.56	0.47	0.503
Water treatment	1	37.34	37.34	0.35	0.564
Water treatment * Time	1	50.40	50.40	0.47	0.504
Residual	16	1725.35	107.83		
Total	19	1863.64	98.09		

5.4.4. Nutrient flux results

5.4.4.1. NO$_x$ flux

To investigate the effects of 'water treatment' and 'experimental time' combined nitrate and nitrite measurements (hereafter known as NO$_x$) were examined. During the experiment NO$_x$ fluxed into the sediment in both the normoxic and hypoxic aquaria (Fig. 5.6 a). Analyses revealed that exposure to hypoxia significantly increased NO$_x$ flux into the sediment, but experimental time had no effect (Table 5.11 a).

5.4.4.2. Ammonium flux

Within all aquaria, irrespective of water treatment, ammonium generally fluxed out of the sediment into the overlying water (efflux). Analyses revealed that 'water treatment' and 'experimental time' had no significant effects on ammonium efflux (Fig. 5.6 b, Table 5.11 b). However, aquaria exposed to hypoxia do show a greater variability in ammonium efflux compared to the normoxic aquaria, possibly indicating the initial effects of hypoxic exposure.

5.4.4.3. Ratios of NH$_4^+$: NO$_x$

Concentrations (µM) of ammonium (NH$_4^+$) and NO$_x$ measured in the experimental aquaria have been presented as ratios [NH$_4^+$: NO$_x$] with the aim of emphasising sedimentary processes such as nitrification and denitrification, that may drive changes in nutrient concentrations (Fig. 5.6 c). Ratio data could not be normalised by any transformation and consequently a PERMANOVA analysis was conducted. Analyses revealed that 'water treatment' significantly affected NH$_4^+$: NO$_x$ ratios, with the normoxic aquaria displaying lower ratio

values compared to the hypoxic aquaria (Fig. 5.6 c, Table 5.11 c). 'Experimental time' had no significant effects on NH_4^+ : NO_x ratio data.
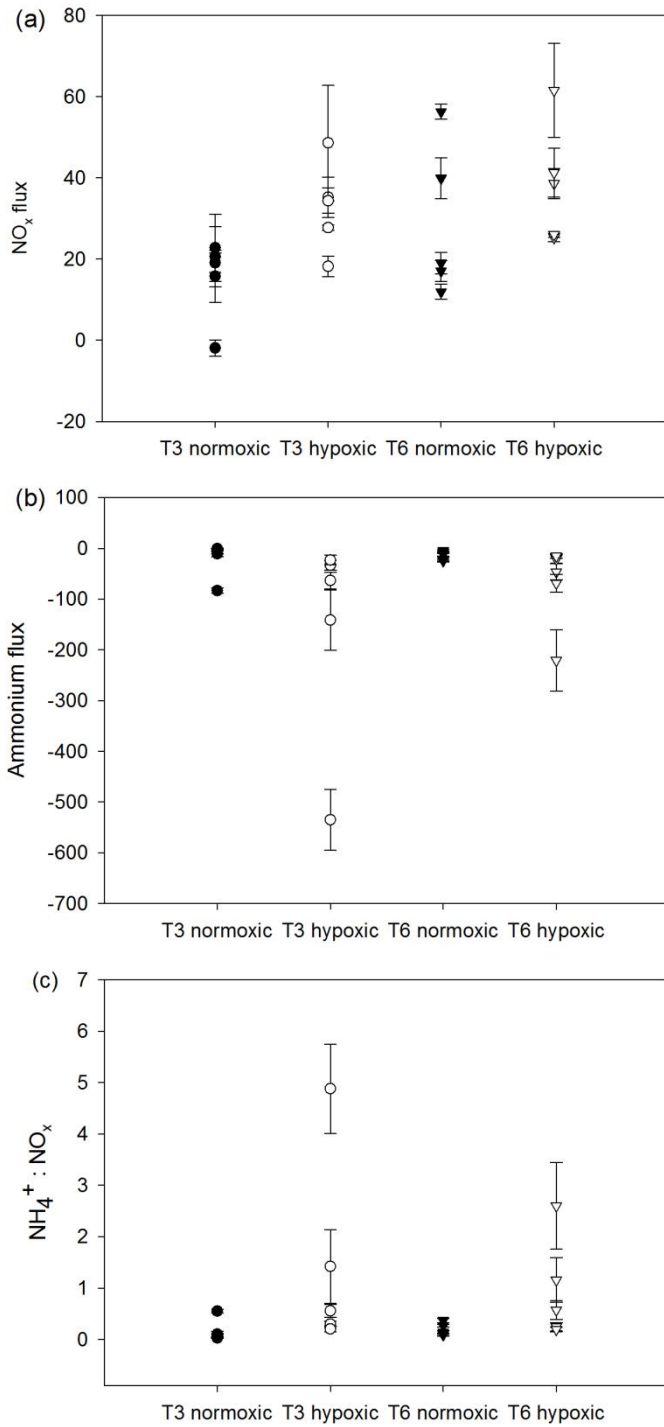
Figure 5.6. NO$_x$ flux (µmol m^{-2} h^{-1}) (a); ammonium flux (µmol m^{-2} h^{-1}) (b); and

NH$_4^+$: NO$_x$ ratios (calculated from concentrations (µM) within the aquaria) (c), in

experimental aquaria at time points T3 and T6. Data are means ± 95 %

confidence intervals. For NO$_x$ and ammonium flux, positive results represent

nutrient influx, whilst negative results represent nutrient efflux.

Table 5.11. Two-way ANOVA for NO_x flux (a); ammonium flux (b); and PERMANOVA test for NH_4^+ : NO_x ratios (c), for experimental samples (normoxic and hypoxic) at time points T3 and T6. Degrees of freedom (DF); sum of squares (SS); mean squares (MS); F-value (F); probability value (p). *Significant p values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	p
(a) NO_x flux					
Water treatment	1	930.16	930.16	4.72	0.045*
Time	1	465.75	465.75	2.36	0.144
Water treatment * Time	1	76.76	76.76	0.39	0.542
Residual	16	3156.55	197.28		
Total	19	4629.22	243.64		
(b) Ammonium flux					
Water treatment	1	1.21	1.21	2.03	0.174
Time	1	0.00033	0.00033	0.00056	0.981
Water treatment * Time	1	0.048	0.048	0.080	0.781
Residual	16	9.53	0.60		
Total	19	10.79	0.57		

(c) NH_4^+ : NO_x (PERMANOVA)

	DF	SS	MS	Pseudo-F	p(perm)	Unique perms
Water treatment	1	5.36	5.36	4.36	0.016*	998
Time	1	0.30	0.30	0.24	0.695	997
Water treatment * Time	1	0.37	0.37	0.30	0.665	996
Residual	16	19.67	1.23			
Total	19	25.69				

5.4.4.4. Phosphate flux

Within the normoxic aquaria, phosphate flux was relatively low, with data points demonstrating both phosphate efflux (release from the sediment) and phosphate influx. Within the hypoxic aquaria, phosphate consistently fluxed out of the sediment (Fig. 5.7). 'Water treatment' significantly affected phosphate flux, with the hypoxic aquaria displaying greater phosphate efflux from the sediments compared to the normoxic aquaria. 'Experimental time' had no significant effects on phosphate flux (Table 5.12 a).

5.4.4.5. Silicate flux

Within the normoxic aquaria, silicate primarily fluxed out of the sediment throughout the experimental period, but some data points indicated silicate influx (Fig. 5.7 b). Within the hypoxic aquaria, silicate consistently fluxed out of the sediment into the overlying water, with a high degree of variability within the results. Analyses revealed that 'water treatment' had a significant effect on silicate flux, with the hypoxic aquaria displaying greater efflux compared to the normoxic aquaria. 'Experimental time' had no effect on silicate flux (Table 5.12 b).
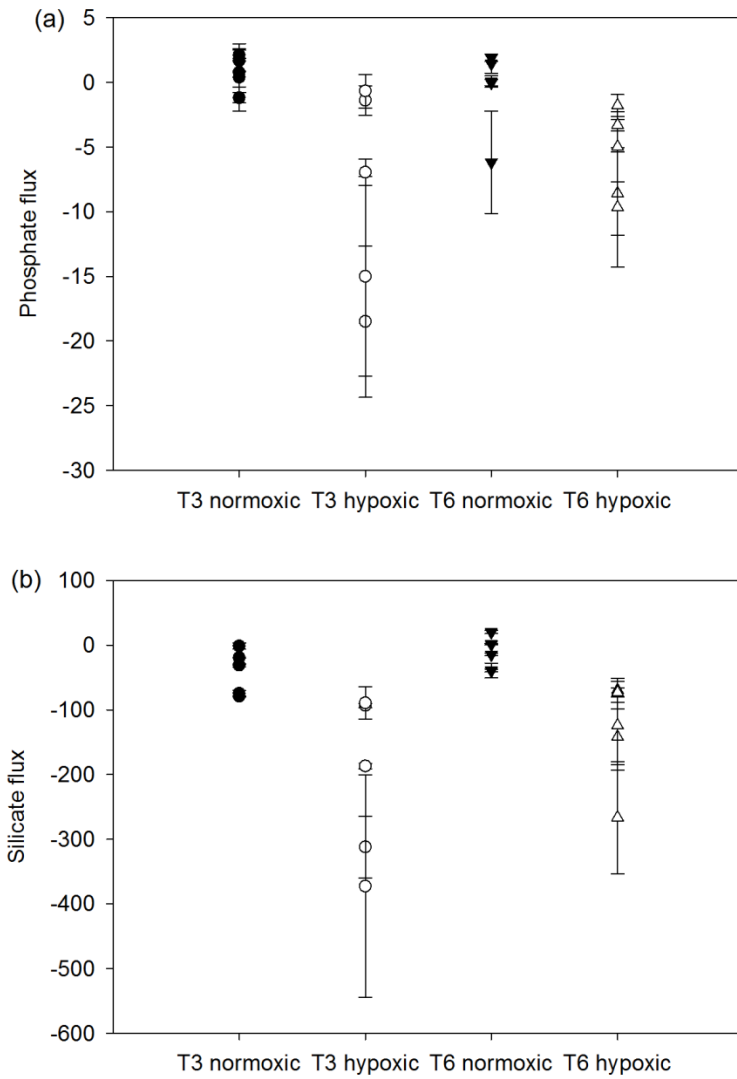
Figure 5.7. Phosphate (PO_4^{3-}) flux (µmol m^{-2} h^{-1}) (a); and silicate (SiO_4^{4-}) flux (µmol m^{-2} h^{-1}) (b), in experimental aquaria at time points T3 and T6. Data are means ± 95 % confidence intervals. Positive results represent nutrient influx, whilst negative results represent nutrient efflux.

Table 5.12. Two-way ANOVA for phosphate flux (a); and silicate flux (b), for experimental samples (normoxic and hypoxic) at time points T3 and T6. Degrees of freedom (DF); sum of squares (SS); mean squares (MS); F-value (F); probability value (*p*). * Significant *p* values (to 95 % significance level).

Source of Variation	DF	SS	MS	F	*p*
(a) Phosphate flux					
Treatment	1	257.71	257.71	11.75	0.003*
Time	1	2.94	2.94	0.13	0.719
Treatment * Time	1	21.43	21.43	0.98	0.338
Residual	16	350.97	21.94		
Total	19	633.05	33.32		
(b) Silicate flux					
Treatment	1	105583.00	105583.00	17.12	<0.001*
Time	1	13177.80	13177.80	2.14	0.163
Treatment * Time	1	2989.50	2989.50	0.49	0.496
Residual	16	98651.15	6165.70		
Total	19	220401.50	11600.08		

5.4.5. Effects of the macrofaunal community on nutrient fluxes

To assess if nutrient fluxes were affected by the macrofauna communities within the experimental aquaria, relationships between nutrient fluxes and bioturbation parameters were examined (Fig. 5.7). Pearson correlation tests were conducted for each nutrient flux against community bioturbation potential (BP_c), maximum luminophore depths (MLD) (cm) and percentage of the sediment surface reworked (% SSR). Analyses revealed no correlations between any bioturbation parameter and nutrient flux measurement (Table 5.13), indicating that any observed differences in nutrient flux results between the normoxic and hypoxic

water treatments could be discussed with reference to impacts on the microbial community rather than the macrofaunal community.

Table 5.13. Pearson correlation analyses between nutrient fluxes and bioturbation measures; NO_x flux (a); ammonium flux (b); silicate flux (c); and phosphate flux (d). Community bioturbation potential (BP_c); maximum luminophore depth (MLD) (cm); percentage of sediment surface reworked (% SSR), n = 20, * Significant p values (to 95 % significance level).

	BP_c	MLD	% SSR
(a) NO_x flux			
Correlation coefficient	0.0903	-0.1	-0.166
p-value	0.705	0.674	0.485
(b) Ammonium flux			
Correlation coefficient	-0.181	-0.143	0.065
p-value	0.444	0.547	0.785
(c) Silicate flux			
Correlation coefficient	-0.194	0.137	0.132
p-value	0.413	0.565	0.58
(d) Phosphate flux			
Correlation coefficient	-0.0837	-0.00463	0.119
p-value	0.726	0.985	0.617
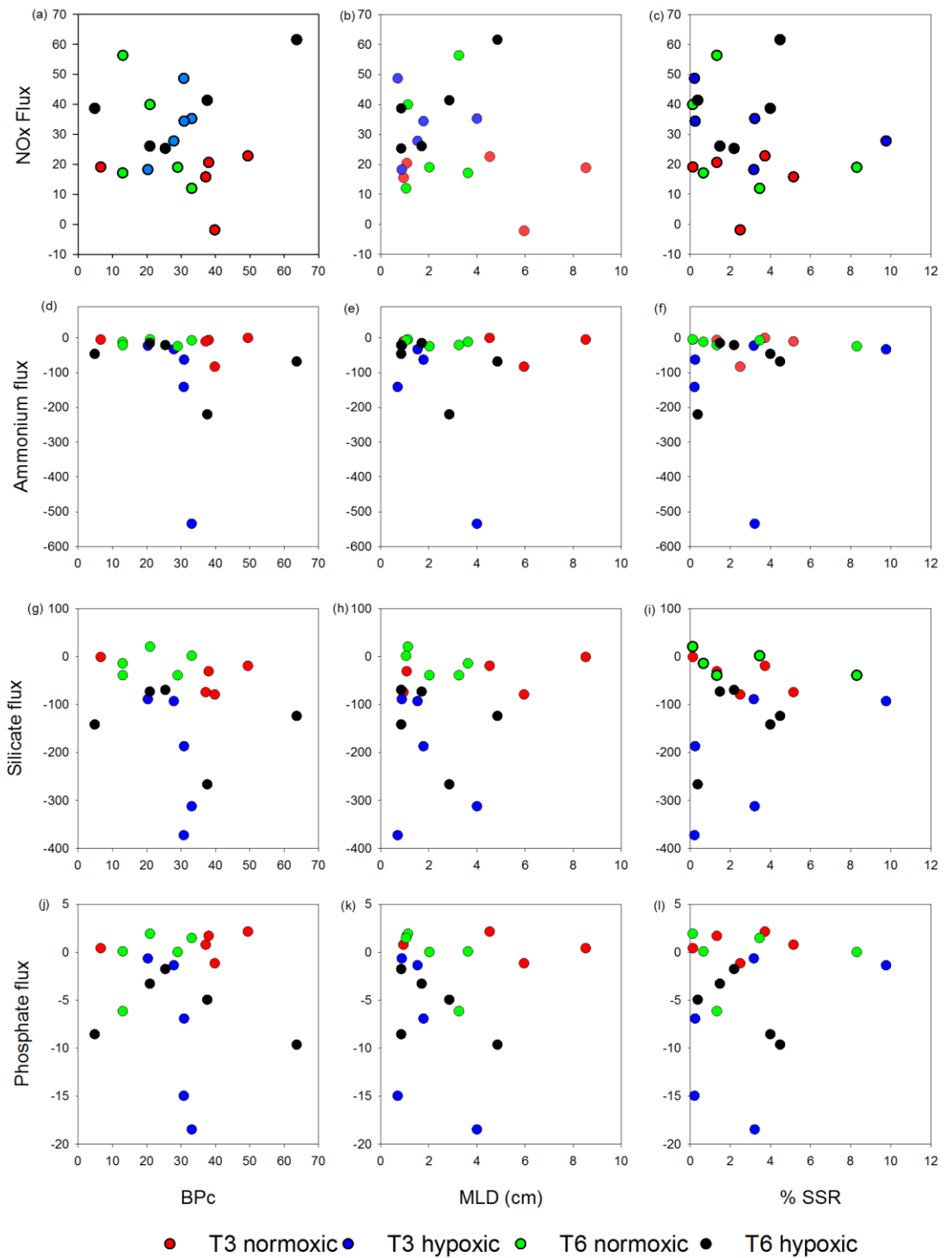
Figure 5.8. Correlation plots between nutrient fluxes and bioturbation measures; NO_x flux (μmol m^{-2} h^{-1}) (a - c); ammonium flux (μmol m^{-2} h^{-1}) (d - f); silicate flux (μmol m^{-2} h^{-1}) (g - i); and phosphate flux (μmol m^{-2} h^{-1}) (j - l). Community bioturbation potential (BP_c); maximum luminophore depths (MLD) (cm); percentage of sediment surface reworked (% SSR).

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5.5. DISCUSSION

Exposure to moderate hypoxia for three and six weeks did not significantly affect the macrofaunal community in terms of behaviour (i.e. position of organisms within the sediment, or bioturbation activity), indicating that the L4 benthic community exhibits considerable behavioural tolerance to moderate hypoxic exposure for these time frames. Most of the ecosystem processes examined (i.e. secondary productivity and community bioturbation potential) were also not affected by hypoxia, but some alterations in nutrient fluxes did occur. On closer inspection there was no evidence to suggest that the macrofaunal community contributed to any of the observed changes in nutrient fluxes, leading to acceptance of both null hypotheses set out in Sect. 5.1. whereby (1) exposure to moderate hypoxia for three and six weeks does not affect the burrowing depth and activity of the L4 benthic community and (2) any consequential changes to macrofaunal community behaviour has no effect on ecosystem processes such as nutrient fluxes and estimated secondary productivity.

5.5.1. Effects of hypoxia on measured bioturbation

Results revealed no hypoxic effects on the measured bioturbation parameter of maximum luminophore depths (MLD) indicating that organism position within the sediment and burrowing depths did not alter during the course of the experiment. Additionally, the second measured aspect of bioturbation, percentage of the sediment surface reworked (% SSR), an indicator of organism activity, was also not affected by hypoxic exposure. These results suggest considerable tolerance, in terms of behaviour, of the L4 benthic community to moderate hypoxia for durations of three and six weeks.

As discussed in Sect. 3.5.2, it is possible that the moderate level of hypoxia (3.09 mg O_2 L^{-1}) used in this experiment and in previous chapters was not severe enough to reduce burrow depths and cause changes in macrofauna behaviour with respect to organism position within the sediment. Sturdivant et al. (2012) found that during *in situ* hypoxic experiments, severe hypoxia (0.1 mg O_2 L^{-1}) reduced burrow length, burrow production and burrow depth within macrofaunal communities. Observations were conducted over a five-month period, with intermittent episodes of severe hypoxia, near anoxia and normoxia. Consequently, although some of the durations of hypoxia were comparable to this study, the severity of hypoxia was much greater than what was manipulated here, resulting in clear behavioural alterations. Rosenberg et al. (1991) conducted laboratory experiments on eight infaunal species and reported that behavioural responses (leaving protected positions within the sediment, and migrating towards the sediment surface) only occurred when oxygen concentrations were slightly higher than those found to induce mortality. More recently, controlled laboratory studies conducted by Weissberger et al. (2009) found amphipod and clam burrows to be shallower when exposed to hypoxia, but polychaete burrow depth did not change. These alterations reported in Weissberger et al. (2009) occurred under hypoxic conditions termed as 'mild', yet the level of dissolved oxygen used (2.0 mg O_2 L^{-1}), was still below the level used here. As described by Weissberger et al. (2009), polychaetes appeared to be fairly tolerant to reduced levels of DO, and did not alter their burrow depths or burrowing behaviour when DO levels were reduced to 2.0 mg O_2 L^{-1}. In this experiment, the dominant phylum within the community samples was the 'Annelida' (as documented in Chapter 4), which may offer some explanation as

to why burrow depth did not alter. Additionally, if certain phyla or groups of species did decrease their burrow depths during this experiment, the luminophore technique used here, may not have detected these distinct separations. This is because luminophores would still have been brought down into the sediment by deeper burrowing, more tolerant species (i.e. the polychaetes) preventing the detection in any shallowing of burrows from other organisms.

Consequently, due to the very nature of their life-mode within oxygen limited sediments, benthic infaunal macrofauna may not show bioturbatory behavioural changes until DO levels are severely reduced. However, alterations in % SSR were observed in Chapter 3 when *Amphiura filiformis* was exposed to moderate hypoxia, but this only occurred when brittlestar densities were high. This current chapter and previous work (Rosenberg et al. 1991, Sturdivant et al. 2012), indicate that behavioural changes within benthic communities generally occur when hypoxia is severe. Consequently, further investigations into the confounding effects of dense aggregations of benthic species and the impacts of moderate hypoxia on bioturbatory behaviour require more attention, as these particular communities or 'aggregations' could be highlighted as being 'at risk' when moderate hypoxia occurs and may need different management strategies in order to protect them and the ecosystem processes they contribute to.

5.5.2. Effects of hypoxia on ecosystem function

5.5.2.1. Effects of hypoxia on secondary productivity

During the unusually large spring bloom at Station L4 in 2012, Zhang et al. (2015) documented a strong trend for benthic macrofaunal production to

increase with response to the spring bloom. It was suggested that the changes observed in macrofaunal abundance and biomass would translate into changes in ecosystem function, such as increased secondary productivity (Zhang et al. (2015). Using abundance and biomass data from Chapter 4, the estimates for secondary productivity calculated here, indicate that experimental hypoxia had no effect on secondary productivity for each mesocosm community. This is perhaps a reflection of the fact that in Chapter 4, hypoxia had no discernible effects on the biomass, number of species or individuals within the mesocosm communities. Field samples collected alongside the experimental samples did have significantly higher secondary productivity values compared to the normoxic experimental communities, but these field samples contained higher abundance and biomass values as documented in Sect. 4.4 and Figure 4.2. It could be argued that comparing results between T3 and T6 field samples with their respective mesocosm samples is unrepresentative as the mesocosm samples were subjected to 'confinement' and a 'closed system', where only death and /or consumption would affect the community, whereas field samples were open to immigration or emigration up until they were collected. Although it is possible that this is the cause of differences between the field samples and mesocosm samples, most of the disparity between communities occurred after the first seven days and in the majority of samples, indicating that it was the translocation into the mesocosm affecting the community composition rather than large predators consuming prey confined within the aquaria for the duration of the experiment. Furthermore, there was no evidence of mass mortalities within the mesocosm samples.

In 2012, the benthic macrofauna community at L4 changed rapidly in terms of abundance and biomass, with consequential changes to estimated secondary productivity, over the period of the spring bloom (Zhang et al. 2015). Given the strong connectivity between the pelagic and benthic systems at this coastal site, and in light of the results presented in Chapter 4, in terms of translocation effects on the community when brought into mesocosm conditions, it would be preferential to examine how secondary productivity is altered under hypoxic conditions during specialised *in situ* experiments at Station L4, when influences of the spring bloom and pelagic dynamics can be incorporated into future investigations.

5.5.2.2. Effects of hypoxia on community bioturbation potential (BP$_c$)

In addition to the measured aspects of bioturbation (i.e. MLD and % SSR), a trait based approach to estimate community bioturbation potential (BP$_c$) was calculated from the species inventories recorded for each community, abundance and biomass data and organism traits associated with sediment mixing, assigned as *per* Queirós et al. (2013). This trait based approach revealed that the majority of bioturbation processes within all field and experimental samples were provided by biodiffusers (R$_i$ = 4), which display slow free movement through the sediment (M$_i$ = 3). Evidence that bioturbation activities at Station L4 are dominated by these two functional groups has previously been provided by Zhang et al. (2015) and this further confirms that, even though the number of species and individuals were reduced in mesocosm communities, translocation did not affect the dominant functional groups present within the mesocosm community samples. Calculations for BP$_c$ within the field samples were significantly higher than the normoxic and hypoxic experimental

samples, but these observed increases likely reflect the higher number of species and individuals found within these samples, as reported and discussed in Chapter 4.

Although the results from Chapter 4 indicate that hypoxia does not affect abundance, biomass, community structure or taxonomic distinctness, within the experimental communities, the effects of hypoxia on specific functional groups associated with sediment mixing were not assessed in that chapter. For example, it could be hypothesised that organisms that live within fixed tubes may be more vulnerable to hypoxia than organisms with free movement within a burrow system, possibly due to the increased mobility and irrigation capabilities of burrow system species. However, in this chapter, similarly to estimates in secondary production, exposure to hypoxia did not significantly affect values for BP_c. Furthermore, there were no significant differences in contributions to BP_c within the functional groups associated with mobility (M_i) or modes of sediment reworking (R_i). Again, this may be due to the moderate levels of hypoxia used in this experiment, with the majority of community members exhibiting tolerance to such DO levels.

BP_c has been shown to be a strong predictor of '*distance'* (the average distance travelled by particles over time) which is estimated using the Schiffer's model (Schiffers et al. 2011, Queirós et al. 2015). This relationship between BP_c (which includes important information about species traits) and sediment particle movement, supports the idea that species rich communities have a greater influence on ecosystem functioning than species poor communities, because they are more likely to contain species with different functional roles

that have larger effects on ecosystem functioning (Loreau 2000, Cardinale et al. 2002, Caliman et al. 2007). However, BP_c relies on changes in organism size and abundance, and cannot account for changes in organism activity, for example, when exposed to sub-lethal stress. Consequently, as hypoxia had no effect on abundance, biomass or functional trait groups, BP_c values remained similar within normoxic and hypoxic experimental communities, and doesn't account for potential alterations in organism activity. However, the inclusion of direct measurements of bioturbation (MLD and % SSR) help to confirm that exposure to moderate hypoxia did not affect organism bioturbation activity in terms of MLD or % SSR, during this experiment.

5.5.2.3. Alterations in nutrient fluxes

It is well accepted that benthic macrofauna influence important biogeochemical cycles, such as nutrient cycling and carbon remineralisation, through the process of bioturbation (Shull 2009, Bertics et al. 2013). However, as previously discussed, hypoxia did not significantly affect any measured aspect of bioturbation (MLD and % SSR) or the BP_c calculated for each community. Furthermore, no correlations between these bioturbation measures and any nutrient fluxes were observed. Therefore, it is reasonable to discuss changes in nutrient fluxes with respect to the potential effects of hypoxia on the microbial community and the effects of low DO levels on redox-dependent chemical reactions. However, unfortunately, redox profiles, oxygen penetration depths, microbial community analysis and bioirrigation rates were not measured during this experiment and are important parameters that could help explain nutrient flux results when under hypoxic conditions. Consequently, future investigations

should incorporate these measures to provide a more complete indication of how hypoxia affects nutrient cycling processes.

5.5.2.3.1. NO_x and ammonium flux during normoxia

For the duration of this experiment, NO_x generally fluxed into the sediment, with one normoxic aquaria at T3 being the exception (Fig. 5.6), whilst ammonium (NH_4^+) consistently fluxed out of the sediment (efflux). Similarly to the NO_x and ammonium flux results presented in Chapter 3, these nutrient sink / source properties have been documented before in nearby sites (Wood et al. 2009, Murray et al. 2013) and suggests that the rates of within sediment nitrification are not sufficient to supply all the necessary nitrate (NO_3^-) and nitrite (NO_2^-) required for processes such as denitrification and anammox. In this experiment, exposure time to hypoxia (three or six weeks) had no effects on NO_x and ammonium fluxes. This could mean that the processes driving NO_x and ammonium cycling, whether it be a change in microbial diversity or efficiency, or alterations in bioirrigation rates, could have occurred before the three-week sampling period, resulting in no 'experimental time' effects being detected.

The NH_4^+ : NO_x ratio data under normoxic conditions were low with minimal variability. This may indicate coupled nitrification and denitrification rates that remained stable during the experimental period. During normoxia, ammonium efflux was low and relatively stable across all experimental aquaria. This indicates that the ammonium available was potentially used within sediment nitrification or annamox processes, with the majority being oxidised to nitrate before efflux could occur. This oxidised ammonium, in addition to the influx of

NO$_x$ from the overlying water may have then been used by denitrifying bacteria during the process of denitrification (Kemp et al. 1990).

5.5.2.3.2. NO$_x$ and ammonium flux during hypoxia

Aquaria exposed to hypoxia had the same NO$_x$ and ammonium (NH$_4^+$) sink / source relationships as the normoxic aquaria, but significantly higher levels of NO$_x$ influx were observed. This could be explained due to amplified levels of denitrification, whereby requirements for nitrate (NO$_3^-$) and nitrite (NO$_2^-$) were increased and sourced from the overlying water. This potential increase in denitrification rates may be due to a rise in the presence of denitrifying bacteria within the hypoxic aquaria, increasing the influx of NO$_x$ into the sediments. Assessments of denitrification rates in the Gulf of Mexico zone of hypoxia, show that denitrification activity was highest at stations with a bottom water DO between 1.0 and 3.0 mg O$_2$ L^{-1}. Denitrification rates were lower at stations where DO levels were less than 1.0 mg O$_2$ L^{-1} or greater than 3.0 mg O$_2$ L^{-1} (Childs et al. 2002). In this experiment, the average DO levels within the hypoxic aquaria were 3.09 ± 0.03 mg O$_2$ L^{-1} (mean ± 95 % CI), falling closely within this optimal DO boundary for elevated denitrification rates.

Formal statistical analyses revealed no significant differences in ammonium efflux between the normoxic and hypoxic aquaria, but variability in ammonium flux within the hypoxic aquaria was much greater compared to the normoxic aquaria, possibly representing the initial signs of reduced nitrification efficiency. Nitrification requires oxygen and as such, occurs under aerobic conditions in the upper sediment layers (Abell et al. 2011). A reduction in DO levels have been shown to decrease nitrification rates in several different studies (Caffrey et al.

1993, Abell et al. 2011). However, due to the moderate level of hypoxia used in this experiment, molecular oxygen would still be available for nitrification processes, allowing the continued oxidation of ammonium. The idea that moderate hypoxia can increase denitrification rates (as discussed above), with nitrification still occurring due to the presence of oxygen, is supported by the NH_4^+ : NO_x ratio data. The hypoxic aquaria, have significantly increased NH_4^+ : NO_x ratios, indicating that nitrification and denitrification are occurring, but the increased variability in ammonium efflux indicates that the two processes are not tightly coupled as suggested within the normoxic aquaria.

Abell et al. (2011) found that decreasing DO levels in the overlying water caused a significant increase in ammonium efflux. This has been recorded in a number of settings including Chesapeake Bay (Kemp et al. 2005), the Baltic Sea (Villnäs et al. 2012), and in Danish waters (Conley et al. 2007). This increase in ammonium efflux is indicative of reduced nitrification efficiency. Abell et al. (2011) also show that even short term exposure to reduced DO levels can alter the microbial community by affecting the ability of bacterial ammonium oxidisers to express a key nitrification gene (ammonium monooxygenase, *amoA*). This rapid change in the microbial community under reduced DO conditions suggests that different groups of ammonia oxidizers demonstrate differential responses to changes in sediment DO, and should be fully investigated in order to understand how the microbial community will react and change to varying severities and durations of reduced DO and hypoxia (Abell et al. 2011).

5.5.2.3.3. Phosphate and silicate flux during normoxia

Phosphate and silicate flux displayed very similar patterns in terms of flux, both within the normoxic and hypoxic aquaria. Under normoxia, phosphate and silicate displayed very low levels of flux, with some aquaria demonstrating influx and others exhibiting efflux from the sediment.

As discussed in Sect. 3.5.5, in oxygenated conditions and oxidised areas of the sediment (i.e. burrow walls) phosphate sorption onto insoluble iron-manganese compounds can readily occur, often resulting in phosphate influx into the sediment. However, the capacity of this process is determined by the supply of Fe(III) in the sediment, which unfortunately was not quantified during this experiment. In an experiment using sediment from a nearby site, Murray et al. (2014) found no significant effects in phosphate flux when brittlestars were present compared to aquaria with no macrofauna. Additionally, in the Baltic Sea under oxic conditions, Koop et al. (1990) found that fluxes of phosphate were consistently low at all stations and were either positive or negative, as seen in this experiment.

Silicate flux within the normoxic aquaria was also low during this experiment, and displayed both influx and efflux from the sediment. Silicate efflux can occur from excretion of silicate rich waste from infauna and diatom decomposition, and silicate influx can be as a result of oxic precipitations into the sediment.

5.5.2.3.4. Phosphate and silicate flux during hypoxia

Phosphate flux out of the sediment (efflux) significantly increased under hypoxic conditions, with experimental time having no effect. When DO levels are

reduced, iron-bound phosphate can be released into pore-water as Fe(III) is reduced to Fe(II), causing efflux of phosphate (Belias et al. 2007). Phosphorous transformations are largely driven by redox-dependant chemical reactions compared to carbon, oxygen and nitrogen transformations, which are greatly influenced by biological processes (Koop et al. 1990). Although redox measurements were not taken during this experiment, the reduction in DO for three and six weeks would likely have affected redox potentials and caused phosphate release. These alterations in redox potentials may have occurred earlier than the three week sampling time, possibly explaining why the duration of hypoxia had no significant effects on phosphate release.

In Chapter 3, there was no difference in phosphate flux between the hypoxic and normoxic aquaria, and it was concluded that the moderate level of hypoxia used still allowed for some oxic absoption of phosphate to take place. However, the species used in Chapter 3 (*Amphiura filiformis*) would have had a greater influence on oxygen penetration depths through its complex burrow structures, bioirrigation activities, and high number of organisms within the aquaria. Therefore, the differences in phosphate flux observed between these two chapters, albeit at similar hypoxic levels, cannot be compared as the effects from the macrofauna present likely play an important role in oxygen penetration depths, and consequently phosphate flux.

Silicate (SiO_4^{4-}) flux out of the sediment (efflux) significantly increased under hypoxic conditions, supporting previous work (Villnäs et al. 2012) and the results presented in Chapter 3. As previously discussed in Sect. 3.5.6, the rise in silicate efflux under low DO conditions could be due to the degradation of

benthic or deposited pelagic diatoms, the release of silicate from surfaces of hydrated oxides of iron due to reduced oxic precipitation into the sediments, or from alterations in bioirrigation within the macrofaunal community. Villnäs et al. (2012) found an increase in dissolved iron (Fe) within the overlying water during hypoxic conditions, confirming the likeliness that silicate efflux was due to the release of silicate from reduced metal ions. Unfortunately dissolved Fe was not measured here, so it is difficult to conclude the exact processes causing the release of silicate, but changes in oxygen penetration depths and redox profiles may have played a large role (Weissberger et al. 2009).

5.6. CONCLUSIONS

The results presented here indicate that the L4 benthic community has exhibited considerable tolerance in terms of behaviour (i.e. bioturbatory activities) when exposed to three and six weeks moderate hypoxia. However, as discussed, the metric 'community bioturbation potential' does not account for changes in organism activity in response to sub-lethal stress, and potential shortfalls of the luminophore tracer technique, may have caused some behavioural alterations for more sensitive phyla or species to be missed. Furthermore, finer-scale investigations were not completed on the L4 benthic community, such as examinations into any potential physiological alterations, including effects to reproductive biology. Changes in organism physiology may affect individual fitness in addition to the biological traits exhibited by each species. Biological traits are the functional characteristics of each organism therefore, ecosystem processes depend greatly on the biological traits within each community assemblage. Biological Trait Analysis uses a series of life history, morphological and behavioral characteristics of the species present

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within the community to indicate aspects of their ecological functioning. The BTA approach is a useful tool to provide a link between species, environments and ecosystem processes (Bremner et al. 2006). Further investigations using a Biological Trait Analysis on the community data collected here would help summarise the biological trait composition of the L4 community assemblage and potentially highlight how changes in DO and other anthropogenic impacts may affect ecosystem functioning (Bremner et al. 2006).

Although the L4 benthic community has demonstrated hypoxic resilience thus far, effects to reproductive biology may have implications for longer-term resilience and benthic-pelagic coupling. The lack of a distinct relationship between the macrofaunal community and nutrient fluxes does not mean important ecosystem processes such as nutrient cycling are robust under hypoxic stress. The observed changes in nutrient fluxes may represent the initial effects of moderate hypoxia on microbial communities, which are essential to nutrient cycling and remineralisation. Consequently, in terms of management and protection of ecosystems and the processes within them, different levels of biological organisation may need different management strategies or 'threshold limits' to protect and maintain the important processes that occur which have a vital role in providing ecosystem services.

CHAPTER 6: GENERAL DISCUSSION

6.1. DISCUSSION

The primary intention of this final chapter is to revisit the aims and objectives set out in Chapter 1, summarising the key and novel findings within and across the chapters. It will do so by asking if there are any common themes between the individual-level and community-level biological and ecological responses to hypoxia and if the responses measured can be used to predict ecosystem level alterations to potential hypoxic events. The chapter ends by highlighting key knowledge gaps and areas for future work that would increase predictive capacity for ecosystem functioning when subjected to hypoxia.

6.2. SUMMARY OF KEY AND NOVEL FINDINGS

The original aims of this thesis were to:

(a) understand the effects of population-level processes (e.g. organism density) on individuals' biological and ecological responses to hypoxia, and

(b) determine the sensitivity of a typical U.K. coastal benthic community to hypoxia and measure the potential ecological effects.

Firstly, what sets the work presented in this thesis apart from traditional 'hypoxia' research is the use of more moderate levels of reduced DO (3.59 ± 0.04 mg O_2 L^{-1} in Chapters 2 & 3 and 3.09 ± 0.03 mg O_2 L^{-1} in Chapters 4 & 5). As discussed in Chapter 1, the term 'hypoxia' is generally applied to situations when levels of DO fall to less than 2.0 mg O_2 L^{-1} (Diaz & Rosenberg 1995, Vaquer-Sunyer & Duarte 2008), often without knowledge of the effects of that particular level of DO on organism or ecosystem function (Seibel 2011). There

is growing evidence that indicates hypoxic effects on organisms can occur at much higher DO levels, indicating the need for new criteria for establishing O_2 tolerance thresholds based on measures of physiological or ecosystem performance (Seibel & Childress 2013).

Seibel and Childress (2013) argue that, based on bioenergetic considerations, cellular metabolism is irreversible and tightly regulated within the cell. As a result, the energy obtained from the oxidation of organic matter is effectivity independent of environmental gas concentrations because, within physiological limits, organisms can maintain gas partial pressures at levels consistent with basic cellular function (Seibel & Childress 2013). Therefore, when establishing limits for oxygen tolerance, a measure of particular importance is the critical oxygen partial pressure (P_{crit}; Grieshaber et al. 1994, Seibel & Childress 2013). The P_{crit} is defined as the oxygen partial pressure (PO_2) below which metabolism cannot be regulated independently of PO_2 and may be indicated by either a reduction in oxygen consumption or an accumulation of anaerobic metabolites (Seibel & Childress 2013). Within Chapter 2, results indicated that moderate hypoxic exposure for 14 days caused reduced oxygen uptake rates in *Amphiura filiformis* with consequences to reproductive development. Although *A. filiformis* is known to be an 'oxyconformer' reducing its metabolic rate with declining PO_2, Seibel (2011) argues that oxyconformation merely describes the metabolic response of organisms to O_2 levels below their P_{crit}. Consequently, the findings from Chapter 2 support the work presented in Seibel and Childress (2013), which argues that the existing oxygen levels are the effective limits for marine life. Therefore, any reduction in PO_2 from the current level requires acclimation, adaption or migration, the capacity for which is unknown for most

species (Seibel & Childress 2013). Consequently, the need to predict how ecosystems and their associated biodiversity will respond to future anthropogenic pressures, especially deviations from current oxygen levels, requires useful criteria (above and beyond physiological limits that cause mortality) for establishing new thresholds to altered DO for species and communities (Seibel 2011). Physiological tolerances, in addition to ecosystem performance measures, provide a more complete representation of how individuals, communities and vital ecosystem processes will fair under varying levels of reduced DO. The investigations presented within this thesis have aimed to do just that.

In **Chapter 2**, impacts of moderate hypoxia on the physiological performance of *Amphiura filiformis* were investigated, whilst also examining if population density resulted in positive species interactions that enhanced survivorship. Exposure to 14 days of hypoxia significantly affected the physiological performance of *A. filiformis*, with reductions in aerobic metabolism, and disruptions to reproductive development. After 14 days exposure to hypoxia, oocyte diameter was significantly reduced and fewer oocytes reached the late-vitellogenic (final) stage of development. However, these effects occurred irrespective of brittlestar density, indicating that brittlestars did not gain any energetic advantage (or presumably have any disadvantages) from being within dense aggregations. The novel aspect of this chapter was gaining a deeper insight into the physiological reasons why hypoxia causes delayed spawning, which has been previously documented by Nilsson & Sköld (1996). This chapter demonstrated that physiological mechanisms can be disturbed during moderate hypoxia, although it is clear that the ecological consequences of hindered oocyte size

and delayed development require further attention, particularly if other species exhibit similar responses.

In **Chapter 3**, impacts of hypoxia were investigated on the bioturbatory behaviour of *A. filiformis*, and subsequently its ability to maintain its role as a key ecosystem engineer, by monitoring its ecological performance with respect to nutrient fluxes. Again, the experiment was designed to investigate the role of population density and assess whether dense populations displayed greater resilience to hypoxic stress by supporting the provision of ecosystem function. Exposure to 14 days moderate hypoxia significantly reduced brittlestar activity, in terms of the percentage of sediment surface reworked. Significant increases to ammonium and silicate efflux were also observed, possibly due to alterations in brittlestar behaviour. However, unlike Chapter 2, the alterations in brittlestar activity and nutrient fluxes only occurred when brittlestar density was high. Consequently, rather than exhibiting greater resilience to hypoxia, dense populations of *A. filiformis* may actually display larger changes in behaviour and shifts in ecosystem function as competition for oxygen and resources heighten. This new insight into the effects of population density on brittlestar behaviour when DO levels are reduced may prove of value when assessing the vulnerability of specific habitat patches to potential hypoxia, and adds further ecological relevance to future investigations. Previous work has highlighted the importance of *A. filiformis* on aspects of bioturbation (Solan et al. 2004a), with community biogenic mixing depth being dependent on whether *A. filiformis* was among the survivors in a simulated extinction event. Consequently, for functionally dominant species, such as *A. filiformis* population density is likely to be a critical factor in determining community functionality. In work examining the

effects of ocean acidification on *A. filiformis*, Wood et al. (2009) found that alterations in seawater pH can affect the relationships between brittlestar density and sediment fluxes of nitrate, ammonium, phosphate and silicate. In contrast to the results presented in Chapter 3, Wood et al. (2009) found no relationships between *A. filiformis* density and ammonium or silicate fluxes. Therefore, predictions about how population density may alter important processes such as nutrient cycling when under environmental perturbations are not straightforward.

Given that the overall provision of ecosystem function can be considered an output of the entire community, **Chapter 4**, examined and documented the effect of moderate hypoxia on a typical U.K. benthic infaunal community. It was concluded that the L4 benthic community was more sensitive to translocation and physical disturbance effects than the effects of altered DO. Nonetheless, detailed investigations into community parameters revealed that the community within the mesocosm samples, albeit that these samples were lower in species abundance and numbers of individuals than concurrently taken field samples, did actually represent the L4 natural community in terms of taxonomic distinctness. This chapter documents the first known investigation on the effects of altered DO on the L4 benthic community, one of the main sites of the Western Channel Observatory (WCO). Knowledge gained about the community's sensitivity to physical disturbance and translocation effects provides valid motives to develop specialised *in situ* hypoxia experiments at site L4, to fully understand how this important marine biodiversity reference site may be affected under hypoxic conditions.

Despite there being no significant effects of hypoxia on the structure of the L4 benthic community demonstrated in chapter 4, it was still considered possible that sub-lethal effects of community function may have occurred. Consequently, **Chapter 5** set out to further our understanding of the ecological effects of moderate hypoxia on the L4 community by assessing potential changes to key behavioural (bioturbation) and functional (nutrient fluxes and secondary productivity) processes. No significant effects of hypoxia were detected on macrofaunal behaviour (bioturbation depth or organism activity), supporting the conclusions from Chapter 4, that indicated the L4 benthic community was considerably resilient to moderately altered DO. Secondary productivity and community bioturbation potential were also not affected by hypoxia, but alterations in nutrient fluxes did occur. There was little evidence to suggest that the macrofaunal community contributed to any of the observed changes in nutrient fluxes, with the conclusion that moderate hypoxia may have rapidly affected the microbial community, causing the observed alterations in nutrient cycling. Like Chapter 4, this was the first known study to investigate how altered DO affected important ecosystem processes occurring at the L4 site. The information gained from Chapter 5 highlights the need for further detailed (and *in situ*) investigations that can assess the 'threshold' limits, in terms of maintaining ecosystem processes, for different levels of biological organisation (i.e. microbial and macrofaunal processes). This information would be invaluable for management purposes and protecting the functionality of this important area.

6.3. ARE THERE ANY COMMON THEMES BETWEEN THE INDIVIDUAL-LEVEL BIOLOGICAL AND ECOLOGICAL RESPONSES TO HYPOXIA AND COMMUNITY-LEVEL RESPONSES?

By investigating an individual species in detail, Chapters 2 & 3 show that moderate hypoxia initiates changes in organism physiology and behaviour that have the potential to impede ecosystem function. It is reasonable to assume that these individual level responses could be manifested within community-level responses, hence causing some commonalities between the results observed in all data chapters. However, Chapters 4 & 5 revealed that there were no significant effects of hypoxia on the macrofaunal community, making it difficult to assess if there were links or commonalities between the two areas of research conducted here. The L4 macrofaunal community displayed resilience to moderate hypoxia in terms of community diversity and behaviour, but no work was conducted on the physiological effects to organisms. This is a major shortfall of the community-level work, because alterations in metabolism, growth rates, reproductive biology or spawning may have major implications for community recruitment levels and biodiversity security, even after a hypoxic event. Consequently, community-level responses may indeed harbour numerous 'expressions' of individual-level responses, but to fully assess the ecological response of a community, a holistic and complete analysis of potential responses should be measured, which includes assessments of reproductive output and fecundity. The ability of organisms to maintain their metabolic rates and energy assimilation for reproductive output will undoubtedly have consequences for an organism's distribution and abundance (Wu 2002, Spicer 2014), in addition to community recovery and the processes it supports.

6.4. CAN ANY OF THE RESPONSES MEASURED BE USED TO PREDICT ECOSYSTEM LEVEL ALTERATIONS TO POTENTIAL HYPOXIC EVENTS?

The results from each chapter presented here clearly indicate that different effects occur at the individual and community level when dissolved oxygen levels are reduced, (~ 3.09 - 3.59 mg O_2 L^{-1}) but not severely hypoxic. Furthermore, shortfalls of the community level investigations, i.e. translocation / mesocosm effects and lack of information on potential physiological responses, mean that the information gained has been valuable, but opens up further avenues for future work. Despite this, the data generated within Chapters 2, 3, 4 & 5 could be used to help predict ecosystem level alterations by contributing to the parameterisation of ecosystem functioning models such as the European Regional Seas Ecosystem Model (ERSEM). ERSEM is one of the most established ecosystem models and has evolved significantly since the early nineties into a generic tool for ecosystem simulations from shelf seas to the global ocean (Butenschön et al. 2016). The model incorporates complex interactions and feedbacks between numerous ecosystem elements with a detailed benthic biological sub-model that includes bioirrigation, bioturbation, nutrient fluxes, distribution of detritus and a food-web structure based on functionality (Blackford 1997). ERSEM does include the O_2 cycle, photosynthesis and respiration of various biological components, air-sea exchange properties and there is some process-dependency based on oxygen concentrations, but incorporating hypoxia as an environmental stressor that could impact on organism abundance, biomass or bioturbation properties is an emerging property of the model that will likely be developed in the near future (Blackford 2016 pers. comms.). Therefore, in short, the knowledge gained from this thesis alongside future experimental work could be used to aid the initial

parameterisations of how ecosystem processes may be altered under various scenarios of hypoxia and improve predicative capacity for ocean systems.

6.5. KNOWLEDGE GAPS AND FUTURE STUDY

6.5.1. Greater knowledge on the effects of 'moderate' hypoxia is required

Further work on the biological and ecological effects of reduced DO, i.e. less than an arbitrary value perceived as 'normal' (Seibel 2011) is urgently required. The justification for this conclusion is as follows; (1) the results presented in this thesis indicate that individual and community level responses are variable and complex and cannot singlehandedly contribute to predictions on the ecological effects of hypoxia, (2) there is a growing consensus and empirical evidence to suggest that the previously defined 'hypoxic' figure of 2.0 mg O_2 L^{-1} is inadequate to describe the onset of hypoxia impacts for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel 2011, Calder-Potts et al. 2015), and (3) hypoxia occurs on different time and spatial scales, ranging from areas with persistent oxygen deficiency to localised short-term events. Therefore, oxygen thresholds should be considered in a dynamic context with appropriate management strategies for specific areas, habitats and biological levels of organisation.

6.5.2. Greater knowledge on the effects of 'moderate' hypoxia on the reproductive systems of functionally important species

The effect of moderate hypoxia on the reproductive and endocrine systems of functionally important marine organisms deserves more attention. Before the investigations documented in this thesis took place, it was already known that hypoxia (1.8 and 2.7 mg O_2 L^{-1}) delayed spawning activity in *A. filiformis* but the

physiological mechanisms behind this response were not uncovered (Nilsson & Sköld 1996). Chapter 2 exposed strong evidence as to why spawning is delayed under hypoxia, namely a reduction in oocyte size and reduced developmental progression. However, it was unclear if these responses were a strategic delay by the organism or if energy assimilation for reproductive output was severely reduced. Delayed spawning and reduced fecundity may impact on how communities recover after a hypoxic event, both in terms of species diversity and functionality. Complete recovery of organism-sediment interactions is a necessary condition for ecosystem functioning recovery (Van Colen et al. 2012), especially if the species affected are functionally dominant. Therefore, information based on physiological alterations that may affect population dynamics can be used to help predict what processes are vulnerable, the effects to benthic-pelagic coupling, and the longer term effects to community dynamics if repeated hypoxic events occur.

6.5.3. Greater inclusion of population dynamics in future investigations

In the context of environmental change, organisms, populations and communities do not respond to approximated global averages (Walther et al. 2002). Rather, regional changes will occur, that will exhibit heterogeneity in their ecological response depending on local dynamics, because responses by individuals are connected through interactions with others at the same or adjacent trophic levels (Walther 2010). Consequently, including parameters such as population density into future studies, not only makes investigations ecologically relevant, it can reveal unexpected consequences as *per* the results in Chapter 3, whereby brittlestars in denser treatments actually exhibited greater behavioural changes than those in sparser conditions. To further our

knowledge on the specific effects of moderate hypoxia on communities, a better understanding of the mechanisms and processes behind the observed species responses are needed. This includes understanding population dynamics and their associated complex interactions, such as how or if density-dependant processes facilitate or hinder individuals within communities. The knowledge gained by including such parameters into future investigations, may help highlight which populations may be at risk from environmental alterations, especially for species such as *A. filiformis* that are functionally dominant and exhibit vast disparities in their population densities.

6.5.4. Increased use of *in situ* hypoxia experiments

Mesocosms are useful tools for the establishment of causal relationships under controlled conditions (Kraufvelin 1998) but as discussed in Chapter 4, have their limitations and may cause effects beyond the investigators control. The development of *in situ* hypoxia experiments is not a new idea. Stachowitsch et al. (2007) developed the '*experimental anoxia generating unit' (EAGU)* which combines photo-documentation with chemo-physical water analyses to capture macrofaunal behaviour and mortality events during oxygen depletion. Villnäs et al. (2012) artificially-induced hypoxia by fixing black low density polyethylene (LDPE) sheets to the seafloor, and Studivant et al. (2012) used a benthic observing system (Wormcam) to transmit *in situ* images and water quality data during naturally occurring hypoxic events. More complex systems such as the 'free-ocean CO_2 enrichment' (FOCE) device have allowed *in situ* perturbation experiments, analogous to the pelagic mesocosms, to assess the long-term impacts of ocean acidification on benthic organisms, communities, and ecosystems. Future directions for FOCE include attempting to combine ocean

warming and decreased DO effects, designs for which are already underway at MBARI, in line with the expected expansion of a 'dead-zone' in Monterey Bay, U.S.A. (Gattuso et al. 2014). Future development and more widespread use of such systems mentioned above would increase our knowledge about how communities in specific locations may respond to hypoxia and other environmental perturbations. However, in addition to community-level alterations, advancements in measuring the functional processes that change would be advantageous, and highly relevant for protecting overall ecosystem functioning.

6.5.5. Considering marine hypoxia in a wider context

Future predictions for marine hypoxia should be considered within a wider context of the changing world. Increasing atmospheric carbon dioxide (CO_2) is the major driver of increasing global temperatures, with a projected increase of ~2.7 °C between the 1990s and the 2090s (IPCC, 2014). Additionally, atmospheric CO_2 levels are affecting ocean pH, pCO_2 levels and carbonate saturation states, a process known as ocean acidification. Ocean pH is predicted to decrease by 0.4 units by the end of this century, and possibly by 0.7 units by 2250 (Caldeira & Wickett 2003). Furthermore, with current global warming predictions, higher temperatures are expected to directly lower oxygen concentrations and enhance stratification, reducing the flow of well-ventilated surface waters to the ocean interior (Gnanadesikan et al. 2011). Ocean models predict declines of 1 to 7 % in global oxygen concentrations over the next century, with declines continuing for thousands of years (Keeling et al. 2010). Deoxygenation is closely linked to acidification through several mechanisms; aerobic respiration uses oxygen and releases carbon dioxide, thus in

metabolically active coastal waters, changes in these two parameters are often correlated and should be considered together, and in the wider context of global change (Breitburg et al. 2015).

Consequently, predicating the effect of an individual stressor in isolation is useful in determining the effect on specific mechanisms, but may also be limiting, given the potential for multiple stressors to interact and occur simultaneously as predicted for the future (Breitburg et al. 2015). Thus, there is a strong need for understanding 'multiple stressors', their interactions, potential effects and prospective management strategies. However, it may be challenging to advance in this area, when there are still considerable knowledge gaps in understanding isolated stressors, i.e. the effects and thresholds of 'moderate' hypoxia on communities and ecosystem processes.

6.5.6. Reconsidering ecosystem management in times of change

Nutrient input reductions are currently the main options for reducing eutrophication in coastal areas and consequently limit the potential for hypoxia to occur. The minimisation of eutrophication effects is specifically mentioned as a requirement in the European Union's Marine Strategy Framework Directive (MSFD) (European Commission 2008). The MSFD requires (EU) member states to apply an ecosystem approach to the management of human activities with the aim to achieve 'Good Environmental Status' (GEnS) by 2020 (Alexander et al. 2015). However, evidence increasingly suggests that ecosystems are 'open, complex and dynamic systems that are characteristically transient and unstable' which makes the goal of ecosystem management harder to define and achieve (Tett et al. 2013). Furthermore, under future global

change scenarios, eutrophication and nutrient inputs may not be the primary determinant governing coastal hypoxia. Therefore, developing an ecosystem-based approach to management strategies will become harder as the need to understand the sources, interactive effects and emerging consequences of co-occurring stressors will increase (Breitburg et al. 2015). If science is to inform policy debate and help implement its outcome, exploring ecosystems in terms of their structure and function, whilst trying to define 'ecosystem health' may be a challenging but logical approach (Tett et al. 2013).

The new knowledge presented in this thesis on the effects of moderate hypoxia, open discussions about what level of reduced DO is acceptable, how should hypoxia be defined for management purposes and what are the end point-effects that should be monitored? Clearly, when considering the effects to organisms, mortality and migratory behaviour is too extreme, and does not offer protection to ecosystem processes. The work conducted here provides evidence that physiological and behavioural changes in macrofaunal organisms can impact on certain ecosystem processes, with further evidence suggesting that the microbial community may be affected prior to the macrofaunal community. Consequently, different levels of biological organisation may have different 'threshold' limits and require different management strategies.

This thesis represents an important step towards a better characterisation of natural systems and how they respond to reduced DO. The results presented here demonstrate that the organism and community response to reduced DO is complex and multifaceted. The assumption that lowered DO will cause a straightforward reduction to community composition and biodiversity, has been

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shown not to be the case. In contrast, this thesis provides evidence of subtle changes at multiple levels that have the potential to effect community dynamics and the functions that they support. Although protecting biodiversity is important, maintaining and protecting the processes that contribute to ecosystem function, in addition to understanding what effects them, will help to minimise the negative impacts of environmental perturbations in the future. Therefore, further effort is needed for the improved understanding of how reductions in DO can cause subtle environmental effects and what these changes mean in relation to ecosystem function in a rapidly changing world.

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APPENDIX A – PUBLISHED MANUSCRIPTS

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