

Unpacking Effects of Multiple Stressors on Estuarine Meiobenthos

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Abstract

Estuaries are increasingly exposed to a multitude of stressors, introduced by anthropogenic activities. Although these stressors often overlap in time and space, ecological studies predominantly consider their impacts in isolation of one another, and often through highly contrived experiments in the laboratory. Of the relatively few multiple stressor studies that have been done, the great majority provide stressors simultaneously, failing to acknowledge that stressors may occur asynchronously, and that the timing and order of stressors may influence their cumulative impact. This thesis considered how two important estuarine stressors – nutrient enrichment and physical disturbance of sediments – independently and interactively influence estuarine meiobenthic communities. The meiobenthos, though important links in food webs and critical to nutrient cycling, are a rarely studied group, especially along the east coast of Australia.

First, to assess the spatial scales across which estuarine meiobenthos naturally vary, and how this relates to estuarine nutrient loading, an observational field study was conducted. Meiobenthos were sampled from seagrass beds at 16 sites, of 8 estuaries, spanning ~1000 km of the coast of New South Wales, Australia. Four of the estuaries had elevated nutrient loadings, relative to pre-European settlement, while 4 were relatively unmodified. I found that across the sites sampled, sediment grain size was a more important determinant of the meiobenthic community structure than nutrient load or other predictor variables such as latitude.

Second, using small-scale field experiments I investigated how nutrient enrichment and physical disturbance interact to influence meiobenthic communities at each of two field sites. Whereas previous mesocosm experiments found that the two stressors have interactive effects, I found predominantly additive effects. Effects varied between the two study sites, demonstrating the importance of environmental factors in mediating stressor impacts.

Finally, I assessed how the synchrony and order of the application of the two stressors (nutrient enrichment and physical disturbance) influences their cumulative impact. The study found that although total abundance and Shannon diversity were insensitive to the timing of stressor application, taxonomic richness was more negatively impacted by simultaneous than sequential stressor application. Individual taxa varied in their response to the

timing and order of stressor application, with responses often site-dependent. This was expected given that taxa vary markedly in their tolerance to particular stressor.

My research has made an important contribution to stressor ecology, providing one of the first empirical tests of ecological theory regarding the effect of the synchrony of multiple stressors on their cumulative impact. It has also addressed a major knowledge gap regarding the sensitivity of meiobenthic communities of south-east Australian estuaries to two major stressors. The knowledge generated by this thesis will assist estuarine managers and policy makers to develop strategies for managing and mitigating a variety of multiple stressor scenarios.

Statement of candidate

I certify that the work in this thesis entitled “Unpacking Effects of Multiple Stressors on Estuarine Meiobenthos” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

_____ Date: 14/10/2019

Ramila Furtado

Contributions:

The content within this thesis has been prepared for publication as follows:

Chapter 1: General Introduction

I completed the literature review and wrote this chapter with constructive feedback from my supervisor, Melanie Bishop

Chapter 2: Sources of spatial variation in meiobenthic assemblages of *Zostera muelleri* in estuaries of New South Wales, Australia

Authors: Ramila Furtado, Lara Ainley, Melanie J. Bishop

This chapter is formatted for submission to *Estuarine, Coastal and Shelf Science*. My contribution to the research paper: concept 70%, data collection 95%, analysis 100%, writing 80%. Total 86.25%. Melanie Bishop provided the original concept for the research. I received constructive help in the analysis and writing from Melanie Bishop.

Chapter 3: Multiple stressor effects of nutrient enrichment and physical disturbance on estuarine meiobenthos – A field study.

Authors: Ramila Furtado, Joseph M. Kenworthy, David M. Paterson and Melanie J. Bishop

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This chapter was presented at the following conference:

Chapter 4: Simultaneous or sequential: the timing and order of multiple stressor applications influences impacts to estuarine meiobenthic communities

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Chapter 5: Discussion

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“Take courage, my daughter; the Lord of heaven grants you joy” Tobit 7:16

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Chapter 1

General Introduction

1.1 The imperative of understanding stressor effects

An exponential increase in the global human population, from 1 billion in 1804 to 6 billion in 1990, growing technological capabilities and a greater standard of living have increased the amount of stress humans are placing on natural resources and the environment (Sanderson et al. 2001, Halpern et al. 2007, Cairns 2013). Excessive environmental stress harms the ecological life support system that provides necessary ecosystem services, such as maintenance of clean air, clean water and food (Breitberg et al. 1999). Such negative effects of humans on the structure and function of biological systems are not only of concern to biological and environmental scientists, and to global economists but to every person on the planet (Parker et al. 1999). Understanding how ecosystems respond to stressors is critical to effective natural resource management, and hence maintenance of biodiversity and its ecosystem functions (Griffen et al. 2016, Van den Brink et al. 2019, Maher et al. 2019).

1.2 Stressor ecology

The terms “stress” and “stressor” were first coined by Hans Hugo Bruno Selye, in the medical field of Physiology. He defined stress at a cell to whole organism level as a “nonspecific response of the biological organism to any demand” and “stressors” as the agents or situations causing such demand (Mason 1975, Fink, 2010). In the ecological literature, definitions of stress and stressor have been the subject of much discussion. Barrett et al. (1976) defined stress “as a perturbation (stressor) applied to a system (a) which is foreign to that system or (b) which is natural to that system but applied at an excessive level”, where ‘the system’ encompasses any level of biological organisation of interest (i.e. cells to ecosystems). Odum (1985) defined “stress” as a syndrome comprising both input and output, where the former is a stimulus and the latter is a response, where the input or stimulus is referred

to as a “stressor”. According to Odum, stress is the effect that is caused by a stressor. Underwood (1989) broadly defined stress as “any environmental change in a factor that causes some response by a population of interest”.

Additionally, within the ecological literature, the terms disturbance, stress and perturbation are often used synonymously (Connell 1978, Bender et al. 1984, Rapport et al. 1985, Rykiel 1985, Parker et al. 1999, Borics et al. 2013). Pickett & White (1985) define disturbance as “any discrete event in time that disrupts ecosystems, community or population structure and changes resources, substrate availability or the physical environment”. An extension was made by Pickett et al. (1989), where he described “Disturbance is a change in the minimal structure caused by a factor external to the level of interest”. Shea et al. (2004) define disturbance as an event which “alters the niche opportunities available to the species in a system”. While According to Sousa (1984), disturbance is defined as “...a discrete, punctuated killing, displacement, or damaging of one or more individuals (or colonies) that directly or indirectly creates an opportunity for new individuals (or colonies) to become established.” Disturbances can have non-significant, positive or negative consequences (Rykiel 1985). Rykiel (1985) defines perturbation as “the response of an ecological component or system to disturbance or other ecological process as indicated by deviations in the values describing the properties of the component or system; relative to a specified reference condition; characterized by direction, magnitude, and persistence”. A perturbation is change in environmental conditions that elicits a change in an ecosystem (Petraitis et al. 1989). Loucks (1975) establishes “perturbation”, wherein the whole system changes direction, due to a change in a parameter that defines the system itself. Accepting the interchangeability of terms, Parker et al. (1999) proposed a working definition of stress as “a detrimental effect on some biological entity (from individual to community level) which occurs following some disturbance (or stressors)”. It is this definition of ‘stress’ and ‘stressor’ that is used in this thesis.

Stressors may impact biological systems at levels of organisation ranging from sub-cellular to whole ecosystems (Sanders 1993, Iwama et al. 2006, Love et

al. 2013). Additionally, stressors may be specific to individual stages of an organism's life history (embryos, larvae, adults) or affect these more generally, with the early life-history stages often the most sensitive (Przeslawski et al. 2015). In some instances, stressors acting at sub-cellular levels may propagate to affect whole populations, by influencing the growth, survival and reproduction of individuals (Sanders, 1993; Götze et al. 2014). There is increasing evidence of stressors affecting the cellular mechanisms to deal with stress (Senst & Bains 2014, Van Oosten-Hawle & Morimoto 2014). Hayward et al. (2014) outlines how subcellular level impacts of extreme cold stress can impact invertebrates at the level of individuals by altering the ratio of saturated to unsaturated lipids in cell membranes, causing death. Population-level impacts are propagated by biological interactions (e.g. competition, facilitation) and networks such as the flow of energy through food webs and detrital pathways. For example, the differential sensitivity of species to sediment hypoxia can result in changes to community structure, with declines in the abundances of some species providing opportunities for others: suspension feeders are replaced by deposit feeders, demersal fish by pelagic fish, macrobenthos by meiobenthos. Microflagellates and nanoplankton also tend to dominate in the phytoplankton community in hypoxic environments (Gray et al. 2002, Wu 2002). At the community level, this may cause changes in biomass, species richness and identity, with flow-on effects to ecosystems functions.

Disturbances or stressors may be environmental fluctuations or discrete events, of natural or anthropogenic origin. They may be caused by biotic (e.g. invasive species) or physical (e.g. dredging) or chemical (e.g. contaminants) abiotic factors (Scheltinga et al. 2004; Vinebrooke et al. 2004). Irrespective of their source, disturbances and stressors can be described according to their spatial and temporal scale, frequency (occurrence number and turnover time), magnitude (intensity and severity), co-occurrence with other disturbances and novelty (i.e. whether they have been experienced by a biological system before) (Paine & Levin 1981, Kelly & Harwell 1989). Where biological systems lack the mechanisms to recover from a stressor, they may completely break down even with small amounts of stress and never recover (Kelly & Harwell

1989). Anthropogenic activities may introduce novel stressors, or produce changes in the disturbance regimes of existing stressors (Barrett et al. 1976, Halpern et al. 2007, 2008, 2015, Crain et al. 2008).

The spatial and temporal scales of disturbances influence the nature of their impacts, and the mechanisms by which affected biological systems can recover (Connell et al. 1997, Svensson et al. 2012). The spatial scale of disturbances can range from global (e.g. sea-level rise) to local (e.g. coastal erosion), or even patch scale (e.g. bait digging) (Kaiser et al. 2001, Roxburgh et al. 2004, Feagin et al. 2005, Nicholls & Tol 2006). The frequency and duration of disturbances can also vary markedly. Some stressors, such as oil spills affect a locality only once, whereas others such as bushfires may reoccur at intervals of several years (Roxburgh et al. 2004). These stressors may be lasting (chronic) or short-term (acute) (Sousa 1984, Pickett & White 1985, Connell 1985). Whereas following small-scale disturbances, that affect only a sub-set of a species' range, recovery may be possible through recolonization or recruitment processes, global disturbances may negatively impact the entire population. Connectivity of affected with unaffected populations is critical to this recovery. Many species exist as metapopulations (*sensu* Levins 1969), whereby they are distributed across a network of habitat patches (spatially separated) that interact at some level. Thus when stressors lead to extinction of local population, the recovery could be overcome by migration or colonisation of species from other spatially fragmented populations (Hanski 1998).

The frequency (time since the last disturbance) of disturbances influences the time available for recovery (Pickett & White 1985). The Intermediate Disturbance Hypothesis posits that community structure is non-linearly influenced by the frequency of disturbances (Connell 1978). At high levels of disturbance, diversity is typically low, because only opportunistic species are able to recolonise before the next disturbance event. At low frequencies of disturbance, diversity is also typically low, because there is sufficient time for competitively superior species to colonise and outcompete opportunistic species. Diversity is posited to be greatest at intermediate frequencies of

disturbance, as the abundances of competitively dominant species are kept in check, allowing opportunists to coexist. The disturbance frequency at which diversity peaks may be influenced by the productivity of the system, with more frequent disturbances required to maximise the biodiversity of productive systems (Dynamic Equilibrium Hypothesis, Huston 1979). Whereas organisms may be able to resist disturbances of short duration (e.g. oysters can shut their valves for periods of up to few weeks to avoid suboptimal environmental conditions during flood events), such resistance mechanisms may break down over longer time scales (i.e. oysters eventually need to open their valves to feed).

The intensity (vigour) of the disturbing stressor is also an important aspect, influencing the way the impacts may progress. For example, the intensity of a cyclone is ranked from a category 1 to 5, where 5 is the most severe. When the disturbance is of low intensity, impacts may be countered by increased growth of affected organisms (Kondoh 2001) but such mechanisms may be insufficient to compensate for loss of species following severe disturbances. The recovery of communities or species is influenced by disturbance intensity as well as productivity of the system (Kondoh 2001, Svensson et al. 2012). Whether a disturbing stressor is press (sustained) or pulse (relatively instantaneous) can also determine effects and response-recovery (Bender et al. 1984).

1.3 Multiple stressor effects

Natural disturbances have always shaped ecosystem processes and evolution, but with rapid human population growth, the number and range of stressor types has increased (Halpern et al. 2007, 2008, 2015, O'Brien et al. 2019). The successful management of these multiple stressors to minimise stress to ecological systems requires understanding patterns of their co-occurrence and the ways in which they interact to influence biodiversity and ecosystem functioning (Frost et al. 1999, Crain et al. 2008,). To the contrary, much of our understanding of stress ecology comes from studies examining effects of single stressors, manipulated in isolation of one another, (Crain et al. 2008, Wernberg et. al. 2012, O'Brien et al. 2019). While these studies have been useful in enhancing our understanding of how aspects of disturbance regime

(i.e. frequency, intensity, scale) interact to influence impact, they may give an oversimplified picture that over or under estimates the resilience of natural ecosystems to stressors because they fail to take into consideration that multiple stressors may have non-additive effects (Breitburg et al. 1999; Lange & Marshall 2017, O' Brien et al. 2019). While the necessity of understanding multiple stressor interactions was identified decades ago (Breitburg et al. 1999), only recently have there been attempts to quantify the underlying interactions to support ecosystem management (Halpern et al. 2008, 2015, Crain et al. 2008).

There is increasing evidence that multiple stressors do not always produce additive effects, and instead often interact to give synergistic or antagonistic effects (Folt et al. 1999, Crain et al. 2008) Additive effects occur where the combined effects of multiple stressors equal the sum of their independent negative effects. For example the effect of increased temperature and reduced pH on the reef building coral *Porites panamensis* are additive because when the stressors are applied together, the reduction in polyps of 45% is equal to the sum of the effects of the stressors when applied separately, with the coral displaying a 21% reduction in polyps in response to the temperature treatment and a 24% reduction in response to the pH treatment alone (Anlauf et al. 2011). Synergistic effects occur when the combined effect of stressors is larger than would be predicted based on additive responses of the individual stressors. For example, elevated levels of CO₂ concentration and Cu exposure produce more negative effects on marine benthic invertebrates than predicted based on the additive effects of the individual stressors (Lee et al. 2017). Finally, antagonistic effects occur when the cumulative effect of multiple stressors is smaller than predicted by the additive effect of individual stressors. An example concerns the marine fish, *Terapon jurbua*, which when exposed to mercury and selenium individually and combination displayed lower concentrations by 15 to 26% in the multiple than single stressor treatments (Dang and Wang 2011).

Although useful progress has recently been towards understanding multiple stressor effects on a variety of physiological and ecological metrics (Sokolova

2013, Todgham & Stillman 2013, Andersen et al. 2015, 2017, Halpern et al. 2015, Ellis et al. 2017, Przeslawski et al. 2015, O' Brien et al. 2019; Kenworthy et al. 2016, Griffen et al. 2016), studies have almost exclusively considered stressors that occur simultaneously. In reality, multiple stressors, can produce cumulative effects without necessarily having synchronous onsets and identical durations. For example, an estuary chronically exposed to metals may also experience acute stress due to additional stressors, for example sediment loading and freshwater pulses following rainfall. Additionally two acute disturbances, for example bait digging and the pulse input of nutrients following a rainfall event may not overlap in time but nevertheless have cumulative effects if the legacy of their impacts exceeds the duration of exposure. The degree to which multiple stressors overlap in time, the interval between the onset of their occurrence, and the order in which they are applied may each influence the way in which they interact (Gunderson et al. 2016).

Additionally, a large number of multiple stressor studies have occurred in simplified laboratory or mesocosm settings (e.g. Widdicombe & Austen 2001). The applicability of the results of such studies to real-world settings remains unclear because they neglect to consider how the full suite of species interactions may exacerbate or weaken multiple stressor impacts on target metrics, or how features of the environment might make organisms more or less susceptible to perturbations (Venebrooke et al. 2004, Godbold et al. 2011, Hicks et al. 2011, in reviews by Wernberg et al. 2012, Przeslawski et al. 2015). In order to be relevant to environmental management, multiple stressor studies need to be conducted in natural settings and have ecologically relevant experimental designs that consider not only the spatial but also temporal dynamics of the multiple stressors (Gunderson et al. 2016).

1.4 Anthropogenic disturbances to estuarine ecosystems:

Estuaries are unique ecotones between terrestrial, marine and freshwater ecosystems, that support biodiversity and resources of considerable value to humans (Constanza et al. 2014, Teichert et al. 2016). They are complex

systems due to their highly variable geomorphology, hydrography, salinity, tidal characteristics, sedimentation, and ecosystem energetics (Kennish 2002). As a consequence of the ecosystem services provided by estuaries, and their location as gateways between land and sea, nearly 40% of the world population lives within 100km proximity of estuaries. Estuaries are consequently focal points of human activities (Paerl 2006; Elliot & Quintino 2007) and as a result are subjected to a multitude of anthropogenic stressors that produce changes to the structure and function of their ecological communities. Anthropogenic stressors to estuaries are rapidly increasing (Alongi 2002, Dafforn et al. 2012), due to increasing human populations, and coastal development, which includes agricultural and industrial activity as well as urbanisation (Kennish 2002). The growing number of anthropogenic stressors to which estuaries are exposed, coupled with climate change, are producing substantive changes to ecological communities, and their functions and services (Sala et al. 2000, Lotze et al. 2006, Worm et al. 2006, Halpern et al. 2008, Hoegh-Guldberg & Bruno 2010, Barbier et al. 2011, Dolbeth et al. 2019).

Among the multitude of stressors to impact estuarine ecosystems, nutrient enrichment and physical disturbance are often singled out as particularly significant drivers of change (Gray 1997, Worm et al. 2000, Carnell & Keough 2014, Kenworthy et al. 2016). As low points in the environment, at the bottom end of catchments, estuaries are particularly prone to nutrient enrichment (Hallett et al. 2019). Furthermore, with estuarine productivity naturally limited by nitrogen (N) and/or phosphorous (P), their ecosystem structure and function is particularly sensitive to changes in nutrient availability. Nutrients may enter estuaries as diffuse runoff from agriculture, urban and industrial land, or in point-source discharges, with fertilizer, cleaning products, animal waste and sewage, are important anthropogenic sources (Nixon 1995, Vitousek et al. 1997). Although moderate nutrient enrichment of oligotrophic estuaries can stimulate primary, and hence, secondary productivity, larger additions, especially when applied to eutrophic estuaries, can produce large blooms of fast growing algae, that shade and kill benthic macrophytes such as seagrass, and can trigger eutrophication by depleting sediment and water-

column oxygen during bacterial decomposition of their substantive biomass (Vitousek et al. 1997).

Physical disturbance of sediment can be caused by a variety of activities, and its impacts depend on its frequency, intensity and scale (Schratzberger & Warwick, 1998). Bait-digging (Howell 1985, Wynberg & Branch 1997, Ferns et al. 2000), anchoring and propeller scars can damage aquatic macrophytes and modify sediment properties at scales of centimeters to meters (Keough & Quinn 1998, Creed & Filho 1999, Bishop 2005). Dredging produces impacts at scales of tens of meters to kilometres (Watling & Norse 1998). Major storm events may impact sediments at scales spanning several kilometres (Morton et al. 1995). Sediment dwelling organisms may be negatively impacted both directly, and indirectly through changes to sediment properties such as grain size and sediment organics (Hall 1994, Bishop 2005). Physical disturbance of sediments can lead to disruption and redistribution of faunal communities through both direct damage to organisms and loss of important habitat, such as burrows, altering density and diversity (Hall 1994, Thrush et al. 1991, Hall & Harding 1997). In some instances, fine sediments may be resuspended by the disturbance, leaving only coarse sands that are unsuitable habitat for some species (Schratzberger & Warwick 1998, 1999). Where physical disturbance causes mortality or damage to organisms, it may enhance organic matter inputs to sediments, inducing sediment anoxia (Duplisea et al. 2001). However, where it oxygenates previously anoxic sediments, positive impacts of fauna may result (Schratzberger & Jennings 2002). In some instances physical disturbance might increase the abundance of benthic species, by favouring opportunistic species colonisation or increasing resources such as food and space or by reducing predation pressure (Sherman & Coull 1980, Dye 2006). However on large spatial scales, disturbance of sediment by extreme events such as storms can make the sediment bed susceptible to erosion, furthering negative impacts (Yeo & Risk 1979).

Although the independent effects of nutrient enrichment and physical disturbance on estuarine ecosystems has been the subject of a large number of studies (e.g. Alongi 1985, Widbom & Elmgren 1988, Warwick 1993, Hall

1994, Hall & Harding 1997, Schratzberger & Warwick 1999, Morris & Keough 2003, Bishop 2005, Posey et al. 2006, Dye 2006b, Santos et al. 2009, O'Brien et al. 2010), comparatively few studies have considered how they may interact to influence benthic community structure (Austen & Widdicome 2006, Kenworthy et al. 2016). Moreover, the majority of studies examining the interacting effects of these stressors have been done in the northern hemisphere, in many instances in mesocosms, and examining impacts to macrofauna (Austen & Widdicome 2006). Temperate Australian estuarine ecosystems may display differential responses to these stressors as compared to northern hemisphere estuaries due to differences in estuarine geomorphology, chemistry and species composition, as well as differences in their history of anthropogenic modification (Scanes et al. 2007, Kenworthy et al. 2016).

Although the estuaries of northern Australia are tide dominated and have comparatively high riverine input, southern Australian estuaries are generally microtidal and are instead wave-dominated (Roy et al. 2001). Overall salinity fluctuation in southern Australian estuaries is driven more by patterns of rainfall than by tides (Heggie & Skyring 1999). Rainfall can be sporadic and in some instances estuaries may become temporarily disconnected from tidal exchange by formation of sand-bar, resulting in long residence times and high rates of evaporation (Currie & Small 2006). Periodic rainfall events can bring an influx of sediments from the catchment into estuarine systems, leading to high sediment loads (Currie & Small 2006, Scanes et al. 2007).

Additionally, temperate Australian estuaries are typically oligotrophic in comparison to their Northern hemisphere counterparts (Scanes et al. 2007). Low nutrient concentrations levels in Australia have been recorded by (Higgins & Thiel 1988, Young et al. 1996, Hauxwell & Valiela 2004, Kelly 2008, Scanes et al. 2007) and are a function of negligible coastal upwelling, biogeography (i.e. nutrient-poor terrestrial soils) and low precipitation (Young et al. 1996, Harris 2001). Australian coastal development is also much more recent when compared to the northern hemisphere, such that estuaries do

not have the same history of sustained anthropogenic nutrient loading, which can shift baseline conditions to a eutrophic state. Consequently, several Australian studies have demonstrated positive effects on productivity of nutrient enrichment (e.g. Bishop et al. 2006, York et al. 2012), that contrasts the negative effects of enriching eutrophic systems. However, in the south-eastern region, estuaries are densely populated by humans (Small & Nicholls 2003, McGranhan et al. 2007), with cities continuing to grow. Hence, temperate Australian estuaries may be particularly sensitive to the effects of nutrient enrichment. There is need to understand how the environmental context of south-east Australian estuaries mediates multiple-stressor effects.

1.5 Meiofauna and their importance:

Ecosystem processes in global sediments are mediated by the diversity of organisms that inhabit them (Snelgrove 1997, 1999). Among these are the “meiofauna” – those organisms that can pass through a 0.5 mm mesh but are retained by a 0.042 mm mesh (Higgins & Thiel 1988). Meiofauna are a highly diverse and abundant group, even compared to macrofauna, are represented by 24 of the 35 known invertebrate phyla and have densities that can exceed 10 million individuals per square meter of sediment surface (Kennedy & Jacoby 1999).

Due to their high density and diversity, ubiquitous distribution, rapid generation time (as short as 1 month) and fast metabolic rates, meiofauna are fundamental contributors to ecosystem functions in all global benthic ecosystems, including those of estuaries (Ansari & Parulekar 1998, reviewed in Coull 1999, Schratzberger & Ingels 2017). They contribute a large proportion of benthic production (Platt & Warwick 1983, Schratzberger & Ingels 2018), serve as an important food source for higher order consumers (Coull 1999) and biomineralize organic matter and enhance nutrient recycling (Tenore et al. 1977, Coull 1999, Vassallo et al. 2006, Semprucci et al. 2015, Schratzberger & Ingels 2018). Two groups within the meiobenthos, Foraminifera (microscopic shelled protozoans) and Nematoda, are the most abundant and diverse groups in sediments, with the former contributing up

to 90% of benthic sediment fauna (Balsamo et al. 2012). Their small size helps them move between the grains of sediment, creating burrows and ingesting and defecating sediment which they excrete along with an extracellular polymerase substrate which glues sediment together (Riemann & Schrage 1978, Kristensen et al. 2012). Through this activity, they can have a major impact on sediment properties (Schratzberger & Ingels 2018).

Compared to many other benthic groups, meiofauna are also particularly sensitive to stressors, and this characteristic, combined with the ease with which they can be sampled, and the extent to which they represent changes to overall communities makes them excellent bioindicators (Coull 1999, Moreno et al. 2011, Alves et al. 2013, Semprucci et al. 2015). As compared to macrofauna, meiofauna display limited dispersal capabilities, with many taxa developing directly rather than following a pelagic larval phase, and with the small size of adults limiting their capacity for migration (Remane 1952, Danovaro et al. 2004, Schratzberger & Ingels 2017). Consequently, meiofauna often display strong and immediate local responses to perturbations (Kennedy & Jacoby 1999). The short generation time of meiofauna means that impacts of stressors can be observed over the entire life cycle (Warwick 1981) and the response of their populations and communities to stressors is often more rapid and of shorter duration than those of macrofauna or animals with longer life cycles (Coull & Channdler 1992). As inhabitants of the sediment, benthic meiofauna are particularly sensitive to changes in sediment properties (e.g. grain size, sorting) and interstitial chemistry, with the various taxa displaying variable sensitivities to stressors that lead to strong community-level responses (Austen et al. 1994, Kennedy & Jacoby 1999).

Meiofauna reflect the overall health of the marine benthos because: (i) in severely impacted sites, meiofauna tend not to occur where other species are absent; (ii) meiofauna participate in the same metabolic and physiological pathways as other fauna and are likely to be exposed to the same types of stressors, and (iii) they share the same habitat with many large fauna and hence are exposed to similar types and concentration of anthropogenic stressors (Kennedy & Jacoby 1999). Meiofauna have consequently been used

extensively to monitor human impacts (Alve 1995, Schonfiel et al. 2002, Mojtahid et al. 2008), including those to coastal ecosystems (Balsamo et al. 2012, Schonfield et al. 2012, Moens et al. 2014). For example, meiofauna are useful indicators of hypoxic conditions both in the field and laboratory (Diaz & Rosenberg 1995, Murrell & Fleeger 1989, Wetzel et al. 2001, Sergeeva & Zaika 2013, Van Colen et al. 2009, De troch et al. 2013), and have contributed to understanding ecological impacts of physical and chemical disturbance (Moore & Bett 1989, Warwick & Clarke 1996, Schratzberger et al. 2002, Schratzberger & Jennings 2002, Lampadariou et al. 2005, Dye 2006a, Liu et al. 2009, Huff 2011, Leduc & Pilditch 2013).

Despite a number of studies documenting the response of meiofauna to nutrient (Coull & Chandler 1992, Garcia & Johnstone 2006) and large-scale physical disturbances such as dredging (Boyd et al. 2000, Schratzberger et al. 2002, Schratzberger & Jennings 2002, Boyd et al. 2003, Schratzberger et al. 2006), these are almost exclusively from the northern hemisphere. Additionally, there are a paucity of studies examining meiofaunal response to multiple stressors, particularly in field settings (but see Widdicombe & Austen 2001, Austen & Widdicombe 2006 for some examples of mesocosm experiments). Changes in the distribution, diversity and abundance of meiofauna have the potential to propagate impacts of stressors up and down food webs, modifying interactions among organisms and between organisms and their environment (Giere 2009). For example, meiofaunal presence can influence macrofaunal diversity, with the macro-meio interaction potentially modifying benthic properties and hence ecosystem services (Piot et al. 2014). Hence, studies on multiple stressor impacts are urgently needed. In general, meiofauna remain under-represented in ecological studies as compared to macrofauna (Schratzberger et al. 2000).

1.6 Thesis outline:

My thesis sought to address knowledge gaps regarding: (1) sources of spatial variation in the structure of estuarine meiofaunal communities of southeastern Australia; (2) multiple stressor impacts of nutrient enrichment

and physical disturbance on meiofaunal communities in field settings; and (3) the effect of the temporal dynamics of multiple stressors on their cumulative impact.

Chapter 2 addresses sources of spatial variation in estuarine meiofaunal communities of seagrass beds across 8 estuaries, spanning 1000km of Australian coastline, and seven degrees latitude. Nutrient enrichment is predicted to be a major determinant of community structure, with four of the estuaries with nutrient loads that have been significantly enhanced post European settlement, and the other four estuaries with nutrient loads that have undergone little anthropogenic change (Roper et al. 2011).

Chapter 3 investigates how at the patch-scale nutrient enrichment and physical disturbance interact to influence meiobenthic communities. Specifically, through experiments at two field sites, it considers (1) whether multiple stressor effects are additive, synergistic or antagonistic and (2) whether the nature of multiple stressor interactions are consistent across environmental settings or vary according to background environmental factors.

Chapter 4 considers how the timing and order of multiple stressor applications influences cumulative effects. It presents the results of a manipulative field experiment examining how the combined effects of nutrient enrichment and physical disturbance depend on whether the two stressors are offered simultaneously or one after the other and whether, within treatments with asynchronously applied stressors, the order of stressor application matters.

Together the three chapters are expected to provide important information for estuarine managers regarding the factors influencing the structure of ecologically important meiofaunal communities, and how multiple stressors should best be managed to minimise negative impacts. This study confirms meiofauna are responsive to anthropogenic stressors, even at a high taxonomic resolution. Depending on how their responses relate to those of other ecosystem components, they could be used as a integrative tool for

investigating stressor impacts in coastal ecosystems, in a similar way as macrofaunal assemblages are used.

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Chapter 2

Sources of spatial variation in meiobenthic assemblages of *Zostera muelleri* in estuaries of New South Wales, Australia.

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Highlights

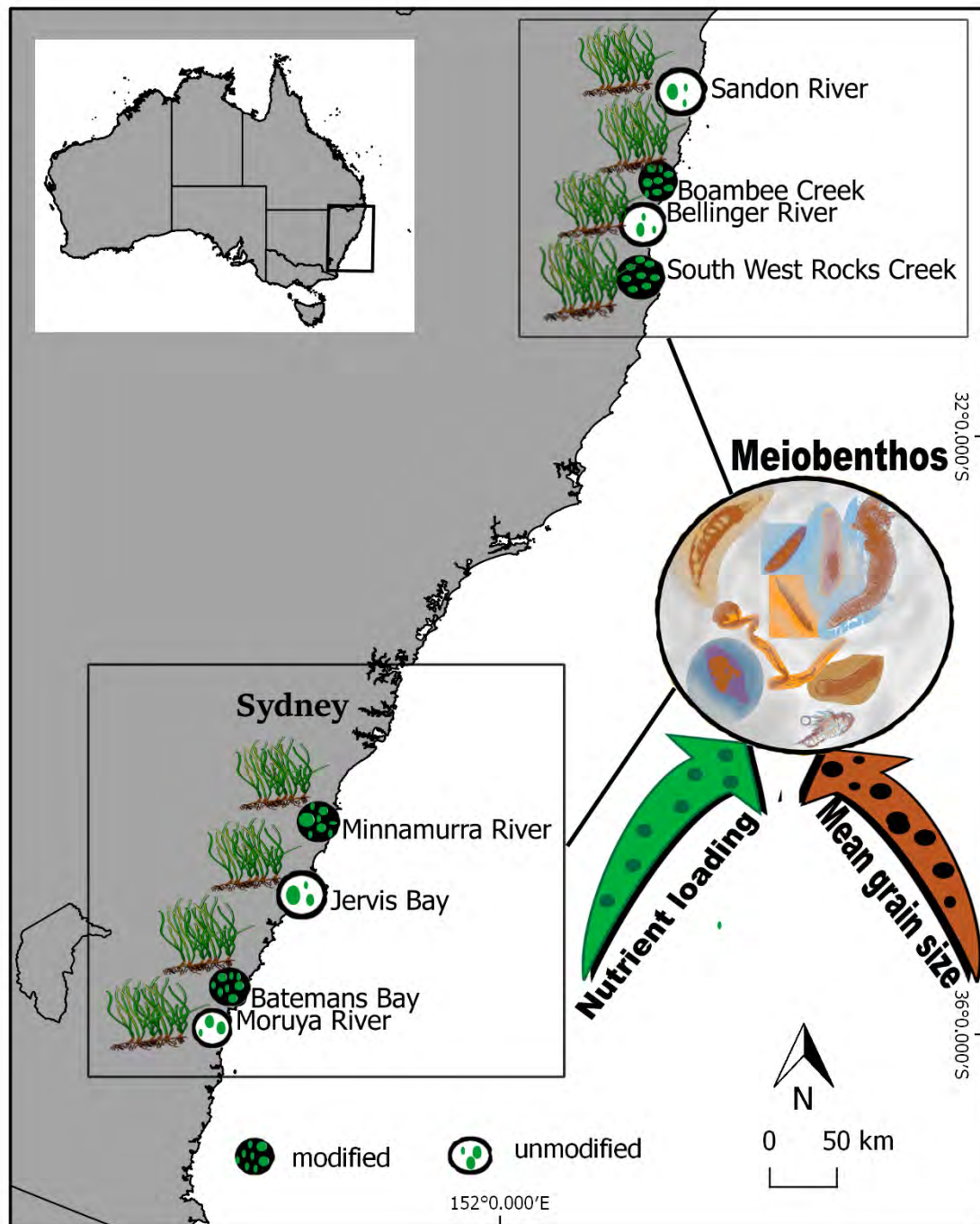
- Sources of spatial variation in SE Australian estuarine meiofauna were examined
- 16 sites, across 8 estuaries of variable anthropogenic nutrient loading were sampled
- Mean grain size was the best predictor of meiobenthic community structure.
- A weak, positive effect of nutrient loading on estuarine meiofauna was found
- Nutrient enrichment is not presently a key driver of SE Australian meiofauna

Abstract

Meiobenthos are a ubiquitous and abundant component of estuarine sediments, that underpin key ecological functions, yet the environmental factors that structure their communities remain poorly described, particularly at larger scales. Along urbanised coastlines, nutrient enrichment is widely regarded as a key stressor to benthic ecosystems. This study conducted sampling of meiofauna and key environmental variables across a gradient of anthropogenic nutrient enrichment, to assess the relative importance of nutrient enrichment, geomorphic, and sediment variables in predicting the structure of meiofaunal assemblages. Sixteen sites vegetated with the seagrass *Zostera muelleri*, distributed over 8 estuaries and 7 degrees of latitude, were sampled. We hypothesized that at the scale of sites, nutrient enrichment would be a more important predictor of community structure than the other variables considered. While our sampling revealed that nutrient loading was indeed a key predictor of meiofaunal community structure, sediment grain size explained more of the variability in community structure at this scale. Although nutrient enrichment is often assumed to negatively affect biota, we found the relationship between nutrient enrichment and meiofaunal abundance was positive. These results suggest that along the oligotrophic east Australian coast, nutrient enrichment is presently below the threshold required to produce deleterious impacts. However, as human populations continue to grow along the coast, manipulative studies are needed to identify tipping points, beyond which nutrient enrichment negatively impact diversity.

Keywords: meiofauna, nutrient pollution, sediment grain size, meiobenthos, seagrass

Graphic Abstract



2.1. Introduction

Meiofauna, broadly defined as those organisms greater than 38 microns in diameter but smaller than 500 microns (Coull 1999, Schratzberger 2012), are

a ubiquitous and important component of estuarine and marine sediments (Higgins & Thiel 1988, Coull 1999, Liu et al. 2007, Giere 2009, Santos et al. 2009, Cooke et al. 2013, Zeppilli et al. 2015). They are key determinants of the physical, chemical and biological properties of sediments (Cullen 1973), and underpin a variety of important ecosystem functions (reviewed by Coull 1999, Zeppilli et al. 2015, Schratzberger & Ingels 2017), including nutrient cycling, trophic energy transfer, and organic matter degradation (Pearson & Rosenberg 1978, Coull 1999, Giere 2009). For example, in irrigating the sediments through their movement and burrow construction, they determine sediment porosity and oxygenation (Kristensen et al. 2012). In ingesting and excreting sediments, they stimulate microbial communities which, in turn, positively influence sediment stability by excreting mucus that binds sediments (Riemann & Schrage 1978, Chandler & Fleeger 1984, Reichelt 1991, Schratzberger & Ingels 2017).

Given the pivotal role meiofauna play in structuring the benthic ecosystem of coastal waters, understanding the scales across which their communities vary spatially, and the key environmental drivers of this variability, is important. Yet, relative to their counterparts, the macrofauna, relatively few studies, have, focused on sources of meiofaunal spatial variability, particularly at larger scales of 100s of kilometers (Morrisey et al. 1992, Schuckel et al. 2013). The relatively few studies on sources of spatial variability in meiofauna suggest that at scales of millimeters to centimeters, meiofauna display patchy distributions that reflect food availability (Findlay 1981, Bodlina et al. 2014). At larger scales of hundreds of meters to kilometers, sediment particle size, temperature, salinity, and nutrient levels have been put forward as key regulators of their numbers (Coull 1999, Lee & Riveros 2012, Bonaglia et al. 2014).

As human activities continue to modify coastal seascapes at escalating rates, there is need to understand how at large spatial scales meiofaunal community structure and function varies as a function of the interacting effects of human stressors and local environmental conditions (Kennish 2002, Halpern et al 2007). Among the multitude of stressors to affect estuarine and coastal

ecosystems, anthropogenic nutrient enrichment is broadly considered one of the greatest threats (Nixon 1995, Fitch & Crowe 2012). Deforestation, agriculture, industrialization, and urbanization have resulted in enhanced nutrient inputs, both from point and non-point sources (Gabric & Bell 1993, Kennish 2002, Niel et al. 2018). As nitrogen and/or phosphorous concentrations are typically limiting of estuarine productivity, nutrient enrichment generally increases gross primary productivity. In some instances, this leads to excessive organic matter production (Nixon 1995, Posey et al. 1999, Bishop et al. 2007), the decomposition of which can trigger sediment anoxia and negative impacts on the benthos (Rossi & Underwood 2002, Bishop & Kelaher 2007).

Previous experimental and observational studies have found variable relationships between nutrient enrichment and meiofaunal community structure (Widbom & Frithsen 1995, Austen & Widdicome 2006, Sundback et al. 2007, 2010, Semprucci et al. 2015, Ravaglioli et al. 2019). In observational studies examining meiofaunal responses to river plumes and phytoplankton blooms, the resulting nutrient inputs to sediments decreased meiofaunal abundance and resulted in the shallower distribution of taxa (Danovaro et al. 2000, Gracia & Johnstone 2006). Extreme organic enrichment from pollution in Mumbai Bay, India, resulted in defaunation of meiofauna (Shaoo et al. 2017). In highly productive *Posidonia* seagrass beds, nutrient addition induced anoxia and reduced meiofaunal taxonomic diversity (Gambi et al. 2008, Mirto et al. 2014). However, moderate nutrient enrichment through decomposition of *Ulva* species or under the cages of fish farms enhanced meiofaunal abundance (Mazzola et al. 1999, Dal Zotto et al. 2016, Balgrihi et al. 2019).

The effects of nutrient enrichment on meiofaunal communities may be mediated by both local and broad-scale environmental conditions. Climatic conditions, such as temperature, can determine the rate at which excess organic matter is remineralised by bacteria, and hence the sensitivity of a system to oxygen depletion (Rabalias et al. 2009). Sediment properties, such as organic content may determine how close a system is to tipping points,

when exposed to additional organic enrichment (Diaz & Rosenberg 2008, Sahoo et al. 2017, Taghon et al. 2017). Additionally, the presence and habitat structure of aquatic macrophytes, such as seagrass, may influence impacts of nutrient enrichment by determining the amount of nutrient input that is biologically uptaken and also by influencing sediment stability, and hence, oxygenation (Gambi et al. 2008, Fonseca et al. 2011).

The key factors structuring the meiobenthic communities of east Australian estuaries remain poorly understood, with relatively few studies conducted over the last two decades (e.g. Dye 2005, 2006, Dye & Barros 2005, Fonseca et al. 2011, Adullah & Lee 2016, 2017). These studies have focused on how estuarine geomorphology and the entrance dynamics of intermittently open lakes and lagoons influences estuarine gradients in meiobenthic communities (Dye 2005, 2006, Dye & Barros 2005) and how local-scale factors such as habitat type, configuration and sediment properties shape community structure (Fonseca et al. 2011, Adullah & Lee 2016, 2017). Despite anthropogenic nutrient loading considered the top-rated local stressor causing degradation to temperate Australian temperate estuaries (Davis & Koop 2006, Nicastro & Bishop 2013), relative little is known of its impacts on meiofaunal communities or how this factor interacts with local factors to influence meiobenthic community structure.

In this study, we assessed sources of spatial variation in the meiofaunal communities of *Zostera muelleri* seagrass beds across 8 estuaries of New South Wales, Australia, varying in anthropogenic nutrient enrichment and spanning 7 degrees of latitude. We hypothesised that (1) nutrient enrichment would explain more variation in benthic meiofaunal community than other environmental variables, including latitude, sediment properties and seagrass habitat structure; but (2) the relationship between nutrient loading and meiobenthic community structure would be modified by local-scale environmental variables, including sediment properties and seagrass morphology.

2.2 Material and methods

2.2.1. Study sites

Benthic meiofaunal communities and environmental variables were sampled in eight wave dominated estuaries distributed along 7° of latitude of the coast of New South Wales, Australia in August 2012 (Fig.1, Table 1). Four of the estuaries were highly modified by humans, with ratios of modelled post- to pre-European settlement Total Nitrogen of greater than 2.5 (Roper et al. 2011). The other four were considered largely unmodified, with post- to pre-European settlement ratios of less than 1.5 (Roper et al. 2011). Within each estuary, sampling was conducted within 10 km of the estuarine mouth. Two sites greater than 100 m apart, each with intertidal *Z. muelleri* were sampled per estuary. All estuaries were permanently connected to the ocean and had a tidal range of ~1.5 meters.

2.2.2. Field and laboratory methods

Sampling of meiofauna and environmental variables was conducted at low tide, during aerial exposure of the seagrass beds. Meiofauna were sampled using a hand-held acrylic corer of 1.9 internal diameter pushed into the sediment to a depth of 10 cm, with five replicate cores per site, at least 2m apart, collected. Meiofaunal samples were fixed with 7% buffered formalin in seawater with Rose Bengal stain. In the laboratory, meiofauna were extracted from samples by washing samples over nested 500µm and 45µm sieves (Dye & Barros 2005), and subjecting material passing through the 500µm but retained by the 45µm sieve to a Ludox HS40 density separation protocol (Burgess 2001). The density of the Ludox solution was made to > 1.15 (Somerfield & Warwick 1996), with the sample mixed thoroughly using a vortex and allowed to settle for 2hrs. The supernatant was poured thorough a 45µm sieve and the organisms retained were washed with distilled water. Ludox was added to the heavy fraction of the sample and the protocol was repeated 4 times. Sub-sampling of the retained meiofauna was done due to high densities. Following the density separation protocol, each sample was made up to 50 ml, homogenized and meiofauna were identified and enumerated in 3-4 subsamples, each, of 3ml until more than 250 individuals

were picked (see McIntyre & Warwick 1984). Separated organisms were preserved in 70% ethanol and these 'meiofauna' were counted using a stereomicroscope to mixed taxonomic resolution. Crustaceans were identified to order, Annelids to subclass, while the remainder of groups were identified to phyla. Previous studies indicate that use of a mixed taxonomic resolution does not compromise the ability to detect impacts (McIntyre & Warwick 1984). The results were expressed per 10 cm² of sediment surface area.

Additional cores (n=3-5 per site and variable) of the same dimensions as described above were obtained for assessment of sediment grain size, total organic content and Chlorophyll *a*. To determine sediment grain size distribution, each of the samples assigned to this variable were treated with 10% hydrogen peroxide to remove any organics, dried to constant weight at 60° C and analysed using a Malvern2000 laser particle sizer. The organic content of designated sediment samples was estimated by first drying samples to constant weight at 60°C, and then determining percent mass loss following ignition at 450°C for 4 hours. Chl *a* was measured as a proxy for microphytobenthic abundance using the Jeffrey & Humphrey (1975) method. Briefly, a well homogenised subsample of about 2g of sediment from each core was taken and the pigments were extracted under dark, refrigerated conditions using 1.5 ml of 90% acetone. After 24 hrs of incubation, samples were mixed using a vortex for 10 s. Chl *a* concentration was then measured after 48 hrs spectrophotometrically. The Chl *a* content of sediment was calculated and expressed per unit area (mg m⁻²).

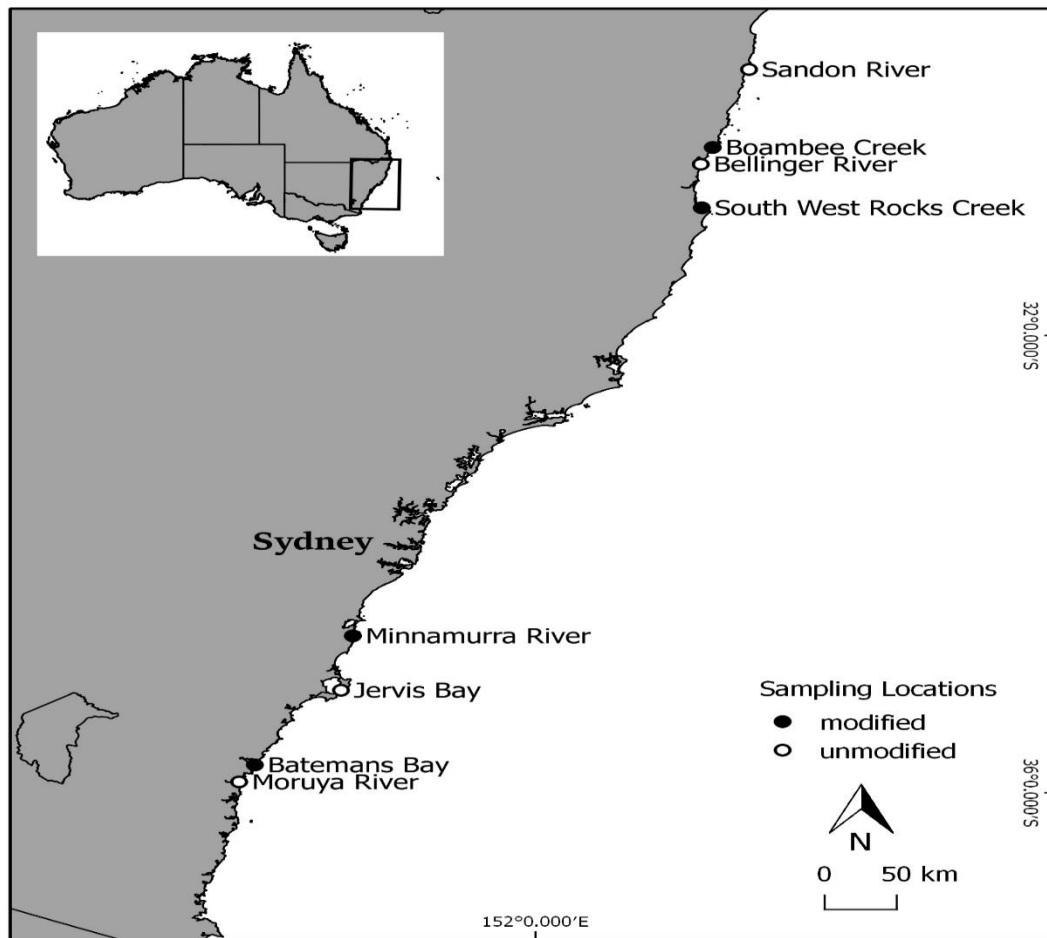


Figure 1. Estuaries of New South Wales, Australia, where sampling occurred. The black filled circles indicate highly modified and the open white circles indicate largely unmodified estuaries.

Table 1. The physical and chemical characteristic of the 8 estuaries that were studied. TN flux= flux of total nitrogen, T: N ratio=ratio of total nitrogen loading pre-European settlement to present, OM= Organic matter, C:N= seagrass carbon nitrogen ratio, N=seagrass nitrogen content, SLA=seagrass Specific Leaf Area, NA=data not available, a data from Roper et al. 2011.

Estuary	Lat	Long	T: N ratio ^a	Actual TN flux (mg m ⁻² d ⁻¹) ^a	Flushing time (d ⁻¹) ^a	Estuary area (km ²) ^a	Catchment area (km ²) ^a	Mean grain size (μm)	Sediment sorting (μm)	OM (%)	Slit & clay (μm)	Chl a (mg. m ⁻²)	N content (%)	C:N ratio	SLA (cm ² . g ⁻¹)
Sandon River	-29° 40'	153°19'	1.0	18	2.3	2.6	109	400.6 ±18.6	2.2 ±0.2	3.8 ±0.7	6.7% ±0.022	6.7 ±0.7	1.6 ±0.0	19.2 ±0.4	163.1 ±11.3
Boambee Creek	-30° 21'	153° 6'	10.5	237	2.4	1.0	45	305.1 ±35.6	3.1 ±0.4	5.3 ±0.9	13.2% ±0.027	6.7 ±1.1	1.4 ±0.1	19.7 ±1.0	178.6 ±7.7
Bellinger River	-30° 30'	153° 1'	1.3	135	3.7	8.2	1110	405.2 ±30.6	2.4 ±0.1	3.8 ±0.3	7.5% ±0.009	15.6 ±2.0	1.9 ±0.0	15.0 ±0.1	126.5 ±13.1
Southwest rocks Creek	-30° 52'	153° 2'	2.6	2.8	1.1	0.9	32	479.6 ±58.0	2.4 ±0.1	4.1 ±0.6	9.0% ±0.026	8.9 ±1.0	1.7 ±0.0	17.2 ±0.2	140.7 ±9.6
Minnamurra River	-34° 37'	150°51'	2.6	67	1.0	1.9	110	531.0 ±25.5	1.9 ±0.2	3.5 ±0.9	3.4% ±0.012	14.1 ±2.5	NA		
Jervis Bay	-35° 6'	150°47'	1.4	0.1	54.4	123.9	410	497.3 ±42.2	1.8 ±0.1	3.2 ±0.4	3.3% ±0.002	11.1 ±1.9	NA		
Batemans Bay	-35° 45'	150° 4'	2.7	0.4	37.8	34.5	28	592.3 ±20.9	1.8 ±0.1	2.0 ±0.2	3.1% ±0.009	3.1 ±0.5	1.8 ±0.1	15.8 ±0.4	163.2 ±9.1
Moruya River	-35° 54'	150° 9'	1.4	80	4.4	6.1	540	597.1 ±8.9	1.6 ±0.1	1.7 ±0.2	2.1% ±0.005	8.3 ±1.1	2.4 ±0.1	12.7 ±0.1	155.8 ±8.3

2.2.2. Statistical Analysis

To test hypotheses regarding the relative importance of nutrient loading versus other environmental variables in influencing meiofaunal community structure, a matrix with physical, chemical and biological attributes of each estuary was constructed. This included site averages of variables sampled during this study (i.e. chlorophyll a concentration, sediment organic content, sediment silt/clay content, sediment mean grain size, sediment sorting and, where available, seagrass C: N ratio, seagrass total nitrogen content and seagrass SLA), latitude, and physical and chemical attributes of estuaries (i.e. flushing time, catchment area, estuary area, TN ratio, TN flux) sourced from Roper et al. 2011 (Table 1). Appropriate transformations were applied to each data set to minimize data skewness. Post transformation, all data were normalized and Principal Component Analysis (PCA) was used to overview the relationship between environmental variables.

To assess patterns in and environmental correlates of variation in meiofaunal community structure, a distance based multivariate regression was run using the DistLM routine (Anderson et al. 2008). The analysis used a Bray-Curtis dissimilarity matrix, calculated using fourth root transformed multivariate meiofaunal abundance data. Sites within estuaries were used as replicates, with meiofaunal community structure and environmental variables averaged across samples within a site. The initial model included all environmental variables, except seagrass traits which were only available for six estuaries. From this, a reduced model with good explanatory power was identified using AICc selection criteria and the BEST procedure in PRIMER (Clark & Warwick 2001). The full model was visualised using a distance based redundancy analysis (dbRDA) (McArdle & Anderson 2001). Vectors, identifying key discriminating taxa with multiple correlation coefficients greater than 0.6, were superimposed on the dbRDA plot. Pearson's correlation coefficients between these key discriminating taxa and individual environmental variables were calculated, as well as between each of total meiofaunal abundance and richness, and T:N ratio.

2.3. Results

The PCA of environmental variables revealed that these fell into 4 groups, within which variables were positively correlated (Fig. 2). The first group included mean grain size, flushing time, latitude and estuary area. The second, which was negatively correlated with the first, included organic matter, silt/clay content and TN flux. The third group, containing Chl *a* and catchment area, was negatively correlated to the fourth, total nitrogen content. Overall, the two dimensional PCA explained 63% of variation in environmental variables.

2.3.1. Meiofauna

Mean site densities of meiofauna ranged from 2402 to 10243 individuals per 10cm², with an average across all sites of $4614 \pm (\text{SE}) 507$ per 10cm². A total of 28 major taxonomic groups were recorded, with mean site richness ranging from 4.4 to 11.6 taxa per 10 cm², and the average richness across all sites, 8.7 ± 0.5 . Nematoda contributed to 79% of total abundance followed by Copepoda (6%), Turbellaria (5%), Polychaeta (4%), Ciliophora (2%), Ostracoda (1%), with other groups present at lesser abundance.

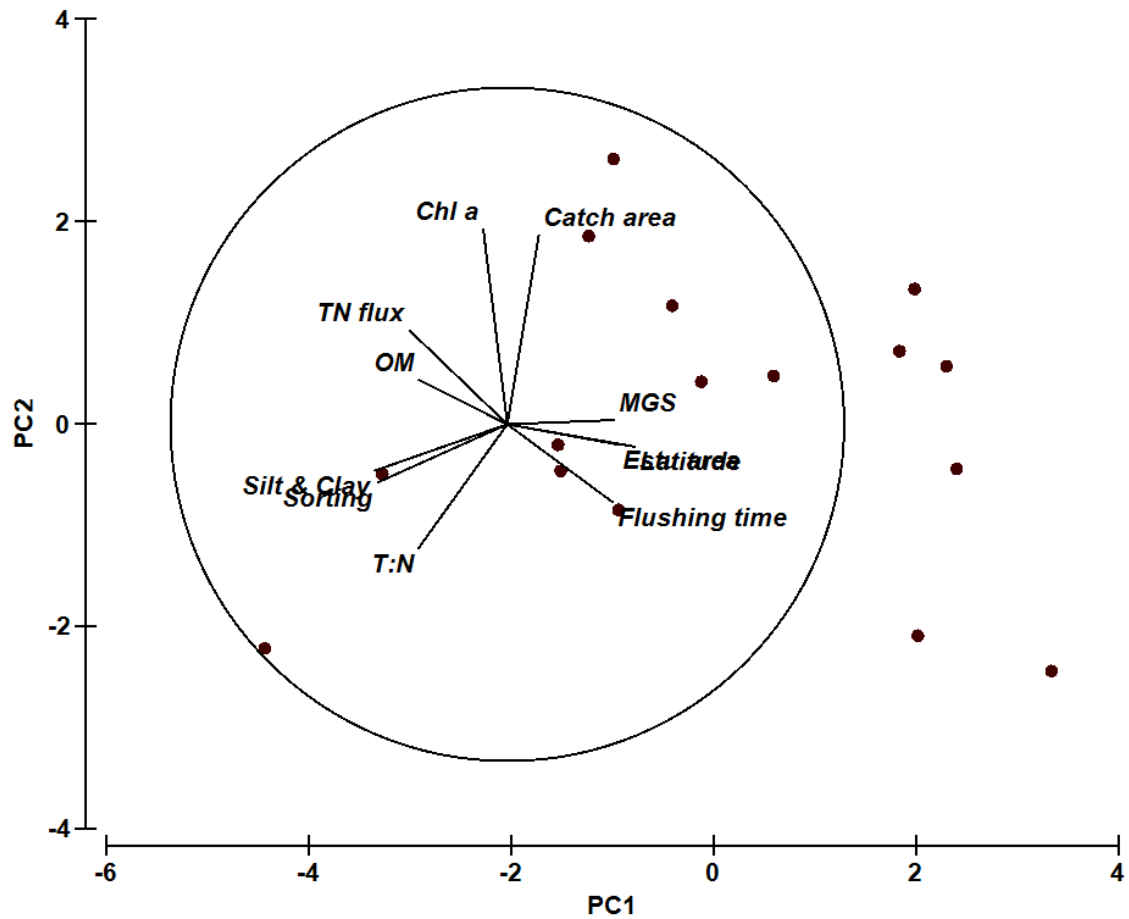


Figure 2. Principle component analysis of the environmental variables listed in Table 1, with the exception of longitude and seagrass metrics, the latter of which were only sampled in 6 estuaries. All variables were transformed and normalized (Table 2). Points represent sites, with two sites in each of eight estuaries (i.e. $n=16$). The variables best explaining variation are denoted with vectors, with the strength of the correlation indicated by the length of the line (circle denotes a correlation of 1.0). Abbreviations: MGS = median grain size, OM = sediment organic matter, T:N ratio= ratio of total nitrogen loading pre-European settlement to present, TN flux= flux of total nitrogen, Chl a = chlorophyll a, catch area = catchment area.

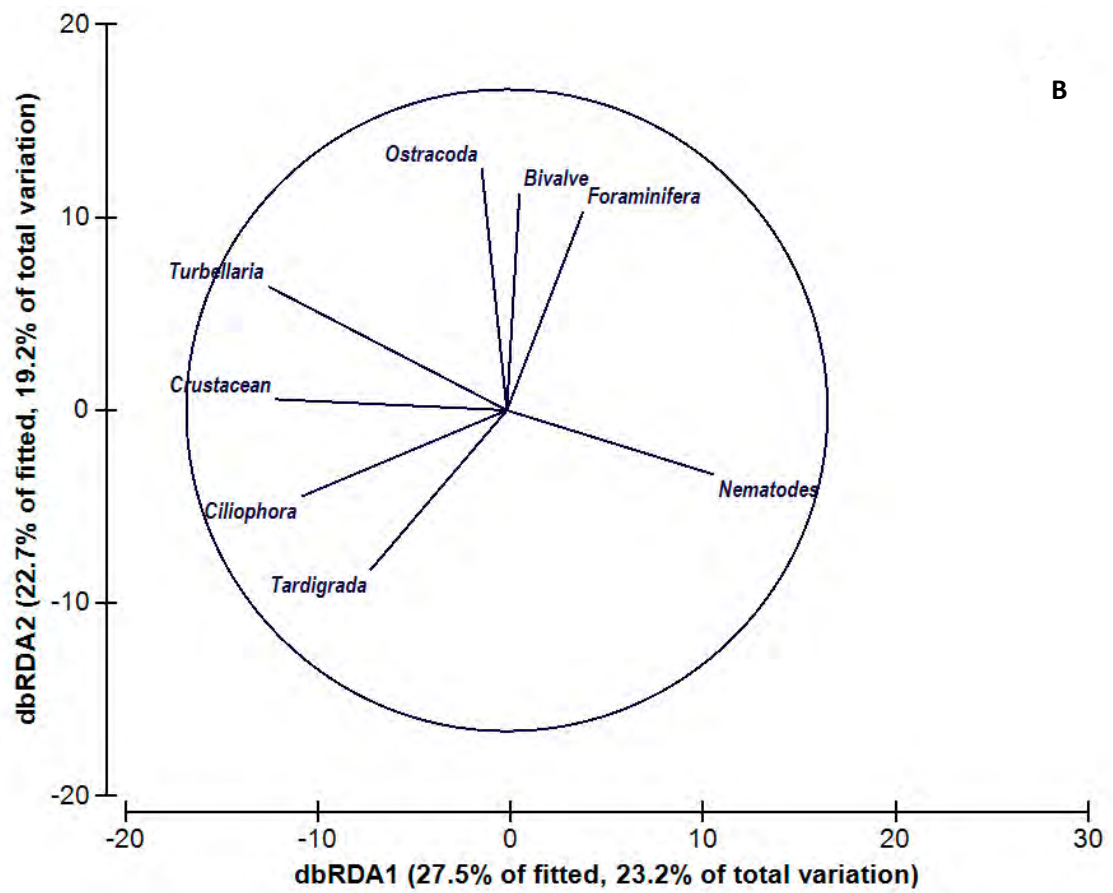
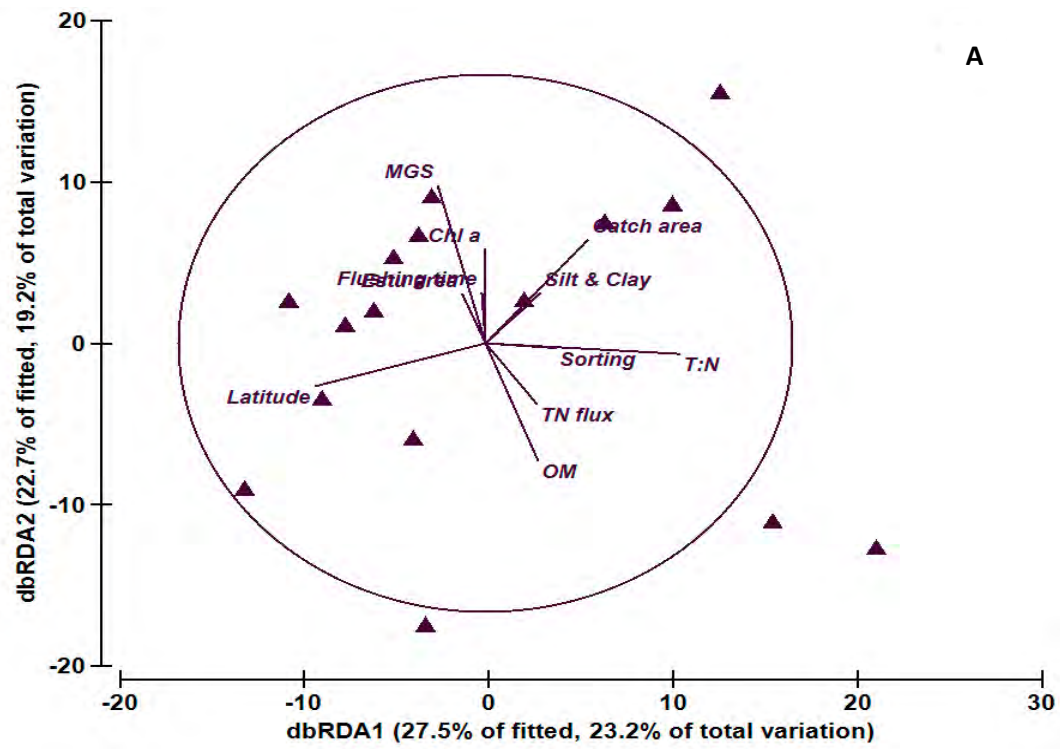


Figure 3. dbRDA plots representing the reduced model of spatial variation in meiofaunal assemblages and its relationship to a) environmental variables and b) the abundance of key taxa significantly correlated with db-RDA axes (multiple correlation > 0.60). Analyses excluded seagrass variables, which were only sampled in six estuaries, and longitude. Points represent sites, with two sites sampled within each of eight estuaries (i.e. n=16). The variables best explaining variation are denoted with vectors, with the strength of the correlation indicated by the length of the line (circle denotes a correlation of 1.0). Abbreviations: MGS = median grain size, OM = sediment organic matter, T:N ratio= ratio of total nitrogen loading pre-European settlement to present, TN flux= flux of total nitrogen.

The BEST procedure (PRIMER) indicated that the individual environmental variables that most strongly correlated to meiobenthic assemblages were sediment organic matter, sediment mean grain size, T: N ratio, TN flux, and catchment area (Table 2). Collectively, these five most strongly correlated environmental variables explained 46.4 % of the total variation in meiofaunal assemblages (Table 2, Fig. 3A). When examined individually, sediment mean grain size, sediment silt and clay ratio, TN flux, Latitude, T:N ratio and estuary area each displayed significant correlations with the meiofaunal data, but sediment organic matter did not (Table 2). Nematoda, Polychaeta, Ostracoda, Crustacea, Ciliophora Turbellaria, Tardigrada and Bivalvia were the taxa that accounted for the greatest proportion of variability in the meiofaunal data (Fig. 3B).

When analysed individually, several of the nine best explanatory taxa showed correlations with individual environmental variables (Fig. 4). Nematoda were negatively correlated with sediment mean grain size, while Crustacea were positively correlated to this variable (Fig. 4). Nematoda exhibited significant positive relationships with sorting, silt/clay content, T: N ratio and Total Nitrogen (Fig. 4). Crustacea and Turbellaria showed a negative relationship with latitude (Fig. 4). Polychaeta was positively linked with flushing time and estuary area and Ostracoda were positively correlated with catchment area. Ciliophora, Bivalvia, Foraminifera and Tardigrada did not show any significant correlations with the environmental variables (Fig. 4).

The total abundance of meiofauna was positive correlated with T:N ratio ($r^2=0.371, df=14, p=0.012$) while richness was negatively correlated with T:N ratio ($r^2=-0.358, df=14, p=0.014$).

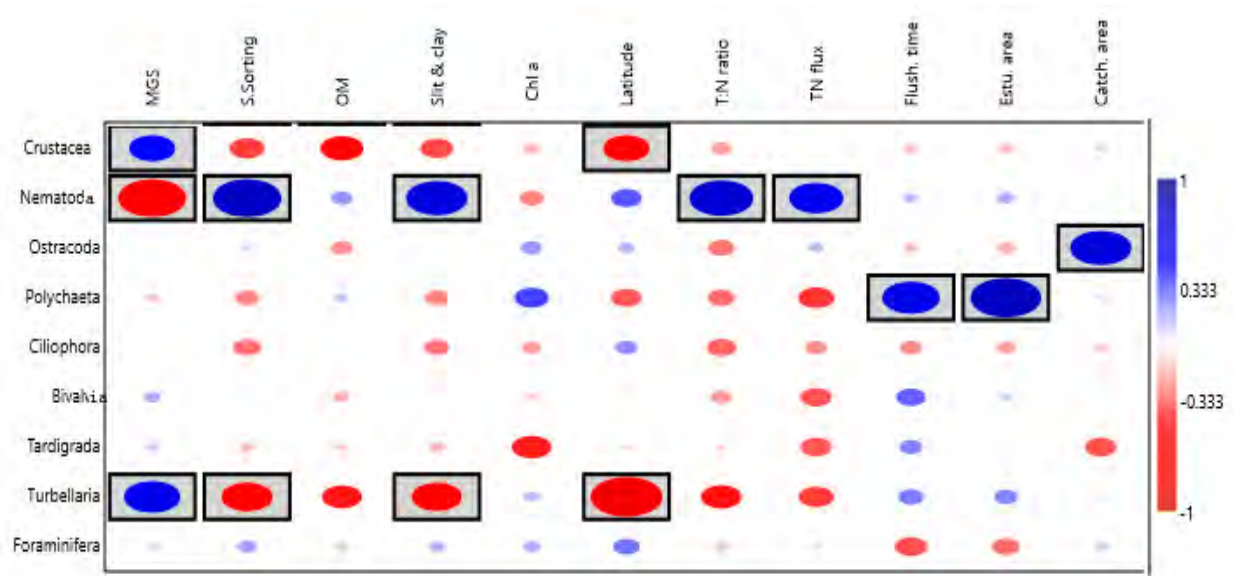


Figure 4. Pearson's Correlations coefficients for relationships between abundances of key meiofaunal taxa and environmental variables (excluding seagrass) sampled across eight estuaries. Blue denotes positive relationships, while red indicates negative relationships. The larger the circle, the stronger the correlation. Significant (at $\alpha = 0.05$) relationships are denoted with boxes. OM = sediment organic matter, T:N ratio= ratio of total nitrogen loading pre-European settlement to present, TN flux= flux of total nitrogen. Chl a= Chlorophyll a, Estu. Area= estuary area, Catch. Area= Catchment area, Flush. Time= Flushing time.

Table 2. Results of multivariate multiple regression analysis (distLM) examining key environmental correlates of meiofaunal community structure across eight estuaries. Analyses excluded seagrass variables, which were only available for 6 estuaries. Prop. = the proportion of variance in meiofaunal assemblages explained by each environmental variable. Significant (at $\alpha = 0.05$) predictor variables are highlighted in bold. Abbreviations: OM = sediment organic matter, T:N ratio= ratio of total nitrogen loading pre-European settlement to present, TN flux= flux of total nitrogen. Meiofauna data were 4th root transformed for analysis. Abbreviations: In order to achieve approximate normally distribution, data were (a) square root, (b) 4th root and (c) log transformed.

Variable	Pseudo-F	P	Prop.
Sediment Organic Matter ^a	1.34	0.23	0.09
Sediment mean grain size ^a	2.91	0.00	0.17
T:N ratio ^c	1.97	0.04	0.12
TN flux ^b	2.11	0.03	0.13
Catchment area ^b	0.99	0.45	0.07
Sediment Silt & Clay ^a	1.92	0.04	0.12
Chlorophyll a ^a	0.52	0.86	0.04
Sediment Sorting ^a	1.93	0.06	0.12
Latitude	2.05	0.02	0.13
Flushing time ^a	1.75	0.06	0.11
Estuary area ^b	1.92	0.05	0.12

2.4. Discussion

As hypothesized, nutrient loading was among the key predictors of meiofaunal community structure across eight estuaries, spanning the coastline of New South Wales, Australia. Yet, whereas nutrient loading emerged as a more important predictor of community structure than geomorphic variables such as estuarine area and flushing time, and seagrass leaf traits, sediment grain size was equally if not more important in predicting community structure at the scale of sites and estuaries. Overall, this study found that meiofaunal densities of east Australian seagrass beds were high as compared to unvegetated estuarine habitats, but within the range of values previously

reported for seagrass beds (Bell et al 1984, Tietjen 1969, Decho et al. 1985, Hicks 1986, Ansari & Parulekar 1994, Troch et al. 2001, Fonseca et al. 2011).

Studies from the heavily developed estuaries of the northern hemisphere typically focus on negative impacts of nutrient enrichment on estuarine ecosystems, arising from the effects of eutrophication (Nixon 1995, Smith 2003, Paerl 2006). Here, however, we observed a positive correlation between nitrogen enrichment and the total abundance of meiofauna. This suggests that nutrient inputs to east Australian estuaries are not at the levels required to produce deleterious impacts. Instead, along this oligotrophic coastline with nutrient-poor soils and weak upwelling, nutrient enrichment may increase productivity of both primary producers and consumers through ratio-dependent trophic responses (Bishop et al. 2006, Scanes et al. 2007, Nicastro et al. 2013, Kelaher et al. 2013). Interestingly, despite correlations between nutrient enrichment and meiofaunal community structure only one of the key taxa, Nematoda, displayed a significant positive relationship with variables of nutrient enrichment (T:N ratio, TN flux). While food availability *per se* is not considered to be an important factor limiting meiofaunal abundance (Coull 1999), the positive relationship between nematodes and enrichment may reflect changes in resource quality. The absence of relationships between other meiofaunal taxa and nutrient enrichment may reflect the coarse taxonomic resolution utilized by this study, necessitated by the poorly described meiofauna of the east Australian coast. Within phyla, taxa can display a diversity of feeding strategies that may lead to divergent responses to environmental change.

Although among urbanized estuaries, nutrient enrichment from diffuse sources would be expected to increase with catchment area, in this study the relationship between anthropogenic nutrient enhancement and catchment area was negative. Along the coastline of Australia, the human population is highly urbanized and concentrated into a few large settlements, focused on highly modified estuaries. As coastal populations increase in presently sparsely developed areas, a stronger relationship between catchment area and anthropogenic nutrient enhancement may develop.

The strong relationship between meiofaunal assemblage structure and sediment variables was as expected given similar studies on macrofauna along the New South Wales Coast (Nicastro et al. 2013), and smaller-scale studies on meiofauna done elsewhere (e.g. Hicks 1986, Troch et al. 2001, Fonseca et al. 2011, Du et al. 2012, Jankowska et al. 2015). In an analysis spanning 16 New South Wales estuaries, including those sampled here, Nicastro et al. (2013) found that among unvegetated habitats, sediment grain size was a better predictor of macrofaunal community structure than estuarine geomorphology or estuarine nutrient loading. Strong linkages between patterns displayed by macro- and meio-fauna are expected given that a component of the meiofauna are, in fact, juvenile macrofauna (McIntyre 1969). Sediment grain size may influence meiofaunal communities by influencing the depth of sediment oxygenation, whether individuals can reside in the interstitial space or must burrow, and by determining sediment organic content (Coull 1999, Lohr & Kennedy 2015). Although spatial variation in sediment grain size is typically reduced in seagrass beds, which trap fine sediments, as compared to unvegetated habitats (Fonseca et al. 2011), our study, in combination with previous smaller-scale studies in seagrass beds (Troch et al. 2001), demonstrates that it is nevertheless an important contributor to spatial variation in meiofaunal communities within this vegetated habitat.

In general northern NSW estuaries had finer sediments, and higher meiofaunal abundances than more southerly estuaries. The northern estuaries have a subtropical climate and receive more rainfall compared to the southern estuaries. Thus, there is more riverine input of fine sediments. Overall, however, the correlation between latitude and meiofaunal communities was weak. This was despite the limited dispersal capacity of the many meiofaunal taxa that display direct development (Bell & Sherman 1980, Palmer 1988) and seasonal patterns in meiofaunal abundance in northern hemisphere estuaries, related to temperature changes (Coull 1999). Although it is possible that latitudinal patterns may merge if larger numbers of estuaries are examined over a large range of latitudes than the 7 degrees

examined here, a previous study spanning tropical to polar latitudes did not find any latitudinal patterns in the diversity in the true meiofauna of sandy beaches when small macrofauna were excluded (Kotwicki et al. 2005).

The various taxa encountered in this study displayed a range of relationships with sediment grain size. As in previous studies (Edgar 1999, Fisher & Sheaves 2003, Fonseca et al 2011), Nematoda and Gastrotricha were more abundant in fine than coarse sediments. In contrast to other taxa, many Nematoda are able to tolerate the hypoxic conditions often associated with finer sediments (Modig & Ólafsson 1998, Wetzel et al. 2001). Turbellaria and Crustacea, by contrast, were more abundant in coarse sediments. Turbellaria are predominantly scavengers and predators so do not share the same dependency on organic-rich, muddy sediments as other, detritivorous groups (Martens & Schockaert 1986). Gallucci et al. (2005) reported that larger predatory meiofauna do not perform well in finer sediments hence are lesser in abundance. Other taxa, including Ostracoda and Foraminifera displayed no relationship with sediment grain size. Yassini & Jones (1987) found negative relationships between the abundance of Ostracoda and shoot density, hypothesizing that this relationship is driven by the lower oxygen level, of the organic-rich, fine sediment in dense seagrass beds. Here, however, organic matter was not a strong correlate of meiobenthic assemblage structure, perhaps because the range in values across the seagrass beds studied was small.

2.5. Conclusion

While this descriptive study suggests that nutrient enrichment and sediment grain size are key determinants of meiofaunal community structure among east Australian estuaries, manipulative experiments are needed to establish cause-effect relationships. Though meiofauna are an essential component of estuarine food webs and are often considered as excellent indicators of environmental change, manipulative field experiments examining key factors structuring their communities across large spatial scales remain rare. As human populations continue to grow along the east Australian coast, it will be important to identify tipping points, below which nutrient inputs should

be maintained, so as to avoid deleterious impacts on meiofauna and the food webs that depend on them.

The strong response of meiofauna to experimental manipulations confirms that they are responsive to anthropogenic stressors, even at coarse taxonomic resolutions, and may be useful indicators for stressor impacts. However, this would also require comparing their response to perturbations against those of other groups commonly used as indicators, such as the macrofauna. They are important contributors to Australian coastal ecosystems and greater understanding of their contribution to ecosystem structure and function is required.

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Chapter 3

Multiple stressor effects of nutrient enrichment and physical disturbance on estuarine meiobenthos – A field study.

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ABSTRACT:

The world's ecosystems are increasingly exposed to a multitude of anthropogenic stressors which may have additive, synergistic or antagonistic impacts. Understanding the cumulative effects of multiple anthropogenic stressors in field settings is one of the most pressing challenges facing environmental managers, yet a large number of studies continue to examine stressor impacts independently of one another, or in contrived aquarium settings. In this study, the interacting effects on sediment meiofaunal communities of two key stressors of estuarine ecosystems, nutrient enrichment and physical disturbance, were examined in a fully orthogonal field experiment replicated at two estuaries in Sydney, Australia. We hypothesized that in line with predictions of the Dynamic Equilibrium Hypothesis, and consistent with the results of a previous mesocosm experiment, effects of physical disturbance on meiofauna would be mediated by the level of nutrient enrichment, with stronger effects of physical disturbance at low levels of nutrient enrichment. Contrary to our predictions, we found generally additive effects of nutrient enrichment and physical disturbance on sediment meiofaunal communities that varied in magnitude between study sites. Of the two stressors, nutrient enrichment had a greater effect on meiofaunal abundance and richness. The divergence of our results from those of previous mesocosm experiments highlights the importance of field experiments, that incorporate the full complexity of ecological systems, in understanding stressor impacts. The site-dependent effects of stressors reinforces the role that background environmental conditions, including pre-exposure to other stressors, and community structure can play in determining the sensitivity of ecological communities to stressors.

KEYWORDS: Multiple stressors, meiofauna, nutrient enrichment, physical disturbance, field study, benthic community.

3.1. INTRODUCTION:

Coastal ecosystems are increasingly threatened by a growing list of anthropogenic stressors (Crain et al. 2008, Darling & Côté 2008; Sundbäck et al. 2010, Ellis et al. 2015, Halpern et al. 2015, Griffen et al. 2016). These stressors rarely occur in isolation of one another but, rather, overlap in time and space. Understanding how these multiple stressors interact to produce ecological impacts is critical to the adoption of appropriate strategies for managing coastal ecosystems (Alsterberg et al. 2012, Piggott et al. 2015, Cote et al. 2016, Van den Brink et al. 2016). Yet, prediction of multiple stressor effects remains a key challenge. A recent study by Rudd, (2014) involving over 2000 scientists in 94 countries identified multiple stressor studies as the most pressing research question for marine systems (Griffen et al. 2016).

Effects of multiple stressors cannot be easily predicted from single-stressor studies because their impacts are not necessarily additive and in many instances are instead synergistic or antagonistic (Folt et al. 1999, Crain et al. 2008, O'Brien et al. 2019). Yet a majority of studies continue to examine the effect of single stressors (Crain et al. 2008, O'Brien et al. 2019) and those that consider multiple stressor effects are frequently focused on single species or a sub-set of taxa (Wernberg et al. 2012) or utilise highly contrived microcosm or mesocosm set-ups (Carpenter 1996). In communities, interactions among species may exacerbate or mitigate stressor effects to individual taxa (Vinebrooke et al. 2004). The full complexity of ecological systems is rarely replicated in aquarium experiments. Although well designed *in situ* mesocosm experiments can be powerful tools for providing insights into how environmental changes influence biodiversity and ecosystem functioning (Steward et al. 2013, Pansch et al. 2016) they nevertheless differ in environmental conditions to natural systems. For example, tides and currents, predation and mixing will differ between mesocosms and natural systems (Pansch et al. 2016), and resultant experimental artefacts (e.g. wall effects, mixing, exposure time) may weaken or exacerbate stressor impacts.

Amongst the most common stressors to affect estuarine sediments are anthropogenic eutrophication and physical disturbances of sediments (Gray

1997, Sundbäck et al. 2010). Land-use change associated with urbanisation and agricultural activities, and use of fertilizers and cleaning products have increased nitrogen and phosphorous inputs into estuaries (Valiela et al. 1992, Nixon 1995). Nitrogen and/or phosphorus are generally limiting resources, and enhanced loadings of these can directly or indirectly effect the physical, chemical and biological properties of aquatic systems (McClanahan et al. 2005, Carreiro-Silva et al. 2009). For example, algal blooms stimulated by enhanced nutrient inputs can shade seagrass, and in enhancing organic matter inputs to sediments can lead to hypoxia and anoxia as a consequence of bacterial decomposition consuming oxygen (Vitousek et al. 1997). Physical disturbance of sediments can occur both at large scales of tens to hundreds of meters, as a result of trawling and resuspension of sediments during storms events (Posey et al. 1995, Tuck et al. 1998) and at small scales of centimetres to meters as a result of boat anchorage, propeller scarring, coastal walking, and bait digging (Wynberg & Branch 1994, Bishop 2005, Rossi et al. 2007). Both small- and large-scale physical disturbance can lead to changes in benthic communities by damaging and displacing taxa, and by modifying sediment properties such as grain size (Yea & Risk 1979, Lindegrath & Hoskin 2001, Bishop 2005).

According to the Dynamic Equilibrium Hypothesis (Huston 1979), nutrient enrichment and physical disturbance should produce non-additive effects. In contrast to the Intermediate Disturbance Hypothesis, which predicts that intermediate frequencies of physical disturbance, by displacing competitive dominants, should maximise biodiversity irrespective of productivity (Svenson et al. 2007), the Dynamic Equilibrium Model (Huston 1979) posits that the effect of physical disturbance on biodiversity will be dependent on productivity. According to the Dynamic Equilibrium Hypothesis, as productivity increases, more severe disturbances will be required to displace competitive dominants, and hence maximise biodiversity. The Dynamic Equilibrium Hypothesis has been supported by mesocosm experiments that assessed the response of meiofaunal communities to the addition of dried *Ascophyllum* seaweed powder to enhance nutrients and racking of sediments

to physically disturb them (Widdicombe & Austen 2001, Austen & Widdicombe 2006). However, in a field study examining macrobenthic responses to fertilizer addition and raking, the effect of the two stressors was predominantly additive (Kenworthy et al. 2016). It is unclear whether the differing conclusions of the two studies reflect differences in their methodology (e.g. mesocosm vs field, organic vs inorganic nutrient addition) or fundamental differences in meiofaunal versus macrofaunal responses.

Meiofauna (32-100µm) are an abundant, diverse and omnipresent component of sedimentary ecosystems (Coull 1999, Giere 2009). They are essential contributors to ecosystem functioning (Coull 1999, Covich et al. 2004, Zeppilli et al. 2015, Schratberger & Ingels 2017), mineralising organic matter and regulating biogeochemical processes. Due to their rapid turnover, and the direct development of many of their constituent taxa, they are highly sensitive to environmental changes and excellent pollution indicators (Coull 1999). The present study assessed how meiofaunal communities respond to nutrient enrichment and physical disturbance in a field setting. It was hypothesised that in contrast to the (Austen & Widdicombe 2006) study which found interactive effects of the two stressors on meiofauna in experimental mesocosms, we would find additive effects of the two stressors as per Kenworthy et al. (2016) who studied effects of these two stressors on macrofauna at our two study sites. We expected that with increasing nutrient enrichment, the availability of microphytobenthos for grazers would increase, but that physical disturbance would lower microphytobenthic biomass.

3.2. MATERIAL AND METHODS:

3.2.1. Study sites:

In order to test for multiple stressor effects, and assess their spatial generality, our study was replicated at two sites in Sydney, New South Wales, Australia: Tambourine Bay, on the Lane Cove River (33° 49'39" S, 151° 09' 38" E); and Taren Point, Woollooware Bay within Botany Bay (34°01'11" S, 151°07'46" E). These sites were randomly selected from a larger pool of sites that met the following criteria: (1) they had a significant area of unvegetated, intertidal sand/mudflat on which manipulations could be performed; (2) they were away

from point sources of nutrient input, such as storm drains; and (3) were not routinely subject to bait digging or raking (a method of bivalve harvest in some parts of the world). Each of the sites were 15 km from the estuary mouth and were adjacent to highly urbanised areas. At both sites, mangroves were present at mid-high intertidal elevations, adjacent to our unvegetated plots. The tidal range at each of the study sites was ~1.5 m.

3.2.2. Experimental set-up:

Our hypothesis was tested with a fully orthogonal field experiment, replicated at each of the study sites and running from May 2012 through until Sept 2012, wherein we manipulated two stressors: nutrient enrichment and physical disturbance. For each stressor there were 3 levels of intensity: zero, low and high. At a tidal elevation of mean low water + 0.5 m, 70 square plots of 0.25 m² area were established and randomly assigned to one of the 9 experimental treatments or the procedural control, with 7 replicate plots for each of the 10 treatments. Plots were at least 2-3 m apart to prevent leaching of nutrients between these.

In order to manipulate nutrient loading, semipermeable membrane coated pellets of slow release fertiliser (Scotts Osmocote Pro, 8-9), with a N:P:K ratio of 16:4.8:8.3, were added to designated experimental plots. Coated nutrient pellets, which slowly leak nutrients over sustained periods, have previously been used to mimic chronic nutrient enrichment in a variety of habitats (Worm et al. 2000). The pellets' slow release mechanism enables enrichment of sediments over prolonged periods without need for replenishment. Plots designated to the high nutrient loading (High) received 1000 g of fertilizer and plots designated to the low nutrient loading (Low), 500 g, with the no nutrient (Zero) treatment receiving none. The high nutrient level mimicked the loading that might result from sewage discharge (Morris & Keough 2002, 2003, O'Brien et al. 2010), with the low nutrient level set at half of this. The fertilizer designated to each experimental plot was evenly divided among five bags, constructed of nylon pantyhose, and enclosed within these prior to burial. The nylon material functioned as a permeable membrane that allowed leaching of nutrients out into the plots. These were buried on either side and

in the centre of each plot in an even grid, so that the tops of the balls were ~2 cm below the sediment surface. The plots assigned to the zero nutrient treatment did not receive any panty hose balls, but were physically disturbed to mimic the burial of these. So as to assess any artefact of the panty hose balls, a procedural control was established, where sediment that had been defaunated by drying in the oven at 80 degrees Celcius was buried in nylon pantyhose in place of the nutrients, with balls distributed within plots as per the High nutrient treatments.

Physical disturbance was manipulated by raking the sediments using a 50 cm wide garden rake to a maximum depth of 4 cm, in a cross-hatched pattern. Plots designated to the high disturbance treatment received six cross-rakings at the start of the experiment and every month thereafter for 4 months. Plots assigned to the low disturbance treatment had two cross-rakings per month. Raking is a common method used for physically disturbing sediments (Cowie et al. 2000, Whomersley et al. 2010), and these high and low levels of disturbance have previously been shown to elicit a response among intertidal communities (Whomersley et al. 2010).

3.2.3. Sampling :

To test how the two stressors interact to influence meiobenthic communities, we sampled meiofauna and sediment variables 1 (June), 3 (August) and 4 (September) months after the application of stressors. The sediment variables were Chlorophyll *a* content and spectral reflectance, each of which served as proxies for the biomass for microphytobenthos (Tolhurst et al. 2005, Kromkamp et al. 2006, Kenworthy et al. 2016), as well as sediment organic content and sediment grain size. At each time point, sample collection was done prior to re-raking of the plots, and from a different position within each plot. To assess whether there were any pre-existing differences in variables of interest among plots that may compromise the interpretation of results, we also collected measurements of spectral reflectance and cores for assessment of meiobenthic community structure from each plot in May 2012, immediately

prior to the application of stressors. A subset of plots (n=25) was also sampled at this time for assessment of background organic content, Chlorophyll *a* and sediment grain size, at each site. Chlorophyll sampling was not possible in the first month at Lane Cove due to large amounts of overlying water caused due to a low-pressure system.

Meiofauna were sampled at low tide using a hand coring tube of 2.5 cm inner diameter that was depressed to a depth of 3 cm. Immediately upon return to the laboratory, samples were preserved in 7 % buffered formalin and stained with Rose Bengal. After a minimum of 48hrs in formalin, samples were wet-sieved through stacked 500 μm and 45 μm sieves, with the material retained on the 45 μm sieve retained. Ludox -HS40 (density of 1.31 g cm⁻³) was used to extract meiofauna from the retained material using the method of Burgess (2001). The extracted fauna was thoroughly washed with fresh water, to remove any Ludox and then stored in 70% ethanol. A pilot study indicated that the extraction efficiency was 94% (determined by examining the remaining sediment of 14 random samples). Meiofauna were identified and enumerated to mixed taxonomic level (*sensu* Warwick & Gee 1984) under a dissecting microscope with 100X magnification. Annelida were identified to class, Crustacea to order and all other taxa to phyla.

Contact coring (Ford & Honeywill 2002) was used to collect sediment for assessment of organic content, Chlorophyll *a* and grain size. The top 2 mm of the sediment was frozen with liquid nitrogen, collected, immediately placed on ice in a darkened cooler and stored in a -80°C freezer. Within 48 hours of collection, photosynthetic pigments were extracted from a 200 mg subsample of each core under refrigerated and darkened conditions using 1.5ml of 90% acetone. After 24 hours, the samples were each thoroughly mixed for 10 s using a vortex mixer. The Chlorophyll *a* concentration of the acetone solution was determined spectrophotometrically using the method of Jeffrey and Humphrey (1975). Chlorophyll *a* content of sediment was calculated per unit area (mg.m⁻²). A second subsample from each core sample (0.4 – 1g after

drying) was freeze-dried and its sediment grain size and particle distribution obtained using a Laser Diffraction Particle Size Analyzer (Mastersizer 2000, Malvern Instruments Limited, England). A third subsample of 2 g of sediment was taken from each core to determine its organic content using the loss on ignition method, with samples combusted at 450°C for 4 h.

In situ sediment surface spectral reflectance was measured using the Ocean Optics USB2000 spectroradiometer. At each sampling time, at least 3 randomly selected plots per treatment were sampled, with 3 measurements per plot collected. The Normalised difference vegetative index (NDVI), was calculated, based upon the reflectance of Chlorophyll *a* in the sediment. The NDVI is a measure of photosynthetically related biomass based on reflectance of Chlorophyll (Kromkamp et al. 2006). Reflectance values (R) in the visible (675 nm) and infra-red (750 nm) parts of the spectra were used to calculate NDVI.

$$\text{NDVI} = (R_{750} - R_{675}) / (R_{750} + R_{675}) \quad (1)$$

The NDVI reflectance measurements per plot were averaged to calculate reflectance and measurements were discarded where there was interference from surface water.

3.2.4. Statistical Analysis:

Hypotheses were tested using multivariate and univariate Permutational Analyses of Variance (PERMANOVA, Anderson et al. 2008). PERMANOVAs can be applied to any distance based matrix and do not make assumptions about the distribution of data (Anderson et al. 2008). Nevertheless, permutational tests for differences in multivariate dispersion among treatments (PERMDISPs) were analysed alongside PERMANOVAs to examine whether treatment differences could be attributed to differences in dispersion. Multivariate analyses used Bray Curtis dissimilarities calculated from data that had been square root transformed to down-weight the effect of dominant species. Univariate analyses used untransformed Euclidean distances.

First, to test for any pre-existing differences in meiofaunal communities among plots assigned to the various treatments that may confound results, two-way PERMANOVAs, with the factors nutrients (fixed, 3 levels: 0, low and high), and disturbance (fixed, 3 levels: 0, low and high) were run on meiofaunal community data (multivariate community structure, as well as univariate analyses of total abundance, Shannon's diversity, and taxon richness) collected from Lane Cove prior to the application of stressors. Issues with storage of samples from Botany Bay prevented such an analysis from being run for this second site. Second, to assess pre-existing differences in environmental conditions between sites, in each of sediment organic content, mean grain size and Chlorophyll *a*, one-way univariate PERMANOVAs were run. Third, to test for any experimental artefact associated with deployment of nutrients in panty hose, a three-way PERMANOVA using multivariate data with the factors time (fixed, 3 levels: 1, 3 and 4 months), Site (random, 2 levels) and treatment (fixed, 2 levels: undisturbed, procedural control) was done.

Four-factor PERMANOVAs tested for interacting effects of the two stressors, physical disturbance and nutrient enrichment, on meiofauna (multivariate community structure, and univariate analyses of Shannon's diversity, total abundance, taxon richness, and key discriminating taxa) and on sediment variables (univariate analyses of each of organic carbon, Chlorophyll *a*, sediment grain size, NDVI). These had the factors site (random, 2 levels), nutrients (fixed, 3 levels: 0, low and high), disturbance (fixed, 3 levels: 0, low and high) and time (fixed, 3 levels: 1, 3 and 4 months following initial stressor application). Time was considered an independent factor as meiofaunal and sediment cores were small relative to the size of plots. Meiofaunal taxa that were key contributors to dissimilarity among treatments were identified by SIMPER (PRIMER software; Clarke 1993) and had dissimilarity to standard deviation ratios greater than 1. PERMANOVAs were followed by post-hoc tests to assess sources of significant treatment effects. Nonmetric multidimensional scaling (nMDS; Field et al. 1982) was used to visualise multivariate differences in meiofaunal community structure among treatments. All analyses were

performed using the PRIMER v6 statistical program with PERMANOVA + addition (Clarke & Gorley 2006, Anderson et al. 2008).

3.3. RESULTS

With one exception (Shannon diversity), none of the metrics of meiofaunal community structure displayed pre-existing (i.e. time 0) differences among experimental plots that corresponded to treatment assignments at Lane Cove (Table 1A). Of the sediment variables, only mean grain size (MGS), displayed systematic variation with respect to plot assignments prior to stressor application (Table 1B). At Lane Cove, there was no variation in MGS among treatments, but at Botany Bay, MGS was significantly coarser in plots that were designated to remain free of nutrients than those assigned to receive low or high nutrient additions (*a posteriori* tests, sig. Si x Nu interaction, Table 1B; ON vs LN: $t=3.27$, ON vs HN: $p=0.005$, $t=4.066$ $p=0.002$). Significant site differences were observed in NDVI and MGS (Table 1B). NDVI was significantly higher at Lane Cove (Mean $\pm$ SE: 0.102 ± 0.004) than Botany Bay (0.074 ± 0.002) and MGS was greater at Botany Bay ($427.98 \pm 47.01 \mu\text{m}$) than at Lane Cove ($211.68 \pm 18.71 \mu\text{m}$). At neither site did any of the meiofaunal nor sediment variables display a difference between undisturbed plots and the procedural controls, at any of the sampling times (Table 2).

Table 1. PERMANOVAs testing for any pre-existing, systematic, variation in (a) metrics of meiofaunal community structure and (b) NDVI among plots assigned to the various experimental treatments prior to their application Nu = Nutrient (3 levels: 0, Low, High); Di = Disturbance (3 levels: 0, Low, High); Si (2 levels: random). Meiofaunal analyses included data from Lane Cove only. Bolding indicates significant results at $\alpha = 0.05$.

(A)	Multivariate		Abundance		Taxon Richness		Shannon Diversity		
Factors	df	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Nu	2	1.68	0.10	1.59	0.18	0.95	0.37	1.90	0.17
Di	2	0.87	0.54	0.23	0.80	0.98	0.39	0.66	0.54
NuxDi	4	1.22	0.23	1.03	0.41	1.39	0.25	2.60	0.04
Res	50								
Total	58								

(B)	NDVI				Mean grain size	
Factors	df	Pseudo-F	P(perm)	df	Pseudo-F	P(perm)
Si	1	36.22	<0.01	1	16.91	<0.01
Nu	2	0.14	0.97	2	0.69	0.74
Di	2	4.86	0.17	2	0.22	0.97
SixNu	2	0.62	0.54	2	10.25	<0.01
SixDi	2	0.42	0.66	2	0.57	0.55
NuxDi	4	2.63	0.18	4	0.18	0.93
SixNuxDi	4	0.95	0.43	4	2.15	0.09
Res	109			69		

Table 2. Three-way PERMANOVAs testing for experimental artefacts associated with the method of nutrient addition. Analyses were run on sediment variables (organic content, NDVI, Chlorophyll a) as well as on metrics of meiofaunal community structure (abundance, taxon richness, Shannon diversity, multivariate). Tr =Treatment (2 levels: Control, Procedural Control), Si = Site (2 levels, random). Mo = Month (4 levels: 0, 1 3 & 4 months following treatment application). Bolding indicates significant results at $\alpha = 0.05$.

Organic Content				NDVI			Chlorophyll a		
Source	Df	Pseudo-F	P(perm)	Df	Pseudo-F	P(perm)	Df	Pseudo-F	P(perm)
Tr	1	0.005	0.665	1	0.041	0.508	1	7.588	0.507
Mo	2	1.289	0.458	2	1.116	0.420	2	4.066	0.279
Si	1	2.927	0.082	1	15.848	0.001	1	0.959	0.330
TrxMo	2	0.431	0.679	2	0.155	0.856	2	2.147	0.334
TrxSi	1	2.227	0.126	1	1.416	0.244	1	0.113	0.727
MoxSi	2	1.000	0.373	2	4.422	0.014	2	2.536	0.081
TrxMoxSi	2	0.675	0.521	2	0.416	0.669	2	0.299	0.720
Res	53			61			51		
Total	64			72			62		

Abundance				Taxon Richness		Shannon Diversity		Multivariate	
Source	df	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tr	1	21.779	0.512	0.152	0.518	0.925	0.502	0.679	0.493
Mo	2	0.514	0.665	0.448	0.678	2.717	0.348	0.732	0.662
Si	1	25.508	0.001	54.023	0.001	40.096	0.001	35.660	<0.001
TrxMo	2	0.409	0.710	16.488	0.082	1.907	0.338	0.827	0.564
TrxSi	1	0.189	0.671	0.362	0.549	1.479	0.241	1.743	0.101
MoxSi	2	4.375	0.013	3.390	0.046	0.752	0.482	4.530	<0.001
TrxMoxSi	2	0.850	0.419	0.027	0.973	0.524	0.612	1.656	0.072
Res	71								

Table 3. Four-way PERMANOVAs testing for the interacting effects of nutrient enrichment (Nu; 3 levels: zero, low, high) and disturbance (Di; 3 levels: zero, low, high), on environmental variables at two sites (Si, random), at three sampling times (mo; month - 1, 3 and 4 mo following stressor addition). n=3-7. Significant results (at $\alpha = 0.05$) are highlighted in bold.

Source	Organic Carbon			NDVI			Mean grain size			Sediment Sorting		Chlorophyll <i>a</i>		
	Df	Pseudo-F	P(perm)	Df	Pseudo-F	P(perm)	df	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Df	Pseudo-F	P(perm)
Nu	2	0.178	0.801	2	2.245	0.291	2	0.289	0.854	0.348	0.759	2	4.540	0.194
Di	2	1.408	0.499	2	0.095	0.916	2	0.338	0.801	0.059	0.901	2	1.781	0.398
Mo	2	5.947	0.201	2	0.543	0.656	2	0.933	0.595	0.384	0.793	1	0.920	0.658
Si	1	44.218	<0.001	1	9.419	0.002	1	113.700	<0.001	27.383	<0.001	1	4.322	0.040
NuxDi	4	0.758	0.610	4	0.784	0.591	4	2.195	0.229	1.311	0.396	4	2.004	0.259
NuxMo	4	3.478	0.134	4	1.121	0.452	4	1.078	0.472	1.172	0.444	2	19.838	0.053
NuxSi	2	0.368	0.699	2	3.204	0.040	2	0.597	0.552	0.874	0.415	2	1.132	0.318
DixMo	4	1.079	0.479	4	15.081	0.010	4	0.332	0.847	0.193	0.924	2	0.006	0.992
DixSi	2	1.931	0.148	2	3.979	0.018	2	0.630	0.531	0.988	0.376	2	2.554	0.076
MoxSi	2	1.162	0.309	2	12.450	<0.001	2	1.227	0.295	4.178	0.018	1	6.131	0.014
NuxDixMo	8	2.456	0.112	8	0.860	0.582	8	1.267	0.371	0.691	0.693	4	1.068	0.470
NuxDixSi	4	2.017	0.093	4	2.659	0.036	4	0.152	0.962	0.825	0.505	4	0.352	0.841
NuxMoxSi	4	0.193	0.946	4	1.623	0.171	4	0.378	0.831	0.620	0.650	2	0.104	0.894
DixMoxSi	4	1.546	0.183	4	0.134	0.970	4	0.442	0.776	0.638	0.635	2	1.836	0.158
NuxDixMoxSi	8	0.363	0.934	8	1.533	0.146	8	0.612	0.771	1.384	0.203	4	1.594	0.181
Res	255			303			185					204		

The two proxies for microphytobenthic biomass - NDVI (Normalised difference vegetative index) and Chlorophyll *a* concentration – displayed differing responses to the stressors. In the case of NDVI, site-dependent non-additive effects of nutrient enrichment and physical disturbance were apparent (sig. Site x Nutrient x Disturbance interaction, Table 3). At Botany Bay, there was no effect of physical disturbance on NDVI in zero or low nutrient plots (Fig. 1A). In the high nutrient plots, NDVI was significantly greater in plots with zero physical disturbance than with high disturbance, with plots with low disturbance recording an intermediate value (Fig. 1A). At this site, NDVI generally increased with increasing nutrient enrichment (Fig. 1A). At Lane Cove, effects of physical disturbance on NDVI were seen within all three nutrient treatments. When there was no addition of nutrients, plots receiving low physical disturbance had a significantly lower NDVI than plots receiving high physical disturbance, with the zero disturbance treatment displaying an intermediate NDVI (Fig. 1B). Under both low and high nutrient enrichment, NDVI was greater when low physical disturbance was applied than when there was zero or high physical disturbance, the latter two of which did not significantly differ (Fig. 1B). Chlorophyll *a* concentrations, by contrast, did not display significant differences among nutrient treatments, physical disturbance treatments, or their interaction (Table 3).

Each of organic content, median grain size and sorting did not respond to the addition of either stressor, at either site (Table 3).

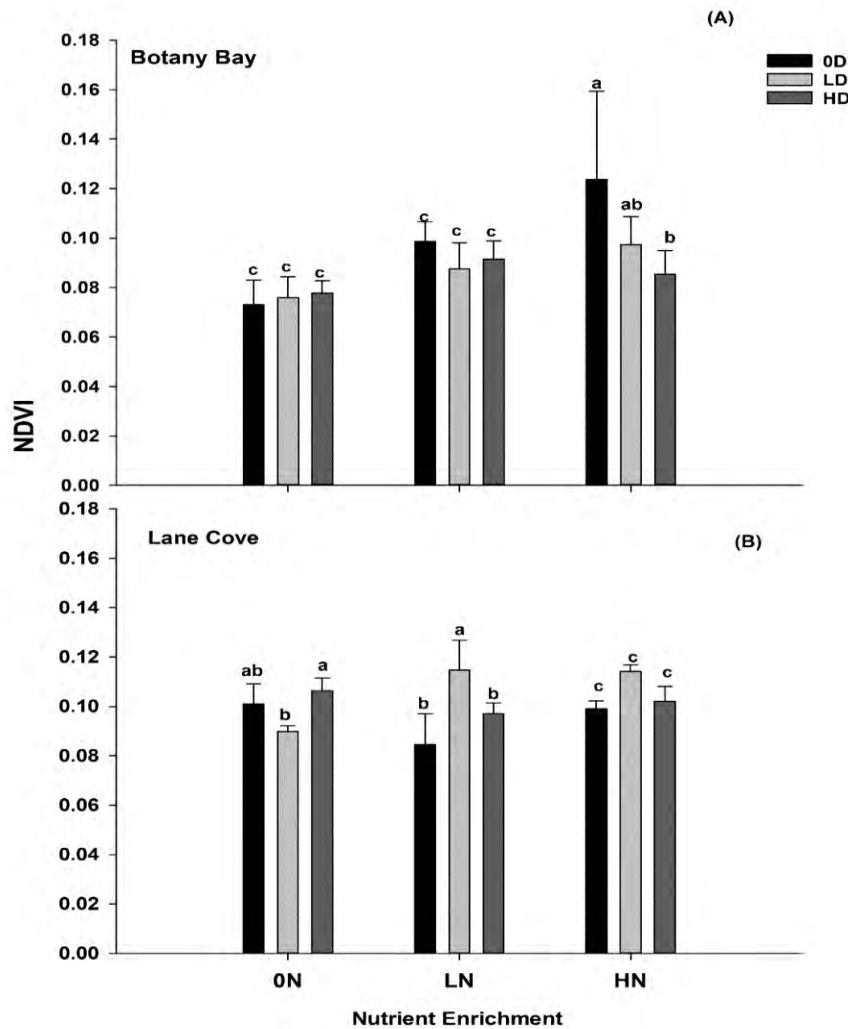


Figure 1.. Mean (± 1 SE) NDVI of sediment at Botany Bay and at Lane Cove, in plots with zero (ON), low (LN) or high (HN) nutrient enrichment and zero (0D), low (LD) or high (HD) physical disturbance. Data were pooled across months as these did not statistically differ significantly. Letters above columns indicate treatments that were found to significantly differ (at $\alpha = 0.05$) when *a posteriori* PERMANOVAs for the significant three-way interaction were run. N =7

3.3.1. Meiofaunal Analysis

Across the two sites, 20 major meiofaunal groups were detected, 14 of which were common to both sites (Supplementary Table S1). At Lane Cove the most abundant taxon was Nematoda, accounting for 59% of total meiofaunal abundance, followed by Copepoda (17%), Ostracoda (16%), Polychaeta (2%) and Turbellaria (2%). At Botany Bay, Nematoda was also the dominant taxon, accounting for 84% of all meiofauna sampled, followed by Copepoda (6%), Tardigrada (3%), Ostracoda (3%), Turbellaria (2%) and Polychaeta 1%. Multivariate analyses revealed that there were no interactive effects of nutrient enrichment and physical disturbance on meiofaunal assemblages at either site (Table 4). Instead, a site-dependent effect of physical disturbance (sig. Site x Disturbance interaction, Table 4, Fig. 2A) and a temporally variable, but site-independent effect of nutrient enrichment on meiofaunal communities was apparent (sig. Time x Nutrient interaction, Table 4, Fig. 2B). At Lane Cove, meiobenthic communities differed between low and high levels of physical disturbance ($t=1.82$, $p(\text{perm})=0.006$), each of which did not differ from the zero physical disturbance treatment. At Botany Bay there was no significant difference between the plots receiving zero and low physical disturbance, but plots receiving high physical disturbance differed from each of these (0 vs H: $t=1.77$, $p=0.004$; L vs H: $t=2.04$, $p=0.002$; Table 4, Fig. 3). Effects of nutrient enrichment were not apparent at month 1, but were at both months 3 and 4. At month 3, the plots with zero and high nutrients varied significantly ($t=1.948$ $p(\text{perm}) < 0.05$), whereas at month 4, plots receiving low and high enrichment were significantly different ($t=1.536$ $p(\text{mc}) = 0.04$).

None of the three univariate measures of meiofaunal community structure – total abundance, taxon richness, and Shannon’s diversity – displayed an effect of physical disturbance, at either of the sites or at any of the sampling times (Fig. 3). Instead, each variable displayed effects of nutrient enrichment that varied through time (Table 4, Fig. 3). One month after nutrient addition, there was no effect of enrichment on any of the three variables. However, by three months, effects of nutrient enrichment were visible. In each of months 3 and 4, meiofaunal abundance was significantly greater in plots receiving zero than high nutrient enrichment, with plots receiving low nutrient enrichment of

intermediate abundance (Fig. 3A). Similarly, 3 and 4 months after stressor application, taxon richness was greater when zero than low or high levels of nutrients were applied (Fig. 3B). Shannon diversity only displayed a significant effect of nutrient enrichment in month 4, and was lower in the low than the zero nutrient treatment (Fig. 3C).

Table 4. Results of 4 factor PERMANOVAs, testing for effects of nutrient enrichment (Nu, 3 levels: zero, low, high), physical disturbance (Di, 3 levels: zero, low, high), month (Mo, 3 levels, 1, 3 and 4 months) and site (Si, 2 levels) on meiofaunal abundance, taxon richness, Shannon's diversity and multivariate community structure. N=5-7. P-values were calculated using Monte Carlo(MC) simulations. Bolding indicates significant results at $\alpha = 0.05$.

Factors	Df	Abundance		Taxon Richness		Shannon Diversity		Multivariate	
		Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)
Nu	2	1.585	0.384	0.164	0.854	1.470	0.416	1.359	0.315
Di	2	4.600	0.179	1.403	0.420	1.288	0.440	1.211	0.381
Mo	2	0.444	0.699	0.261	0.790	2.042	0.322	0.781	0.642
Si	1	73.300	<0.001	190.060	<0.001	180.070	<0.001	129.870	<0.001
NuxDi	4	2.988	0.158	0.559	0.707	2.374	0.221	1.209	0.334
NuxMo	4	28.300	0.004	6.818	0.048	7.451	0.041	2.202	0.047
NuxSi	2	1.412	0.247	1.416	0.240	1.637	0.195	1.410	0.172
DixMo	4	3.810	0.113	1.358	0.395	0.785	0.583	1.015	0.489
DixSi	2	0.445	0.640	2.426	0.086	2.094	0.130	2.240	0.015
MoxSi	2	25.896	<0.001	5.804	0.004	6.051	0.002	16.131	<0.001
NuxDixMo	8	0.601	0.762	0.417	0.884	0.759	0.642	0.598	0.945
NuxDixSi	4	0.305	0.873	1.223	0.303	0.642	0.627	1.375	0.116
NuxMoxSi	4	0.040	0.997	0.352	0.847	0.144	0.965	0.590	0.923
DixMoxSi	4	0.333	0.853	0.769	0.543	0.302	0.878	0.805	0.719
NuxDixMoxSi	8	0.632	0.752	1.496	0.154	0.468	0.875	0.901	0.646
Res	308								

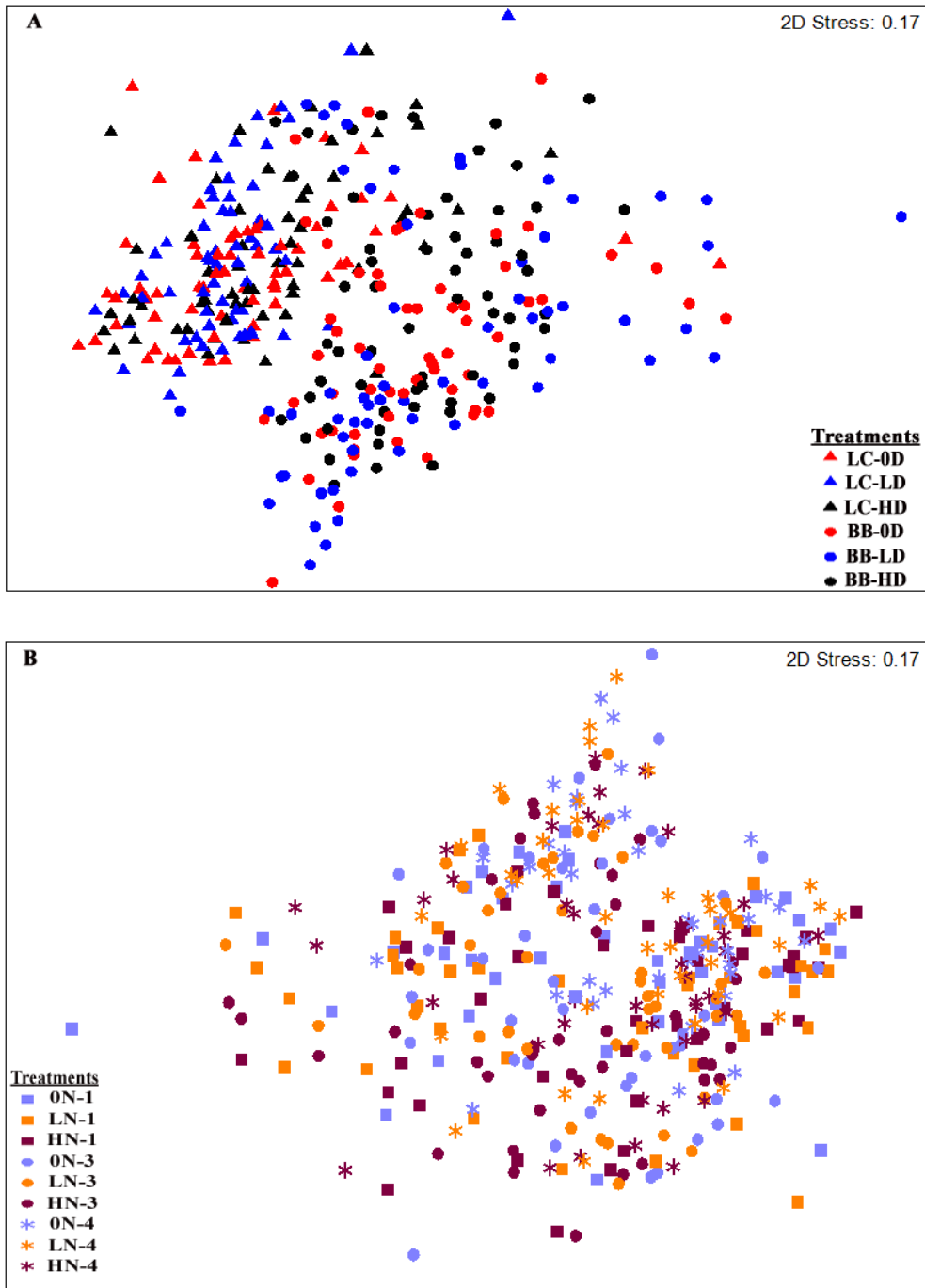


Figure 2. Non metric multi-dimensional scaling (NMDS) plots displaying differences in meiobenthic community composition (A) among levels of physical disturbance (0D: zero, indicated in red; LD: low, is indicated in blue; HD: high, indicated in black) at the two sites (LC: Lane Cove is indicated by filled triangles, BB: Botany Bay is indicated in circles) and (B) among levels of nutrient enrichment (0N; zero, indicated in purple; LN: low, is indicated in orange; HN: high, is indicated in maroon) at each of three sampling times (1 mo: is represented by square shaped symbols; 3 mo: filled circle symbols; 4 mo: by asterisk symbols).

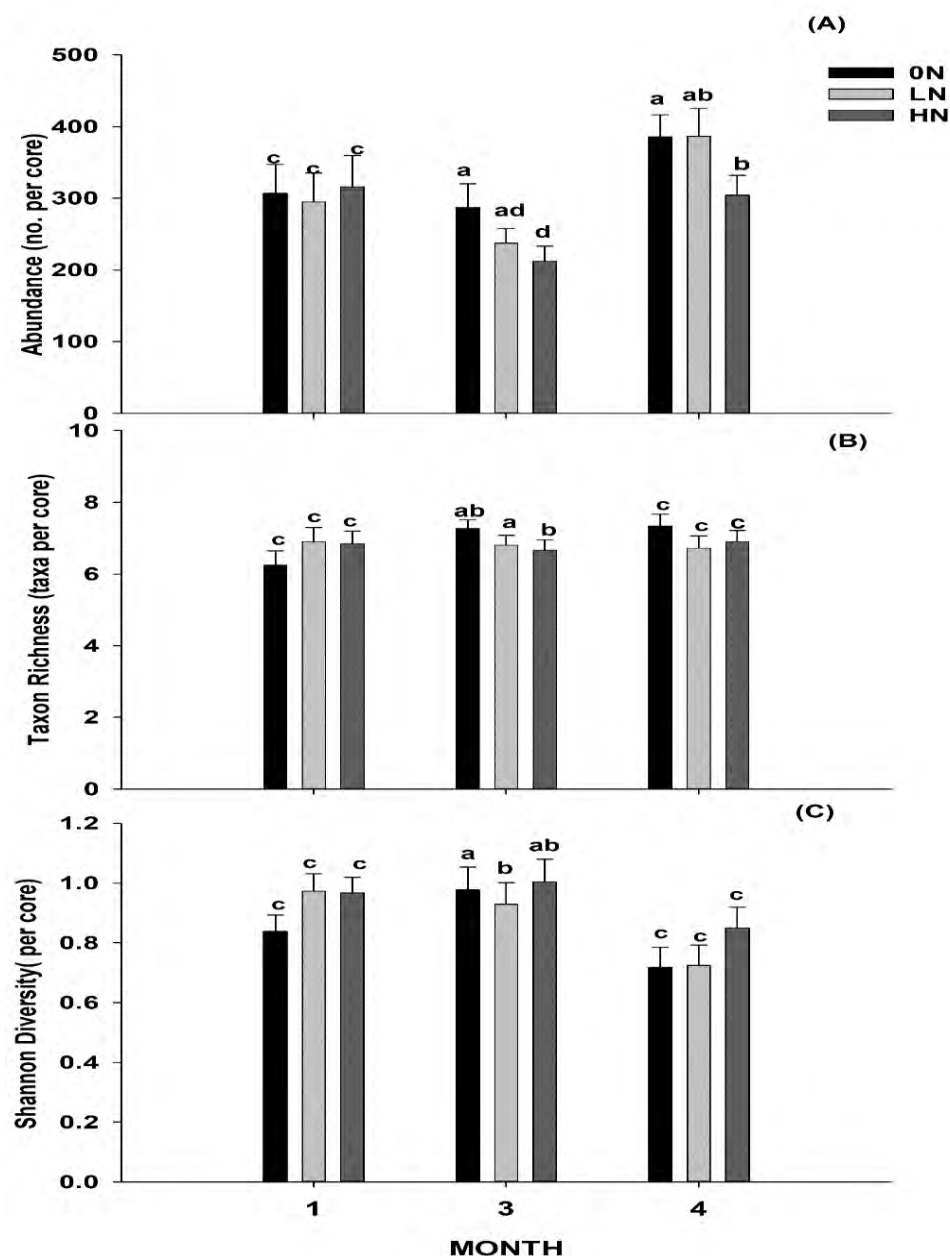


Figure 3. Mean (± 1 SE) (A) abundance, (B) taxon richness and (C) Shannon diversity of meiofauna in plots receiving zero (0N), low (LN) or high (HN) nutrient enrichment, 1, 3 and 4 months following commencement of stressor application. Data are pooled across disturbance treatments and sites as these did not display a significant interaction with nutrients or months. Letters above columns indicate treatments that were found to significantly differ (at $\alpha = 0.05$) when *a posteriori* PERMANOVAs for the significant three-way interaction were run. N=5-7

Table 5. Results of 4-factor PERMANOVAs examining the interacting effects of nutrients (Nu; fixed, 3 levels; 0, low and high), physical disturbance (Di; fixed, 3 levels: 0, low and high), month (Mo; fixed, 3 times: 1, 3 & 4) and site (Si, random) on the key discriminating species common to both sites within Botany Bay and Lane Cove estuaries.

Factors	df	NEMATODA		COPEPODA		OSTRACODA		POLYCHAETA		KINORHYNCHA		TURBELLARIA	
		Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)
Nu	2	2.321	0.302	0.156	0.869	0.251	0.795	0.062	0.942	1.043	0.496	3.035	0.248
Di	2	8.432	0.107	0.659	0.600	0.520	0.669	0.035	0.966	0.741	0.574	8.297	0.110
Mo	2	0.823	0.550	4.281	0.192	0.522	0.658	3.672	0.211	1.104	0.475	1.085	0.484
Si	1	11.443	0.001	243.380	<0.001	276.640	<0.001	150.240	<0.001	470.220	<0.001	23.812	<0.001
NuxDi	4	19.589	0.007	0.236	0.907	0.389	0.809	0.779	0.597	1.236	0.423	0.730	0.619
NuxMo	4	12.095	0.018	0.350	0.830	0.332	0.844	1.372	0.383	1.448	0.366	1.566	0.350
NuxSi	2	1.460	0.238	2.116	0.124	2.053	0.129	5.908	0.004	5.082	0.007	0.504	0.614
DixMo	4	1.153	0.442	1.550	0.342	1.584	0.333	1.457	0.365	0.908	0.529	0.399	0.802
DixSi	2	0.340	0.716	2.857	0.063	0.939	0.391	2.159	0.129	0.699	0.499	0.248	0.776
MoxSi	2	26.779	<0.001	1.794	0.173	18.930	<0.001	4.492	0.013	9.311	<0.001	21.721	<0.001
NuxDixMo	8	0.501	0.826	0.452	0.855	1.064	0.467	0.748	0.652	1.161	0.426	0.209	0.981
NuxDixSi	4	0.090	0.983	2.770	0.029	1.872	0.113	1.767	0.136	0.956	0.431	2.548	0.038
NuxMoxSi	4	0.121	0.975	1.803	0.124	0.283	0.889	0.924	0.449	1.196	0.317	1.364	0.246
DixMoxSi	4	0.464	0.769	0.873	0.479	0.932	0.461	0.899	0.479	0.542	0.713	1.034	0.387
NuxDixMoxSi	8	0.536	0.834	1.035	0.412	0.860	0.557	0.513	0.853	0.941	0.479	1.212	0.300
Res	308												
Total	361												

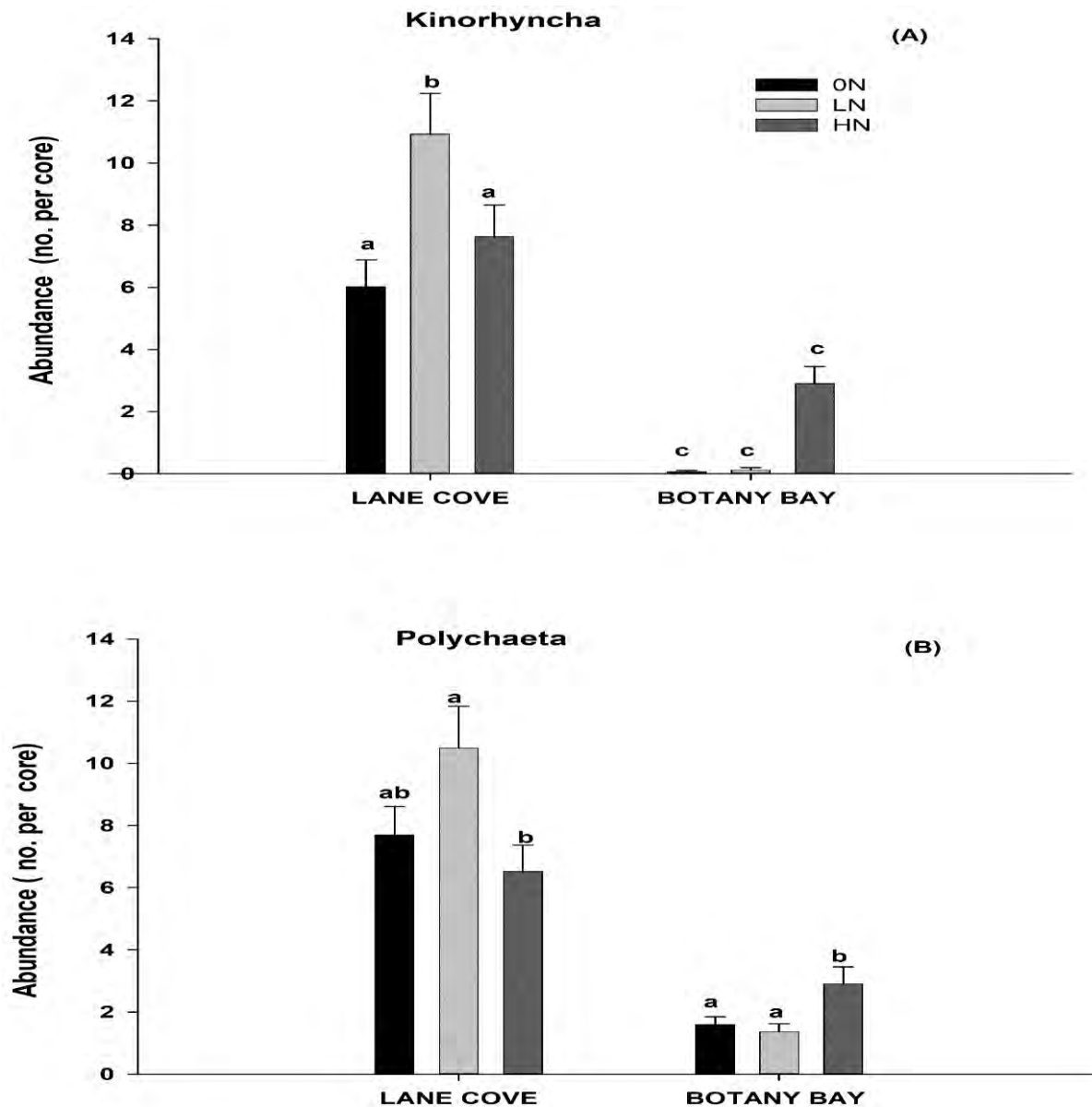


Figure 4. Mean (± 1 SE) abundance of (A) Kinorhyncha and (B) Polychaeta in plots receiving zero (ON), low (LN) or high (HN) nutrient enrichment, at each of two sites. Data are pooled across physical disturbance treatments and months as these did not display significant interactions with nutrient enrichment or site (PERMANOVA, Table 5). Letters above columns indicate treatments that were found to significantly differ (at $\alpha = 0.05$) when *a posteriori* PERMANOVAs for the significant two-way interaction were run. N =5-7

SIMPER analysis identified six key discriminating taxa, contributing most to dissimilarity between nutrient and disturbance treatments in Botany Bay and Lane Cove. These were Nematoda, Copepoda, Ostracoda, Polychaeta, Kinorhyncha and Turbellaria.

Polychaeta and Kinorhyncha displayed site-specific effects of nutrient enrichment (sig. Nu x Si interaction, Table 5). At Lane Cove, each of these taxa were more abundant in plots receiving the low than the zero or high nutrient addition, with the latter two treatments not significantly differing (Fig. 4). At Botany Bay, by contrast, where each of the groups was less abundant, Kinorhyncha did not significantly differ among nutrient enrichment treatments and abundances of Polychaeta were greater in the high than the low or zero nutrient enrichment treatment (Fig. 4 A, B).

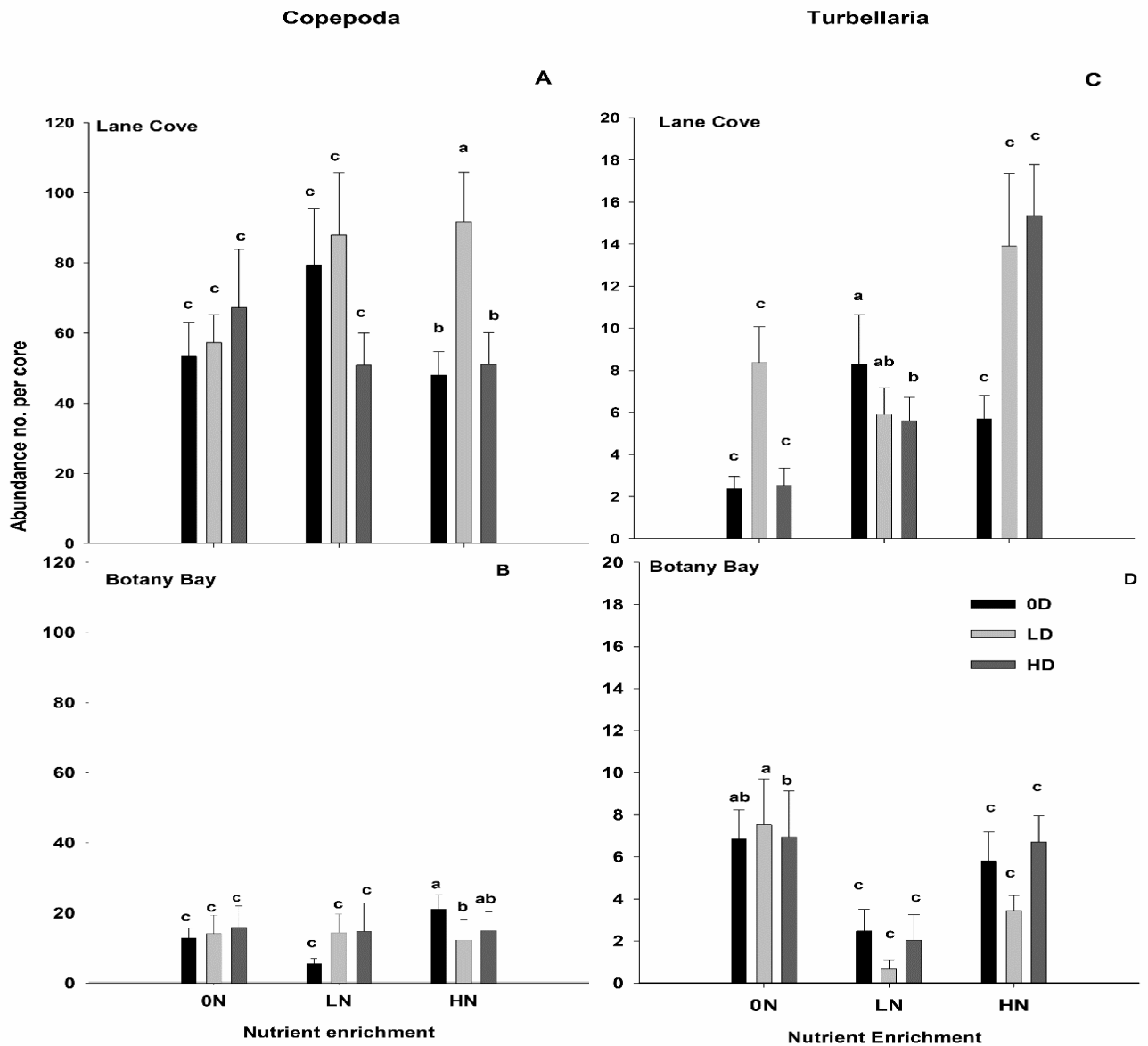


Figure 5. Mean (± 1 SE) abundance of (A, B) Copepoda and (C, D) Turbellaria in plots receiving zero (0N), low (LN) or high (HN) nutrient enrichment, and zero (0D, black bars), low (LD, light grey bars) or high (HD, dark grey bars) physical disturbance, at each of two sites (A, C: Lane Cove; B, D: Botany Bay). Data are pooled across months as these did not display significant interactions with nutrient enrichment or physical disturbance (PERMANOVA, Table 5). Letters above columns indicate treatments that were found to significantly differ (at $\alpha = 0.05$) when *a posteriori* PERMANOVAs for the significant three-way interactions were run. N =5-

Copepoda and Turbellaria displayed interactive effects of nutrient enrichment and physical disturbance that varied between the sites (sig. Nu x Di x Si interaction, Table 5). Physical disturbance had no effect on Copepoda abundance under conditions of zero or low nutrient enrichment at either site (Fig. 5A, B). However, at Lane Cove, in the plots receiving high enrichment, abundances of Copepoda were greater when exposed to Low than zero or high physical disturbance (Fig. 6A). At Botany Bay, by contrast, Copepoda abundance was greater in the high nutrient enrichment plots receiving zero than low physical disturbance, with other pairwise comparisons of physical disturbance treatments non-significant (Fig. 5B). Turbellaria displayed more idiosyncratic patterns between the sites (Fig. 5 C, D). At Lane Cove, effects of physical disturbance were seen only in the plots receiving low nutrient enrichment, among which abundance was greater in the zero than the high physical disturbance treatment, with all other pairwise comparisons non-significant (Fig. 5C). At Botany Bay, effects of physical disturbance were seen in the both the zero and low nutrient enrichment treatments (Fig. 5D). In the plots receiving zero nutrient enrichment, the abundance of Turbellaria was significantly greater in plots receiving low than high physical disturbance, with no significant differences between other pairs of treatments (Fig. 6D). In the plots receiving low nutrient enrichment, there were significantly fewer Turbellaria in the Low than the zero physical disturbance treatment, with no other pairwise differences (Fig. 5D).

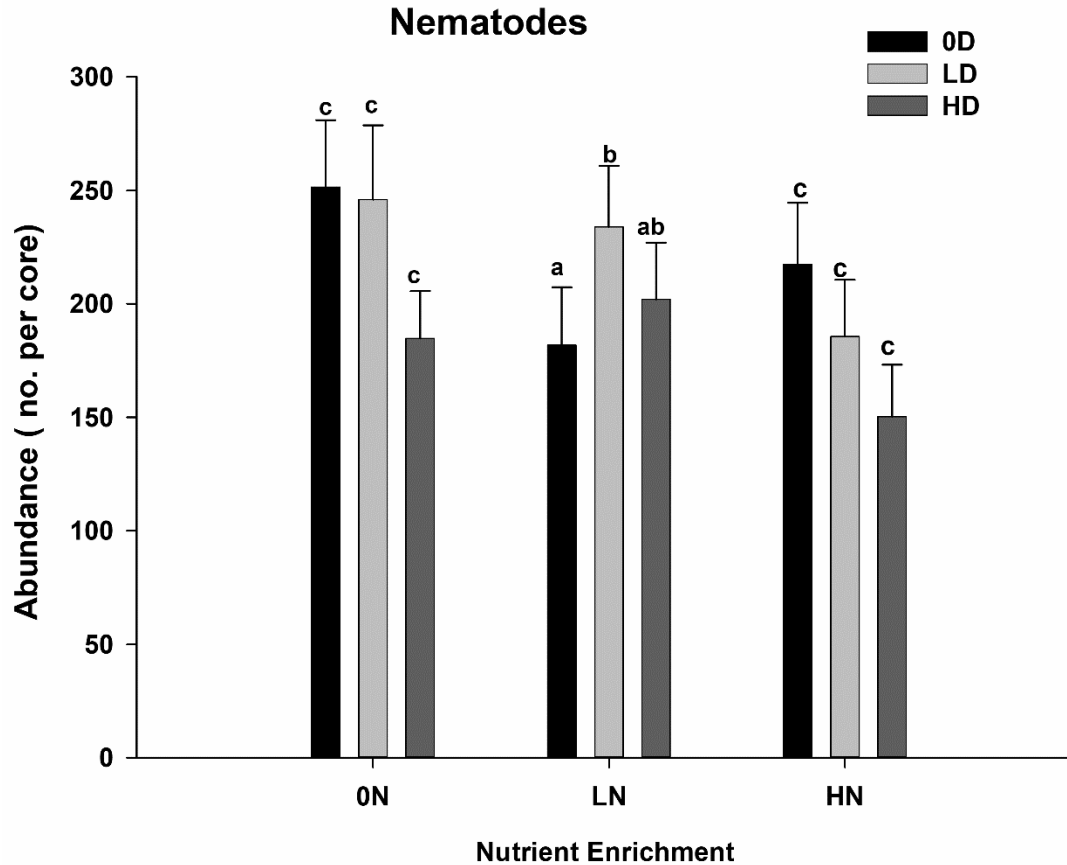


Figure 6. Mean (± 1 SE) abundance per core of Nematoda in plots receiving zero (ON), low (LN) or high (HN) nutrient enrichment and zero (0D), low (LD) or high (HD) physical disturbance, after stressor application. Data are pooled across sampling times and months as neither factor displayed a significant interaction with nutrient enrichment and physical disturbance (Table 5). Letters above columns indicate treatments that were found to significantly differ (at $\alpha = 0.05$) when *a posteriori* PERMANOVAs for the significant two-way interaction were run. N =5-7.

Nematoda displayed a two-way interaction between nutrient enrichment, and physical disturbance that was independent of site or sampling time (sig. Nu x Di interaction, Table 5) and a main effect of nutrients that was dependent on month (sig. Nu x Mo interaction, Table 5). At zero and high levels of nutrient enrichment, there was no significant effect of physical disturbance (Fig.6). However, under conditions of low nutrient enrichment, there were significantly ($t=14.643$,

$p(\text{mc})=0.045$) more nematodes in plots receiving low than zero physical disturbance, with other pairwise comparisons non-significant (Fig. 6).

When the month by nutrient enrichment interaction was examined, only in month 1 was an effect of nutrient enrichment apparent. Plots receiving low enrichment differed from those receiving high ($t= 15.813$, $p=0.04$) levels of nutrient enrichment, with the other treatments not significantly differing with higher abundances at low enrichment levels.

The two other key discriminating taxa, Ostracoda and Targdigrada, did not respond to either the manipulation of nutrients or physical disturbance, with only differences in abundance among sampling times and sites apparent (Table 5).

3.4. DISCUSSION

Contrary to the predictions of the Dynamic Equilibrium Hypothesis (Huston 1979), our field manipulations demonstrated generally additive effects of the two stressors, nutrient enrichment and physical disturbance, on meiobenthic communities. Only three of the taxa examined responded in abundance to the interacting effects of nutrient enrichment and physical disturbance, and for two of these, the nature of the interaction was site-dependent. The predominantly additive effect of the stressors on meiofaunal communities is counter to the results of a mesocosm experiment, where physical disturbance subdued the effects of high levels of nutrient enrichment on meiofauna (Widdicombe & Austen 2001). Overall, meiofaunal responses to the two stressors were largely concordant with previous assessments of macrofauna at our study sites (Kenworthy et al., 2016).

The differing results of this field study and previous mesocosm studies on the combined effects of nutrient enrichment and physical disturbance on meiofaunal communities (Austen & Widdicombe 2006, Widdicombe & Austen 2001) may reflect differences in the methodology of the two experiments, differences in environmental conditions, or alternatively the taxonomic resolution used. Both

the source of nutrients and the frequency of physical disturbance differed between the present and previous experiments. Whereas we manipulated nutrients through addition of inorganic fertiliser, the mesocosm experiments added enriched sediments via application of dried and ground-up *Ascophyllum* (Austen & Widdicombe 2006, Widdicombe & Austen 2001). Our study raked sediments monthly, whereas the mesocosm experiment physically disturbed sediments daily to monthly. In the intertidal zone, biological responses to nutrients depend on its source, with inorganic nutrients assimilated more slowly than organic nutrients (O'Brien et al. 2011). The frequency of disturbance is, in combination with its area and severity, a key determinant of impact (Austen & Widdicombe 2006, Picket & White 1985).

Additionally, field experiments may give differing results to mesocosm experiments for several reasons. First, mesocosm experiments are unable to replicate the inherent patchiness of natural systems, which may influence stability as well as recovery processes by determining availability of larvae and adults for recolonization (Crain et al. 2008). Second, collection and containment of animals for use in mesocosm experiments can introduce an additional source of stress, that amplifies effects of stressors of interest (Cowie et al. 2000). Third, mesocosm experiments are unable to fully replicate the biological and environmental complexity of natural systems, that may serve to buffer organisms from the effects of stressors (Vinebrooke et al. 2004, Stachowicz et al. 2008). Finally, whereas the previous mesocosm studies identified taxa to species, our study used the coarser resolution of phyla due to a paucity of local taxonomic expertise on the meiofauna. Although in many instances, phyla level analyses are adequate for detection of ecological impacts (Olsgard et al. 1998) it is generally acknowledged that the ability of assessments to reliably represent multivariate distances between samples decreases with coarsening resolution (Vanderklift et al. 1996).

Our sampling techniques did not distinguish between permanent meiofauna (species that remain in the meiofaunal size class their whole life cycle) and transient meiofauna (species that start out as meiofauna but grow into macrofauna or emerge out of the benthos). Hence, the concordance of our results with those of Kenworthy et al. (2016) who tracked macrofaunal responses to the two stressors at the same study sites may reflect impacts to macrofauna that occur during their early life-history stages. Nevertheless, several of the taxa (e.g. Kinorhyncha) which responded most strongly to the stressors were permanent meiofauna. Similar responses of meiofauna and macrofauna to environmental stressors is common, though not universal (Austen & Widdicombe 2006).

Of the two stressors considered, nutrient enrichment generally had greater impacts on meiofaunal communities than physical disturbance. Effects of physical disturbance on meiofauna were weak. It has been suggested that in comparison to macrofauna, meiofauna may be less sensitive to changes in their position in surface sediments, or able to rapidly re-establish their position (e.g. Austen & Widdicombe 2006). East Australian estuaries are generally considered highly oligotrophic, with phosphorus, in particular, a limiting resource (Bishop et al. 2006, Scanes et al. 2007, Kelaher et al. 2013). In these oligotrophic systems the addition of nutrients can, by stimulating productivity, enhance the biomass of consumer species (Bishop et al. 2006, York et al. 2012). Such an effect was seen at Botany Bay, which, according to our measurements of NDVI, had the smaller starting biomass of microphytobenthos of the two sites. At this site, NDVI and the abundance of several taxa, most notably Kinorhyncha and Polychaeta, increased with increasing nutrient enrichment. Kinorhyncha feed on either diatoms or organic matter, depending on species (Dal Zotto et al. 2016) and in several other studies done at oligotrophic locations have responded positively to moderate nutrient inputs from aquaculture, presumably through bottom-up processes (Posey et al. 1995, 2002, De Paula et al. 2006, Dal Zotto et al. 2016). At Lane Cove, where higher NDVI readings suggested greater background levels of microphytobenthos, however, the effect of nutrient enrichment was non-

linear. NDVI and abundances of Polychaeta and Kinorhyncha were enhanced by low but not high nutrient input. This may be because under more eutrophic conditions the addition of more nutrients can result in excess primary production, the decomposition of which can lead to deterioration of sediment conditions by oxygen-consuming bacteria (Bulling et al. 2008, Fitch & Crowe 2010, 2012, Botter-Carvalho et al. 2014). In a mesocosm experiment, Kinorhyncha were found to respond negatively to high nutrient enrichment (Nitrogen, Phosphorus and Silica) but increase in numbers in response to low levels of enrichment (Widbom & Elmgren 1988). In that experiment, the decline in their abundance at high levels of enrichment was attributed high sulphide content as a byproduct of enhanced primary productivity (Widbom & Elmgren 1988).

In the few instances in which non-additive responses to the two stressors were found, the nature of these interactions varied among taxa and between study sites. The dynamic equilibrium hypothesis predicts that greater magnitudes of physical disturbance will be required to control competitive dominants as levels of productivity increase. Consistent with this, significant effects of physical disturbance were most apparent at low, as opposed to high levels of nutrient enrichment. It is also possible that at the highest level of nutrient addition some deterioration of sediment conditions occurred via the effects of over-enrichment, preventing an effect of physical disturbance from being seen. The site-specificity of interactions in two of the three taxa in which they were apparent reinforces the context-dependency of multiple stressor effects. Differences between sites in faunal responses may reflect differences in the key species contributing to coarse taxonomic groupings, differences in meiofaunal assemblage composition that buffer or exacerbate responses of individual taxa to stressors (Bulling et al. 2008, Godbold & Solan 2009), and environmental differences between Botany Bay, which is a largely industrial area with a history of contamination, and Lane Cove which is surrounded by bushland and residential development (Kenworthy et al. 2016). Even within orders or families, species can display marked differences in their sensitivity to stressors (Lenihan et al. 2003). Background environmental

conditions can select for species that are more (or less) resilient to stressors and influence the detectability of stressor effects against a background of spatial and temporal variability (Bulling et al. 2008).

3.5. CONCLUSIONS

Overall, our study suggests that patch scale disturbances of sediment through nutrient enrichment and raking have generally additive effects on sediment meiofaunal communities along the east coast of Australia, with nutrient enrichment being the more influential stressor of the two. Hence, management of nutrients in line with the recommendations of single-stressor studies may, in the absence of interactive effects with other stressors not examined here, lead to predictable outcomes for meiofaunal communities. Studies, are however, needed to confirm whether nutrient application also has additive effects with other common stressors of estuarine environments, such as metal contamination and poly-aromatic hydrocarbons. As the way in which stressors interact is likely to be scale-dependent, and determined by the proximity of sources of colonists, studies are also needed to confirm how results apply to larger scale perturbations.

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The following is the supplementary material for

Multiple stressor effects of nutrient enrichment and physical disturbance on estuarine meiobenthos – A field study.

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Table S 1. List of the major meiofaunal groups observed at each of the two experimental sites, and their contribution (%) to meiofaunal abundance across all treatments.

Taxa	Botany Bay(%)	Taxa	Lane Cove(%)
Nematoda	83.719	Nematoda	59.262
Copepoda	6.413	Copepoda	16.924
Tardigrada	2.716	Ostracoda	15.810
Ostracoda	2.663	Polychaeta	2.139
Turbellaria	2.139	Kinorhyncha	2.132
Polychaeta	0.892	Turbellaria	1.954
Bivalvia	0.436	Halacaroidea	0.660
Gastropoda	0.431	Amphipoda	0.315
Isopoda	0.144	Isopoda	0.298
Rotifera	0.144	Gastropoda	0.194
Unidentified	0.121	Bivalvia	0.163
Oligochaeta	0.081	Oligochaeta	0.109
Halacaroidea	0.040	Rotifera	0.022
Kinorhyncha	0.038	unidentified	0.016
Cladocera	0.010	Arthropoda(Insecta)	0.001
Amphipoda	0.008		
Cumacea	0.003		
Arthropoda(Insecta)	0.003		

Chapter 4

Simultaneous or sequential: the timing and order of multiple stressor applications influences impacts to estuarine meiobenthic communities

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Highlights of the research:

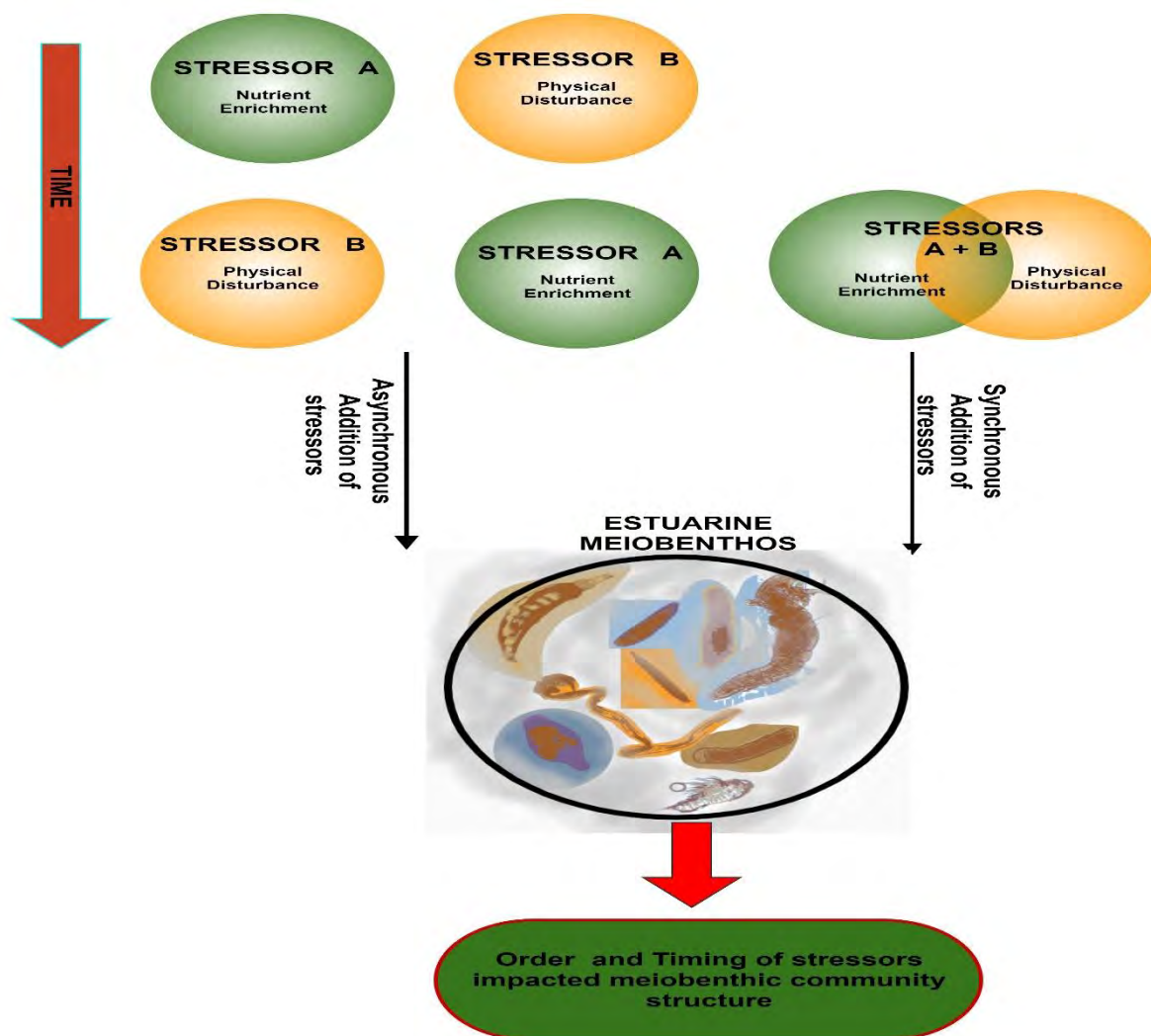
- Estuarine benthic meiofauna were exposed to nutrient and physical disturbance
- Impacts of synchronously and asynchronously applied stressors were compared
- The timing and order of multiple stressor applications influenced their impacts
- Responses were taxon- and site-specific.
- Models of multiple-stressor effects need to account for temporal dynamics.

Abstract

There is growing recognition that anthropogenic stressors rarely operate in isolation and effective management of these requires understanding the way in which they interact. Multiple stressor studies have almost universally considered cumulative impacts of stressors that are applied simultaneously despite many stressors acting asynchronously. Theoretical models predict that the spacing between multiple stressors will determine whether they have additive, synergistic or additive effects. Using a manipulative field experiment, replicated across two sites of Sydney, Australia, we examined whether the cumulative impact on sediment meiofaunal communities of two major anthropogenic stressors of estuarine systems - nutrient enrichment and physical disturbance - are affected by whether the stressors are applied synchronously or asynchronously, and whether among asynchronous treatments, the order of stressor application matters. Our results indicate that asynchronous rather than simultaneous application of stressors generally had stronger effects on meiofaunal community structure. Nevertheless, responses were highly site and taxon dependent. Whereas the abundance of Turbellaria was more negatively impacted when the two stressors were applied synchronously than asynchronously at one of the two study sites, Copepoda displayed the reverse pattern of being less negatively impacted in the synchronous application. These results suggest that species interactions and differential environmental tolerances may mediate multiple stressor impacts. Overall, the results demonstrate that the temporal dynamics of stressors needs to be included in predictive models of multiple-stressor impacts. Additional field studies are needed to disentangle how the identity, magnitude, and timing of multiple stressors influences their cumulative impact.

Keywords: multiple stressors, nutrient enrichment, physical disturbance, sequential stressors, asynchronous stressors, meiofauna, meiobenthos

Graphic Abstract



4.1. Introduction

The growing human population is placing an increasing number of pressures on the world's ecosystems (Cote et al. 2016) which, in causing stress, threaten ecological structure and function (Crain et al. 2008, Doney et al. 2012, Bennet & Chaplin 2016). Consequently, a chief concern of ecologists has and continues to be understanding the ecological impacts of stressors, and how these vary in time and space, according to variation in biological and environmental contexts (Crain et al. 2008, Ellis & Schneider 2008, Molinos & Donhue 2010, Griffen et al. 2016, Gunderson et al. 2016, Bible et al. 2017, Van De Brink et al. 2019). While early studies focused on understanding impacts of single stressors on

ecosystems, there has been growing recognition that stressors rarely occur alone, and that understanding the interactive effect of multiple stressors is imperative to building the predictive models required to manage their impacts (Crain et al. 2008, Przeslawski et al. 2015, Belarde & Railsback 2016, Cote et al. 2016, Griffen et al. 2016, Gunderson et al. 2016). Because multiple stressors may have additive, synergistic or antagonistic effects, predicting the effects of multiple stressors from single stressor studies is not a straight-forward process (Crain et al. 2008).

Multiple stressor studies have, almost ubiquitously, been limited to considerations of how stressors interact when applied simultaneously (reviewed by Gunderson et al. 2016). However, in real systems, stressors may act synchronously or asynchronously at a given location, with full, partial or no overlap in their timing (Gunderson et al. 2016). For example, whereas sediments, heavy metals, and polyaromatic hydrocarbons are simultaneously delivered to estuaries in stormwater following heavy rain, the timing of physical disturbances such as bait-digging and boat run-ups may be largely independent of the timing of pollutant delivery. Basing predictions of multiple stressor effects solely on studies that apply these simultaneously may, where asynchronous stressors have smaller impacts than synchronous stressors, be at best misleading and, where impacts of asynchronous stressors are greater than those predicted from simultaneous, synchronous applications (Molinos & Donohue 2010), at worst be dangerous.

Conceivably the relative timing of multiple stressors may influence their combined impact in several ways (Darling & Cote 2008, Molinos & Donohue 2010, Berga et al. 2012, Maggi et al. 2012, Sokolova et al. 2013, Oliveira et al. 2014, Cimon & Cusson 2018). First, where stressors occur asynchronously, earlier stressors may weaken the effect of later stressors by selecting for a community that is more resilient to stressor impacts. Increased resilience may be conferred by compositional changes in the community (e.g. transition to a new stable state; Thrush et al. 2012) or alternatively through behavioral and/or

physiological adaptations of organisms (Clavier et al. 2005, Wu et al. 2017). Alternatively, earlier stressors may exacerbate the effects of later stressors by reducing the fitness of and/or weakening the condition of organisms comprising the community and/or by producing compositional changes (i.e. reductions in species richness) that render the community more unstable in the face of additional change (Vinebrook et al. 2004, Darling et al. 2013, Piggott et al. 2016).

The few empirical studies that have addressed how the temporal dynamics of multi-stressor applications influence their impacts have produced variable outcomes (Benedetti-Cecchi et al. 2006ab, Cardoso et al. 2008, Molinos & Donohue 2010, Pincebourde et al. 2012, Muthukrishnan & Fong 2014, Halpern et al. 2015), perhaps reflecting variation in the interval between the application of multiple, asynchronous stressors (Gunderson et al. 2016). Gunderson et al. (2016) proposed a conceptual framework for predicting the outcome of multi-stressor interactions, whereby the interval between stressors influences the nature of their interaction. According to the framework, stressors that are applied synchronously or in rapid succession produce synergistic effects, whereas those applied asynchronously have antagonistic effects if the interval between stressors is hours or days, or additive where the interval between stressors is weeks or month. However, empirical tests of the model remain rare (but see Todgham et al. 2005, Benedetti-Cecchi et al. 2006b, Molinos & Donohue, 2010, Pincebourde et al. 2012, Todgham & Stillman 2013, Halpern et al. 2015, Bible et al. 2017), and whether the order or sequence in which stressors are applied, as well as the interval between them, influences their cumulative ecological impact remains unknown.

Here, we examine how the interval between and order of application of two common estuarine stressors – nutrient enrichment and physical disturbance of sediments - influences their cumulative impact on meiobenthic communities. Nutrients enter estuarine systems through a number of pathways, as a consequence of the application of nutrient-rich fertilizers to land, sewage discharge, and runoff of animal waste, among others. Enhanced nitrogen and/or

phosphorus loads stimulate primary productivity, and can lead to eutrophication where the microbial breakdown of excess primary producer biomass depletes oxygen concentrations in the water and/or sediments below (Nixon 1995, 2009). Physical disturbance of estuarine sediments can be large scale, such as following a storm event or trawling (Tuck et al. 1998, Posey et al. 1996), or small scale such as caused by boat wake, anchorage, propeller scarring, bait pumping and sediment bioturbation by organisms (Austen et al. 1998, Bishop 2005, Rossi & Underwood 2002, Rossi et al. 2007, Kenworthy et al. 2016). Physical disturbance can cause changes to community assembly by damaging, killing and dislodging organisms. Changes in the habitat can result in decrease their abundances and richness (Schratzberger & Jennings 2002). Meiofauna (45- 500 microns) provide a key trophic linkage between primary producers and higher trophic levels and can, through their bioturbation, enhance mineralization of organic matter and modulate sedimentary biogeochemical processes by nutrient regeneration (Bonaglia et al. 2014, Nascimento et al. 2012). Due to their short life-cycle, and limited mobility meiofauna are highly sensitive to anthropogenic stressors and are considered excellent environmental indicators (Coull & Chandler 1992, Schratzberger & Warwick 1999, Danovaro et al. 2000, 2004, Fleeger et al. 2015, Balsamo et al. 2012, Moens et al. 2014, Moens & Beninger 2018).

A number of studies have investigated, through field or mesocosm studies, how nutrient enrichment and physical disturbance interact to influence the community structure of macro- or meiobenthos when simultaneously applied (Chapter 3, Widdicombe & Austen 2001, Austen & Widdicombe 2006, O'Brien et al. 2009, Kenworthy et al. 2016). However, how their interactive effect is influenced by the interval between their application remains unknown. The main aims of this study are to ascertain: (1) how the interval between the application of nutrient enrichment and physical disturbance influences the way in which estuarine meiobenthos respond to their combined effect; and (2) how the order in which these two stressors are applied to a system influences their interaction. According to the predictions of Gunderson et al. (2016) we expect that whereas

the simultaneous application of stressors will produce synergistic effects, asynchronous stressors spaced by several months will produce additive effects. Additionally, we expect that the effects of the second stressor will overwhelm the effects of the first.

4.2. Materials and Methods:

4.2.1. Study sites

Experiments were conducted on two unvegetated intertidal estuarine mudflats within the greater Sydney metropolitan region, New South Wales, Australia, between May and September 2012. The first was within Tambourine Bay, Lane Cove River (33° 49' 39" S, 151° 09' 38" E) while the second was within Woollooware Bay, Botany Bay (34° 01' 11" S, 151° 07' 46" E). Both mudflats (hereafter 'sites') were situated adjacent to mangroves, around 20 km from the estuary mouth, in highly urbanized estuaries. Each was away from point-source nutrient inputs such as stormwater drains and had minimal activities causing sediment disturbance. The sediment at Lane Cove (mean $\pm$ grain size: 240.72 SE $\pm$ 11.55 μ m) was finer than Botany Bay (316 $\pm$ 35.89 μ m), but the two sites had a similar sediment organic content (Lane Cove: 1.86 $\pm$ 0.17%; Botany Bay: 1.57 $\pm$ 0.26%) and Chlorophyll *a* concentration of surface sediments (Lane Cove: 47.12 $\pm$ 6.30 mg.m⁻², Botany Bay: 51.96 $\pm$ 4.49 mg.m⁻²).

4.2.2. Experimental design

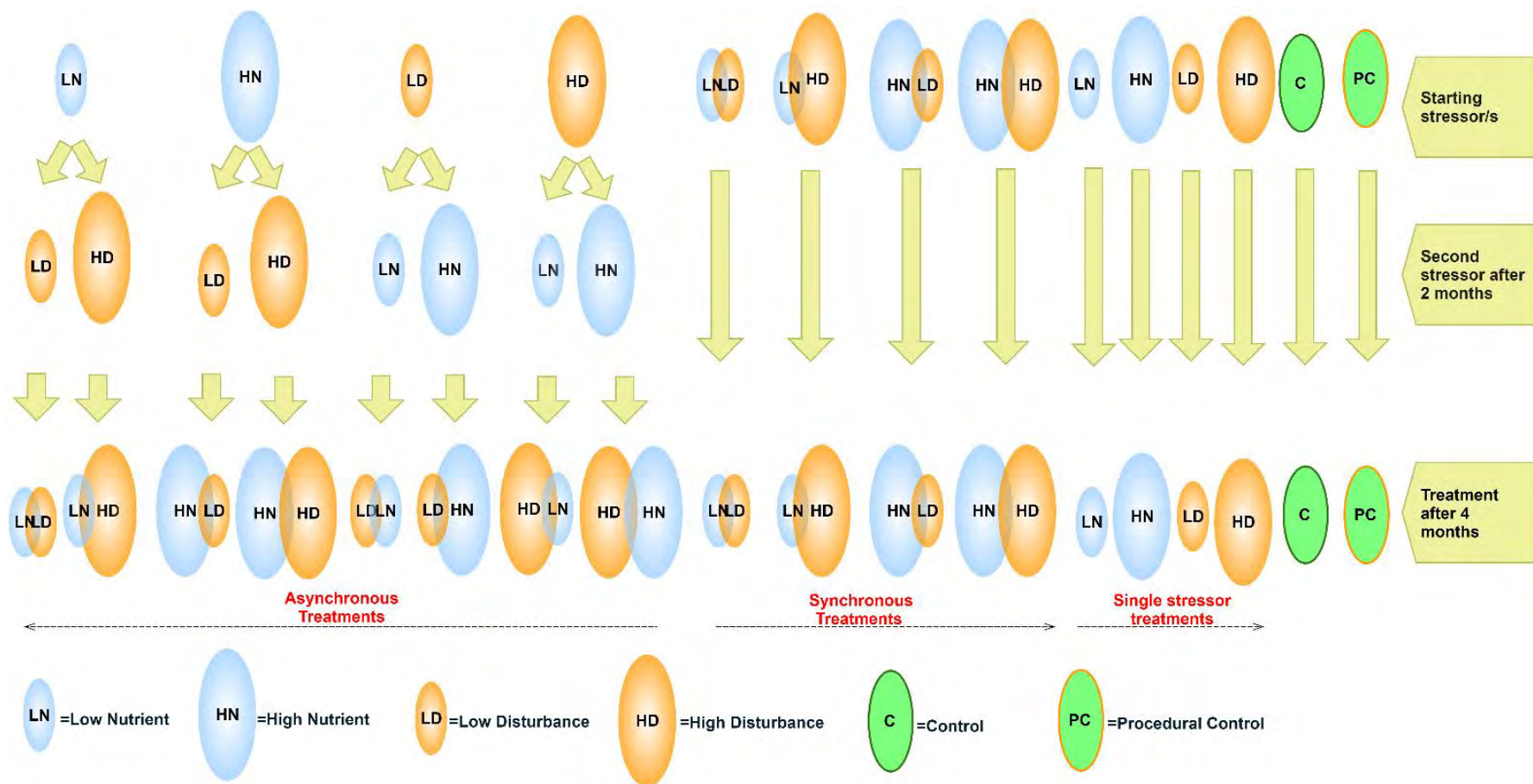


Figure 1. Summary of the experimental treatments in which multiple stressors were applied synchronously, simultaneously, or singularly, as controls.

At each of the two study sites, one hundred and twenty six 0.5 x 0.5 m experimental plots were established, each 2-3 m apart. Twenty eight of the plots (with $n = 7$ per treatment) were randomly assigned to receive one of four simultaneous multi-stressor applications, giving all possible combinations of low and high physical disturbance, and nutrient enrichment in a fully orthogonal design (Fig. 1): (1) low physical disturbance, low nutrients (LDLN); (2) low physical disturbance, high nutrients (LDHN); (3) high physical disturbance, low nutrients (HDLN); and (4) high physical disturbance, high nutrients (HDHN). Fifty six plots were randomly assigned to receive one of eight asynchronously applied multi-stressor treatments (Fig. 1, again, each with $n=7$): (1) low physical disturbance, followed by low nutrients (LD-LN); (2) low nutrients, followed by low physical disturbance (LN-LD); (3) low physical disturbance followed by high nutrients (LD-HN); (4) high nutrients, followed by low physical disturbance (HN-LD); (5) high physical disturbance, followed by low nutrients (HD-LN); (6) low nutrients, followed by high physical disturbance (LN-HD); (7) high physical disturbance, followed by high nutrients (HD-HN); and (8) high nutrients, followed by high physical disturbance (HN-HD). Hence, by the end of the experiment, the plots receiving asynchronously applied stressors had received the same stressors as one of the treatments with simultaneous application (1-4), allowing effects of stressor timing and order to be disentangled. Finally, to allow multiple stressor effects to be compared to single stressor effects, 28 of the plots ($n=7$ per treatment) received a single stressor (Fig. 1, low nutrients, high nutrients, low physical disturbance or high physical disturbance), at the first time-point only. The remaining 14 plots were assigned to an undisturbed control ($n=7$) or procedural control ($n = 7$) treatment (Fig. 1).

Nutrient enrichment was manipulated through the addition of Scotts Osmocote Pro, 8–9 mo coated fertiliser pellets (N:P:K ratio of 16:4.8:8.3), slow release water-soluble fertilizer. Plots designated to the high nutrient treatment received 1000 g of fertilizer, which produced nutrient concentrations in adjacent sediments that were commensurate with those at sewage discharge points (Worm et al.

2000, O'Brien et al. 2010). The low nutrient treatment was set at half this loading (i.e. 500 g of fertilizer). Fertiliser was added to plots enclosed within five semipermeable nylon pantyhose bags, across which the total amount of fertilizer was evenly divided. Fertiliser balls were even spaced across the plots and buried to 2-4 cm depth. A procedural control treatment was established, where defaunated sediment was buried in nylon balls in place of fertiliser.

Sediment was physical disturbed through raking with a 50 cm wide rake, depressed to 4 cm depth, in a cross-hatched pattern (Cowie et al. 2000, Whomersley et al. 2010). Plots assigned to the high physical disturbance treatment received 6 strokes, while those assigned to the low physical disturbance treatment received 2 strokes monthly from the date of initial stressor application.

4.2.3. Sampling and laboratory processing

Meiofauna were sampled at low tide, immediately prior to the initial application of stressors and 4 months after the start of the experiment, by hand coring with a tube of 2.5 cm inner diameter, depressed to a depth of 3 cm. Upon collection, samples were fixed with 7 % buffered formalin and stained with Rose Bengal. Following at least 48hrs of fixing, formalin was washed from samples, and each was sieved over stacked 500 μm and 45 μm sieves. To extract meiofauna from the sediment (Somerfield & Warwick 1996), material retained on the 45 μm sieve was transferred to a centrifuge tube, made up to a volume of 50 ml by adding Ludox-HS40 (specific gravity of about 1.15), vortexed for 5 seconds then centrifuged for 5 mins at 4400 rpm. The samples were rested and the supernatant was passed through the 45 μm sieve. The process of Ludox addition, vortexing and centrifugation was repeated. The extracted fauna, retained on the 45 μm sieve, were thoroughly washed with fresh water to remove remaining Ludox and stored in 70% ethanol. Meiofauna were enumerated to a mixed taxonomic resolution under a stereo microscope. Crustacea were identified to order, Annelida to subclass and other taxa to phyla (*sensu* Warwick & Gee 1984)

4.2.4. Statistical analyses

Effects of the magnitude, order and synchrony of stressors on meiofaunal communities were assessed using permutational analyses of variance (PERMANOVA: Anderson et al., 2008). Multivariate analyses, using Bray-Curtis dissimilarities, were run on meiofaunal community data while univariate analyses, using Euclidean distances matrices, were run on the total abundance, taxon richness, and Shannon diversity of meiofauna in each sample, as well as the abundance of meiofauna taxa that were found to be key contributors to multivariate differences among treatments. Prior to analysis, community data were square root transformed to down-weight the influence of dominant taxa, and were visualized using non-metric multidimensional scaling (nMDS).

First, to test for experimental artefacts of nutrient application, two-way analyses, with the factors treatment (2 levels: undisturbed, disturbed control) and sites (random 2 levels: Lane Cove and Botany Bay) were run on samples collected 4 months after experimental initiation. A second set of PERMANOVAs, with two factors, (1) treatment and (2) site assessed how within particular stressor combinations (i.e. LNLD), impacts among plots receiving a single stressor (LN, or LD), the two stressors simultaneously (LNLD) or the two stressors sequentially (LN-LD or LD-LN) varied as compared to undisturbed treatments. There were four sets of this analysis run: one for each of the four stressor combinations (LNLD, LNHD, HNLD, HNHD). Third, to assess how the timing and order of stressor application influences their impact, and how this interacts with stressor magnitude, four-way PERMANOVAs, with the following factors, were run: (1) timing of stressor application (fixed; 3 levels: simultaneous, S; nutrients first, N; or physical disturbance first, D); (2) the magnitude of the nutrient stressor (fixed; 2 levels: low, LN; high, HN); (3) the magnitude of the physical disturbance stressor (fixed; 2 levels: low, LD; high, HD) and (4) site (random; 2 levels: Botany Bay, B; Lane Cove, L). Separate analyses were run for time 0 (Lane Cove only due to sample loss), to assess any pre-existing variation among plots, unrelated to treatments, that would influence the interpretation of results, for month 4.

Pairwise post hoc tests examined sources of significant treatment effects (at $\alpha = 0.05$). These used Monte Carlo (MC) simulations. SIMPER analyses were used to identify taxa that were key discriminating taxa (defined having a dissimilarity/standard deviation ratio >1 between treatments), contributing to multivariate differences in meiofauna community structure among treatments.

4.3. Results

The analyses of controls and procedural controls revealed no experimental artefacts associated with the method of nutrient addition, at any sampling time (Table 1). Additionally, there were no pre-existing differences in meiofaunal community structure, total abundance, Shannon diversity, or taxon richness among experimental treatments prior to experimental manipulation that needed to be considered in the interpretation results (Table 2).

Table 1. Two factor PERMANOVAs testing for experimental artefacts associated with the method of nutrient addition-(Treatment, Tr; two levels - Control and Procedural Control) at each of the two study sites (Si), Lane Cove and Botany Bay, at Month 4. Bold indicates significant results at $\alpha = 0.05$.

Multivariate						Univariate			
Factors	df			Abundance		Taxon richness		Shannon diversity	
		Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tr	1	0.137	0.664	0.203	0.649	2.910	0.495	0.651	0.651
Si	1	13.748	0.001	3.361	0.079	10.558	0.003	14.259	0.002
TrxSi	1	2.241	0.080	1.525	0.240	0.087	0.769	2.394	0.132
Res	23								

Table 2. Three-way PERMONOVAs testing for any pre-existing variation in meiofaunal communities, with respect to assigned treatments, prior to stressor application (i.e. at time 0) at Lane Cove. Factors were: timing of stressor application (Or; simultaneous, nutrient first, disturbance first), the level of nutrient enrichment (Nu; low, high) and the level of physical disturbance (Di; low, high). N = 7.

Multivariate						Univariate			
Factors	Df			Abundance		Taxon richness		Shannon diversity	
		Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)
Nu	1	2.185	0.069	0.993	0.324	0.305	0.588	3.712	0.057
Di	1	0.269	0.923	0.211	0.647	0.041	0.848	0.194	0.662
Or	2	0.701	0.677	0.060	0.943	1.148	0.324	0.582	0.566
NuxDi	1	0.253	0.934	0.005	0.944	0.190	0.668	0.903	0.334
NuxOr	2	0.613	0.773	0.363	0.692	1.451	0.245	2.874	0.062
DixOr	2	0.916	0.483	0.333	0.728	2.529	0.089	0.294	0.747
NuxDixOr	2	1.078	0.349	1.796	0.171	0.894	0.404	0.311	0.734
Res	68								

Table 3. Multivariate PERMANOVA analyses testing for significant treatment (Tr) effects on meiofaunal communities, between control plots, not receiving stressors, plots receiving single stressors, and plots receiving multiple stressors, synchronously or asynchronously. A separate analysis was run for each combination of nutrient (N) and physical disturbance (D) treatments. L = low magnitude; H = high magnitude.

	LNLD			LNHD			HNLD			HNHD	
Factors	df	Pseudo-F	P(perm)	Df	Pseudo-F	P(perm)	df	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tr	5	2.003	0.051	5	1.252	0.284	5	2.437	0.050	1.251	0.336
Si	1	27.574	0.000	1	28.428	0.000	1	30.720	0.000	25.621	0.000
TrxSi	5	1.047	0.405	5	1.524	0.046	5	0.817	0.698	1.001	0.458
Res	61			62			65				

Whether meiofaunal communities displayed a response to stressors varied according to the magnitude of stressor application (Table 3). Following four months of experimental manipulation, plots receiving combinations of the HN and/or HD treatments did not display any treatment effects (Table 3). By contrast plots receiving combinations of the LN and HD treatments displayed site-dependent treatment effects. At Botany Bay, community structure differed between undisturbed control plots, plots receiving the LN and HD treatments asynchronously and plots receiving either a singular disturbance or the two synchronously, the latter two of which did not differ (REFER TO SUPPLEMENT MAT Table. S1). At Lane Cove, control plots did not significantly differ from disturbed plots, with the only treatment difference between plots that received low nutrients followed by high disturbance, sequentially, and plots receiving only the high physical disturbance (REFER TO SUPPLEMENT MAT Table. S1).

Plots receiving the HN and/or LD treatments displayed a main effect of treatment, and plots receiving the LN and/or LD treatments displayed a marginally significant treatment effect (Table 3). In neither instance did controls differ from any of the other treatments. In both instances, when nutrient

enrichment (Low or high) was the first stressor followed by low disturbance, the community composition differed to the plots receiving just the low disturbance on its own ($t=2.592$, $p=0.04$ and $t=3.528$, $p=0.02$).

Table 4. PERMANOVA analyses testing for significant treatment (Tr) effects on total meiofaunal abundance, between control plots, not receiving stressors, plots receiving single stressors, and plots receiving multiple stressors, synchronously or asynchronously. A separate analysis was run for each combination of nutrient (N) and physical disturbance (D) treatments. L = low magnitude; H = high magnitude.

Factors	df	LNLD				LNHD				HNLD		HNHD	
		Pseudo-F	P(perm)	df		Pseudo-F	P(perm)	df		Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tr	5	11.589	0.008	5		4.378	0.065	5		3.761	0.097	6.434	0.037
Si	1	1.215	0.276	1		7.714	0.006	1		3.295	0.075	7.080	0.011
TrxSi	5	0.317	0.902	5		0.857	0.524	5		0.630	0.675	0.322	0.898
Res	61			62				65					

The total abundance of meiofauna displayed a significantly main effect of treatment when plots received a combination of the LN and LD disturbance, or the HN and HD disturbances (Table 4) but treatment effects were non-significant for the analyses examining combinations of the LN and HD or HN and LD treatments. Nevertheless, for all combinations except LN and LD, there was a trend for total meiofaunal abundance to be greater in control plots and those receiving single stressors, than those receiving multiple stressors. For LN and LD combinations, meiofaunal abundance was greater for synchronous stressor addition than the other treatments. The lowest abundance was generally seen when plots received the two stressors asynchronously (i.e. LN-LD, LN-HD & HN-LD), although HN-HD did not conform with the general trend (Fig. 2).

Pair wise tests for the LN and LD combinations found that multiple stressor treatments did not significantly differ from single stressor or control treatments, however plots receiving single stressors of low nutrient and low disturbance were significantly different ($t= 35.222$, $p(\text{MC})=0.018$,) and the low nutrient plots were different to control plots ($t=12.509$, $p(\text{MC})= 0.055$). The pair wise tests for the HN

and HD combinations did not show any significant difference among plots receiving single or multiple stressors, but plots receiving HN followed by HD were marginally different from controls ($t=11.015$, $p(\text{MC})=0.056$).

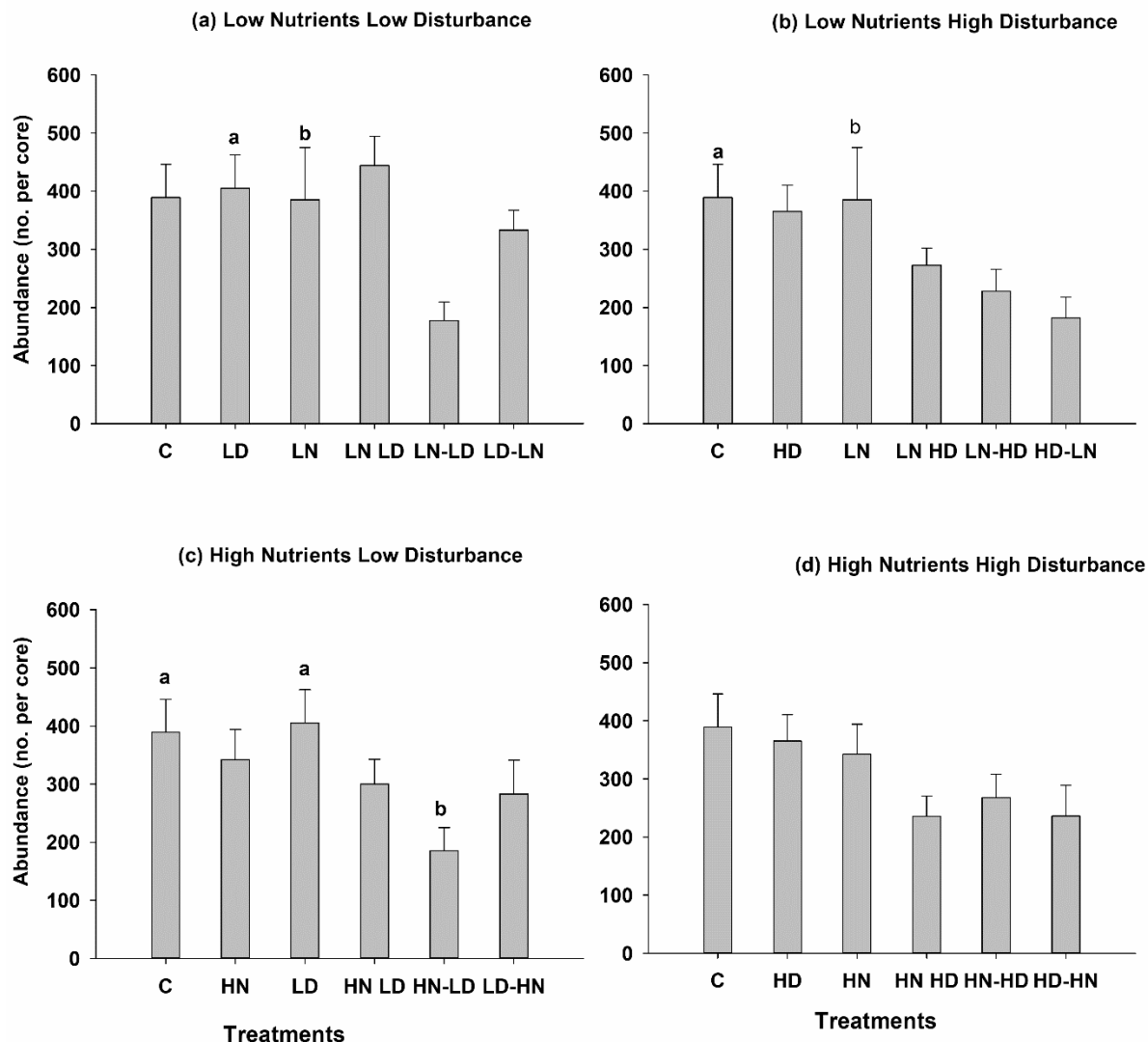


Figure 2. Mean ($\pm$ SE) abundance of meiofauna in plots that received two stressors, nutrient enrichment (N) and physical disturbance (D) individually or together in multiple stressor treatments. C = control plots, not receiving stressors. H = high magnitudes and L = low magnitudes of the disturbances. Asynchronously applied multiple stressors are denoted by hyphens, while simultaneous multiple stressor treatments are unhyphenated. $N = 7$. Letters indicate treatments that differed significantly.

Table 5. PERMANOVA analyses testing for significant treatment (Tr) effects on meiofaunal taxonomic richness between control plots, not receiving stressors, plots receiving single stressors, and plots receiving multiple stressors, synchronously or asynchronously. A separate analysis was run for each combination of nutrient (N) and physical disturbance (D) treatments. L = low magnitude; H = high magnitude.

Factors	Df	LNLD			LNHD			HNLD		HNHD	
		Pseudo-F	P(perm)	df	Pseudo-F	P(perm)	df	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tr	5	0.967	0.522	5	0.847	0.563	5	17.863	0.004	3.827	0.080
Si	1	41.148	<0.00	1	39.346	<0.00	1	38.744	<0.00	22.467	<0.00
trxSi	5	1.621	0.168	5	1.797	0.130	5	0.094	0.994	0.272	0.926
Res	61			62			65				

In all four groups, there was a trend for taxonomic richness to be highest in control plots and least in plots receiving multiple stressors asynchronously, with the exception of plots where low physical disturbance was followed by low nutrients (Fig. 3). However, these differences were only significant for the subset of treatments receiving the HN and LD treatments (Table 5). The pair wise tests difference between treatment of HN & LD showed borderline significant difference among plots receiving high nutrient synchronously followed by low disturbance and high nutrients on its own ($t=11.00$, $p(\text{MC})=0.057$).

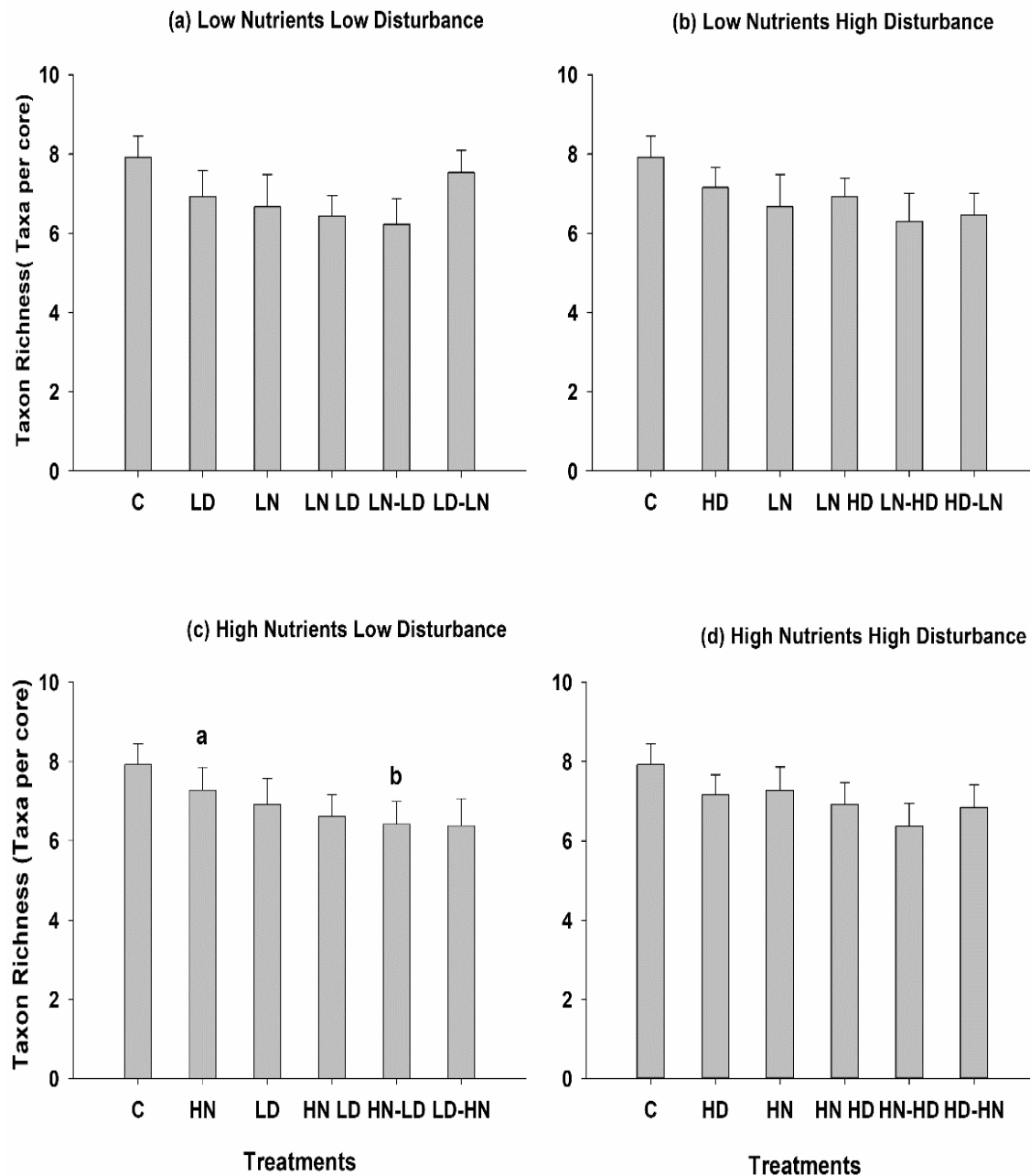


Figure 3. Mean ($\pm$ SE) taxon richness of meiofauna in plots that received two stressors, nutrient enrichment (N) and physical disturbance (D) individually or together in multiple stressor treatments. C = control plots, not receiving stressors. H = high magnitudes and L = low magnitudes of the disturbances. Asynchronously applied multiple stressors are denoted by hyphens, while simultaneous multiple stressor treatments are unhyphenated. N = 7. Letters indicate treatments that differed significantly.

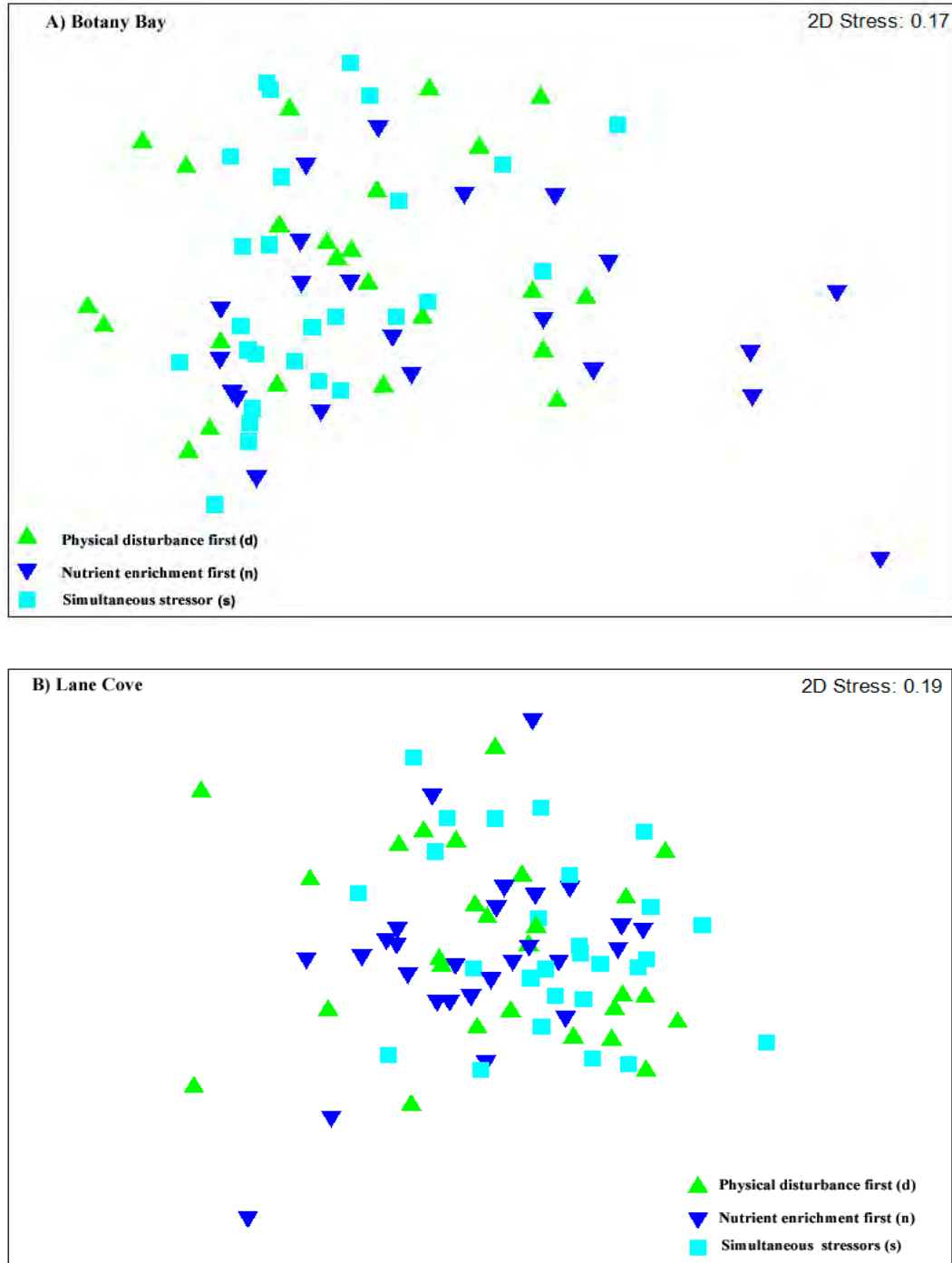


Figure 4. Non-metric multidimensional scaling showing sources of variation in meiobenthic composition 4 months after the start of stressor application at each of two sites, (a) Botany Bay, and (b) Lane Cove. Plots were subjected to nutrient and physical disturbance stressors simultaneously (s) or sequentially, with the nutrient (n) or physical disturbance (d) stressor applied first. The ordination uses Bray-Curtis dissimilarities produced from square-root transformed data. Points represent individual plots (n=5-7).

Table 6. Results of four-way PERMANOVAs testing for interacting effects of the timing of stressor application (Or; simultaneous, nutrient first, disturbance first), the level of nutrient enrichment (Nu; low, high) and the level of physical disturbance (Di; low, high) on aspects of meiofaunal community structure at each of two sites, four months after initial stressor application. N = 5-7. Bold indicates significant effects at $\alpha = 0.05$.

Multivariate				Univariate Abundance		Taxon Richness		Shannon diversity	
Factors	Df	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)	Pseudo-F	P(MC)
Nu	1	5.268	0.021	494.010	0.029	2.687	0.351	4.163	0.283
Di	1	1.825	0.668	15.022	0.163	0.156	0.757	0.061	0.849
Or	2	0.702	0.279	6.609	0.125	1.522	0.390	1.457	0.398
Si	1	88.614	<0.001	8.789	0.004	46.338	<0.001	52.743	<0.001
NuxDi	1	1.591	0.298	122.780	0.058	1.429	0.448	16.782	0.152
NuxOr	2	0.394	0.347	9.392	0.093	0.432	0.698	0.143	0.877
NuxSi	1	0.193	0.936	0.003	0.964	0.130	0.722	0.243	0.623
DixOr	2	2.602	0.201	2.584	0.283	1.973	0.344	4.882	0.163
DixSi	1	0.949	0.208	0.248	0.610	2.261	0.136	3.801	0.050
OrxSi	2	2.049	0.014	0.911	0.402	1.176	0.312	0.580	0.560
NuxDixOr	2	0.323	0.436	0.446	0.691	39.715	0.024	4.131	0.187
NuxDixSi	1	0.527	0.422	0.020	0.888	0.236	0.629	0.089	0.766
NuxOrxSi	2	0.943	0.829	0.239	0.787	1.424	0.245	1.002	0.367
DixOrxSi	2	0.624	0.159	2.262	0.112	0.162	0.853	0.268	0.766
NuxDixOrxSi	2	2.880	0.895	0.557	0.577	0.013	0.988	0.294	0.752
Res	13								
	0								

At the conclusion of the 4 month experiment, meiofaunal communities displayed site-dependent responses to the timing of stressor application (Table 6; Fig. 4). At Lane Cove, the meiobenthic community differed between plots where stressors were applied simultaneously versus asynchronously, irrespective of the order in which the asynchronous stressors were applied (*a posteriori* tests, sig. Order effect, Table 6: nutrient first vs simultaneous: $t=1.866$, p (MC) =0.006; disturbance first vs simultaneous: $t=1.563$, $p=0.0369$; nutrient first vs disturbance first: $t=0.980$, p (MC) =0.440). A similar effect was observed at Botany Bay, but community composition only significantly differed between the simultaneous stressor application and the asynchronous treatment in which nutrients were applied first ($t= 2.127$, $p=0.002$), with the other pairwise

comparisons non-significant (disturbance first vs simultaneous: $t=1.4605$, $p(\text{MC})=0.0639$; disturbance first vs nutrient first: $t=1.307$, $p(\text{MC})=0.131$).

Whereas multivariate community structure varied according to the timing of stressor application, total meiobenthic abundance and Shannon diversity did not (Table 6). Instead, total abundance displayed a main effect of nutrient enrichment, whereby abundances were greater at low than high levels of enrichment (Table 6). Shannon diversity displayed a significant disturbance by site interaction (Table 6), although the level of physical disturbance did not significantly affect diversity at either Botany Bay ($t=1.323$, $p(\text{MC})=0.178$) or Lane Cove ($t=1.465$, $p(\text{MC})=0.154$). By contrast, taxon richness displayed a significant 3-way interaction between timing, nutrient level and disturbance level (Table 6). There was no difference between synchronous and asynchronous treatments for any of the combinations of nutrient enrichment and physical disturbance (a posteriori tests, $p > 0.05$). However, when plots received a low level of nutrient enrichment simultaneously with a disturbance treatment, there were fewer meiofaunal taxa present in plots receiving low than high physical disturbance ($t=47$, $p=0.012$; Fig. 5).

SIMPER analysis revealed 8 key discriminating taxa, contributing most to multivariate differences among treatments. These were: Nematoda, Copepoda, Kinorhyncha, Turbellaria, Polychaeta, Mites (Halacoridae), Ostracoda and Amphipoda. All taxa, with the exception of Nematoda, varied significantly in abundance ($p<0.001$) between sites, with Amphipoda less abundant at Botany Bay than Lane Cove, and Polychaeta, Mites, and Kinorhyncha each displaying the reverse pattern (Table 7).

The response of these key discriminating taxa to the timing of stressor

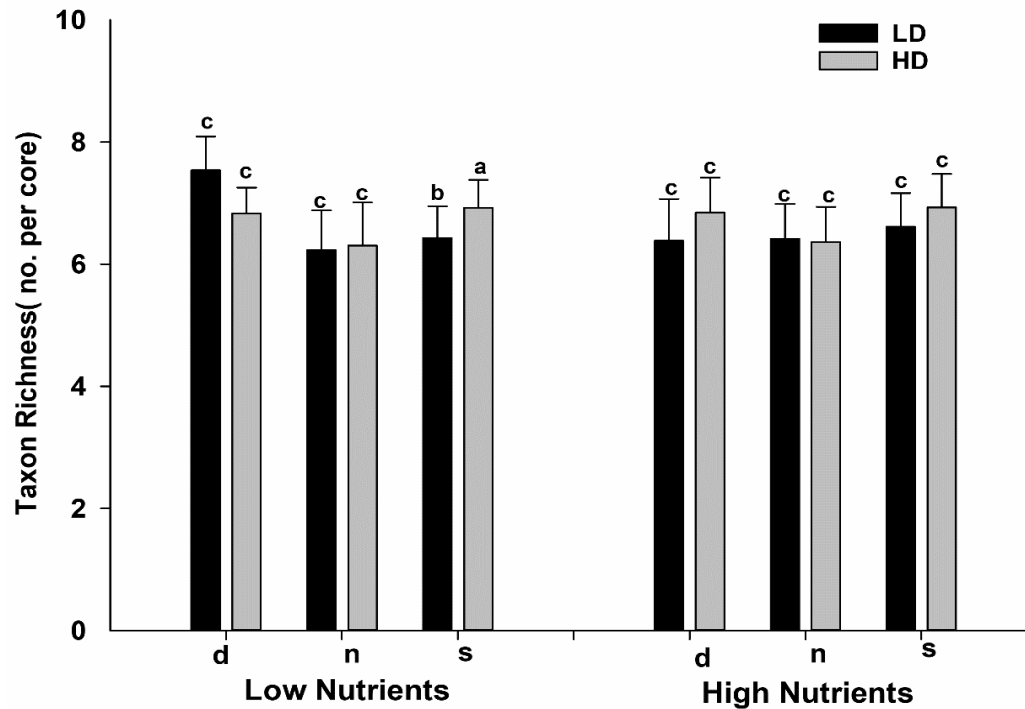


Figure 5. Mean ($\pm$ SE) taxon richness of meiofauna in plots that received two stressors, nutrient enrichment (low and high) and physical disturbance (LD = low disturbance, HD = high disturbance, simultaneously (s) or asynchronously (d = disturbance first, n = nutrients first)). $N=5-7$. Letters indicate treatments that differed significantly.

application varied (Table 7), with three of the eight taxa (Copepoda, Turbellaria and Nematoda) displaying either main or site-dependent effects of the timing of stressor application. Copepoda and Turbellaria each displayed effects of timing that varied between the sites, but the nature of the site differences varied between these (Table 7, Fig. 6). For Copepoda, there was no significant effect of the timing of stressor application at Botany Bay, but at Lane Cove, their abundance was greater in plots receiving the two stressors simultaneously than in those receiving the stressors asynchronously, irrespective of whether physical disturbance ($t=2.551$, $p=0.015$) or nutrient enrichment ($t=2.240$, $p=0.03$) was applied first (Fig. 6A).

Table 7. Results of four factor PERMANOVAs examining the interacting effects of nutrients (Nu; fixed, 2 levels- low and high), disturbance (Di; fixed, 2 levels-low and high), order (Or; fixed, 3 levels - disturbance first, nutrients first and simultaneous stressors) and site (Si; random) on the key discriminating species common to both sites within Botany Bay and Lane Cove estuaries.

Factors	Df	Nematoda Pseudo- F	P(MC)	Copepoda Pseudo- F	P(MC)	Kinorhyncha Pseudo- F	P(MC)	Turbellaria Pseudo- F	P(MC)	Polychaeta Pseudo- F	P(MC)	Halacaroida Pseudo- F	P(MC)	Ostracoda Pseudo- F	P(MC)	Amphipoda Pseudo- F	P(MC)
Nu	1	29.616	0.113	6.420	0.237	0.690	0.902	1.307	0.464	40.748	0.100	125.590	0.061	0.690	0.564	1.000	0.517
Di	1	1.047	0.485	3.769	0.303	0.013	0.787	0.232	0.708	0.054	0.852	18.423	0.145	0.013	0.920	1.000	0.520
Or	2	55.797	0.018	0.688	0.583	4.430	0.879	0.283	0.786	4.587	0.176	0.670	0.597	4.430	0.183	1.000	0.473
Si	1	0.578	0.447	85.380	<0.001	94.862	<0.001	7.035	0.009	17.526	<0.001	79.162	<0.001	94.862	<0.001	44.505	0.001
NuxDi	1	534.640	0.027	30.528	0.108	216.280	0.348	0.672	0.564	1.750	0.408	1.075	0.492	216.280	0.042	1.000	0.524
NuxOr	2	2.592	0.280	2.625	0.278	0.906	0.366	1.094	0.474	0.003	0.995	0.671	0.594	0.906	0.521	1.000	0.515
NuxSi	1	0.114	0.734	0.407	0.526	0.015	0.589	0.226	0.638	0.024	0.875	0.021	0.886	0.015	0.908	0.004	0.955
DixOr	2	2.577	0.284	2.682	0.272	0.017	0.391	0.448	0.702	0.277	0.782	4.806	0.172	0.017	0.984	1.000	0.515
DixSi	1	0.722	0.393	0.529	0.470	6.487	0.149	0.384	0.542	2.989	0.084	0.023	0.875	6.487	0.012	0.745	0.366
OrxSi	2	0.092	0.909	3.159	0.047	0.254	0.569	8.278	0.001	0.232	0.794	2.042	0.129	0.254	0.779	0.121	0.891
NuxDixOr	2	0.949	0.510	0.175	0.849	1.503	0.411	1.197	0.458	2.614	0.279	0.764	0.570	1.503	0.405	1.000	0.512
NuxDixSi	1	0.002	0.964	0.076	0.782	0.004	0.502	0.295	0.582	2.038	0.154	2.101	0.149	0.004	0.951	2.086	0.138
NuxOrxSi	2	0.493	0.613	0.280	0.760	0.902	0.872	0.227	0.796	2.339	0.104	2.814	0.064	0.902	0.408	0.443	0.643
DixOrxSi	2	1.843	0.171	1.071	0.345	1.701	0.581	2.303	0.106	0.936	0.395	0.025	0.978	1.701	0.184	0.904	0.397
NuxDixOrxSi	2	0.580	0.563	0.484	0.613	0.971	0.848	0.337	0.709	0.070	0.932	0.811	0.440	0.971	0.385	0.352	0.693
Res	130																

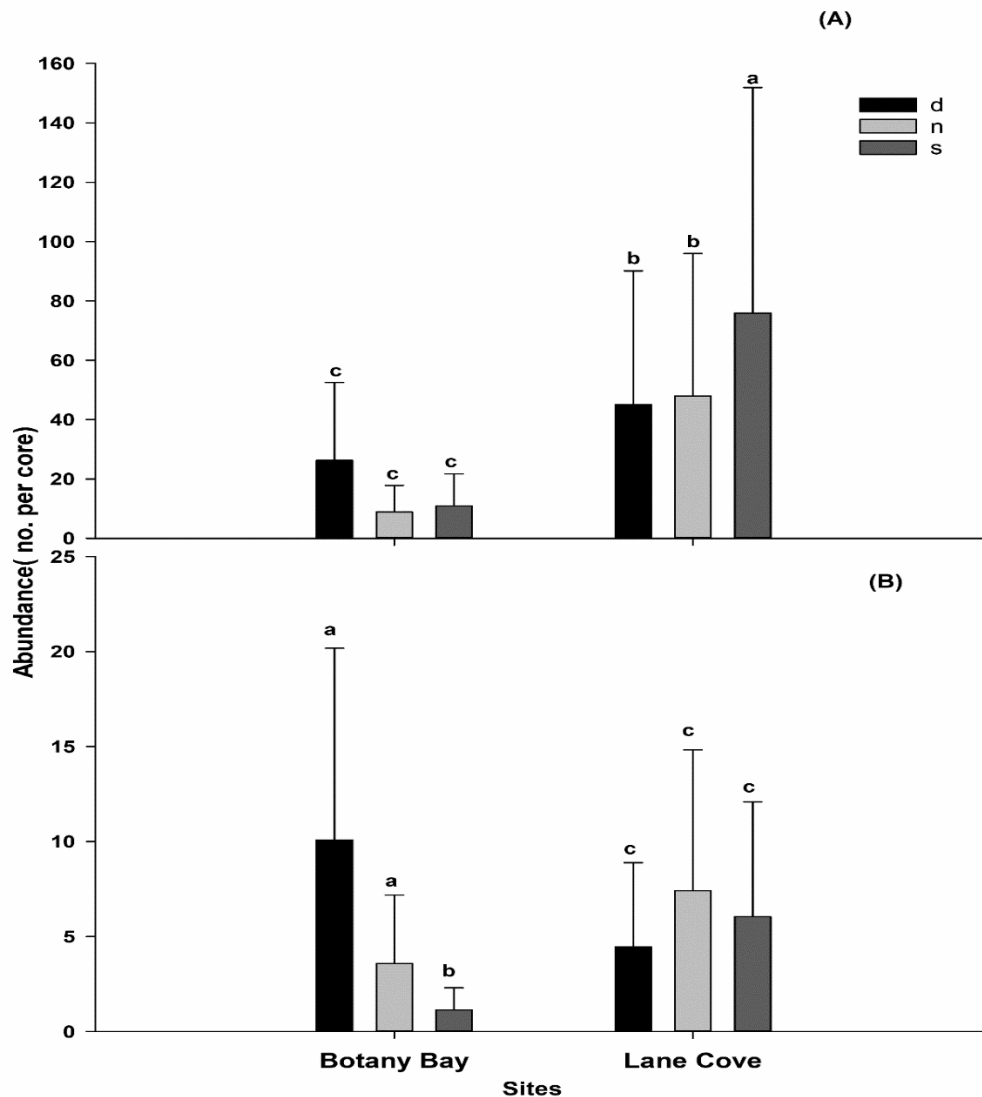


Figure 6. Mean ($\pm$ SE) abundance of (A) Copepoda and (B) Turbellaria in plots that received two stressors, nutrient enrichment and physical disturbance simultaneously (s) or asynchronously (d = disturbance first, n = nutrients first). N = 5-7. Letters indicate treatments that differed significantly.

By contrast, Turbellaria displayed an effect of the timing of stressor application only at Botany Bay (Table 7, Fig. 6B) whereby abundances were greater in plots receiving stressors sequentially than simultaneously (disturbance first vs simultaneous: $t=3.58$, $p=0.002$; nutrient first vs simultaneous: $t=2.716$, $p=0.009$) Fig. 6B), with the two sequential treatments not significantly differing ($t=1.459$, $p=0.155$).

Nematoda was the only taxon that displayed a significant main effect of the timing of stressor application (Table 7, Fig. 6). Their abundance was significantly greater in plots receiving stressors simultaneously than sequentially (*a posteriori* tests: nutrient first vs simultaneous: $t = 3.444$ $p = 0.0182$; disturbance first vs simultaneous: $t = 5.023$ $p = 0.0129$), and between the two sequential treatments, was significantly less in plots where the nutrient stressor was applied first ($t = 60.443$, $p(\text{MC}) = 0.011$), than where the physical disturbance stressor was applied first.

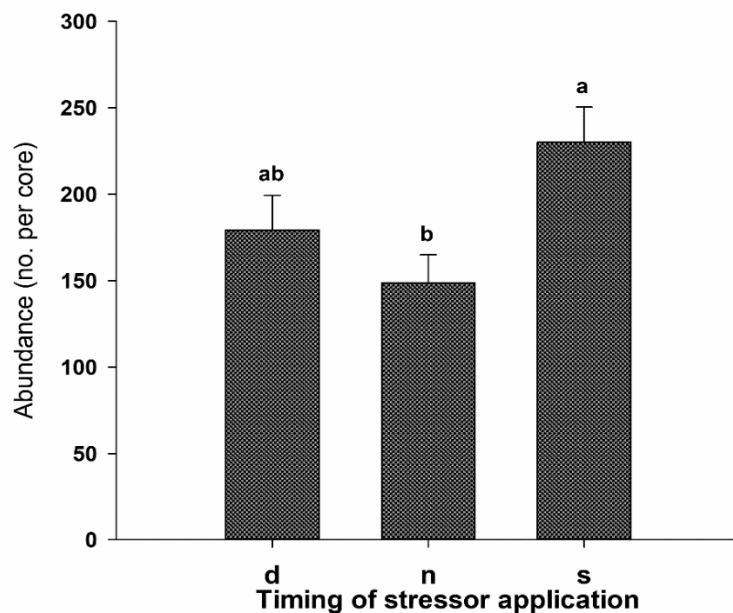


Figure 7.. Mean ($\pm$ SE) abundance of nematodes in plots that received two stressors, nutrient enrichment and physical disturbance simultaneously (s) or asynchronously (d = disturbance first, n = nutrients first). $N = 5-7$. Letters indicate treatments that differed significantly.

4.4. Discussion

Providing a generalized conceptual framework for predicting the effects of multiple stressors on ecosystems is crucial for their effective management (Halpern et al. 2007, 2008, Van den Brink et al. 2019, O'Brien et al. 2019). Various attempts have been made to understand multiple stressor effects

(Crain et al. 2008, Cote et al. 2016, Stock et al. 2018), with the vast majority of studies exposing systems to multiple stressors simultaneously (Darling & Côté 2008). This study advances predictive frameworks for understanding multiple stressor effects on ecosystems by experimentally addressing the questions of how the relative timing (i.e. synchronous or asynchronous) and order of stressor application influences ecological community response. Specifically, we examined how the timing and order of nutrient enrichment and physical disturbance stressors influenced meiobenthic community responses in intertidal estuarine mudflat habitats.

We found that whether the two stressors, nutrient enrichment and physical disturbance, were applied synchronously or asynchronously to our two study sites influenced meiobenthic community response. Although total abundance and Shannon diversity were insensitive to the timing of stressor application, taxonomic richness was more negatively impacted by simultaneous than sequential stressor application. This result is consistent with Gunderson et al.'s 2016 model, which predicts that multiple stressors that are applied synchronously or in rapid succession will produce synergistic effects whereas asynchronously applied stressors, applied hours or days apart will produce antagonistic effects or applied days or months apart will produce additive effects. Given that the majority of multiple stressor studies have exposed systems to stressors simultaneously, they are likely to be biased towards synergistic effects. Hence, the conclusion of meta-analyses that multiple stressor effects are generally synergistic (e.g. Crain et al. 2008) may reflect the design of the studies reviewed, and be limited to simultaneous stressor application. As the number of studies examining interactive effects of asynchronous stressors grows, a greater range of responses may be seen, altering this conclusion.

Individual taxa varied in their response to the timing and order of stressor application, with responses often site-dependent. This is expected given that taxa vary markedly in their tolerance to particular stressors (Warwick 1988), and background characteristics of the environment can influence impacts by determining the traits of species comprising ecological communities, their

background stress levels, as well as ecological interactions that may dampen or accentuate impacts (Vinebrook et al. 2004, Crain et al. 2008, Sundback et al. 2007). For example, whereas Copepoda displayed greater abundances in treatments receiving stressors simultaneously than asynchronously at the Lane Cove site only, Turbellaria displayed the reverse pattern of smaller abundances in the simultaneous stressor treatments, and at Botany Bay only. Copepoda are generally most abundant in well oxygenated sediments (Giere 2009, Riera et al. 2011) such that at Lane Cove, the muddier of the two sites, a possible mechanism for the enhancement of their abundance was the combined effect of mechanical churning oxygenating sediments and the nutrient enrichment enhancing food supply. Previous studies have shown that Turbellaria are abundant and diverse in coarse sediments and are very sensitive to anoxic conditions in sediments (Reise 1983). Although we did not measure sediment redox potential in the present study, we hypothesise that the addition of nutrients either first or in combination with physical disturbance facilitated algal growth causing sediment anoxia, and resulting in a decline in Turbellaria numbers. When physical disturbance was first and nutrient enrichment second, however, there was insufficient time for algal mats to establish. Physical removal of algal mats on a large scale in a field experiment by Reise (1983) saw a 5-fold increase in Turbellaria in barren sediments.

Overall, asynchronously applied stressors had more negative impacts on Nematoda than simultaneously applied stressors. Although, among asynchronously applied stressor treatments, the order in which stressors were applied had relatively little influence on overarching community structure, Nematoda were more negatively affected by the stressors when nutrient enrichment was applied first than second. Previous research has found that whereas high nutrient enrichment negatively influences Nematoda abundance (Ferreira et al., 2015), physical disturbance can enhance Nematoda abundance where it eliminates larger macrofauna, which may serve as predators and/or competitors (Pranovi et al., 2000; Ingels et al., 2011, 2014; Zeppili et al., 2015). The more negative effect of adding nutrients

first than second may be because this stressor had longer to act in the former (4 mo) than the latter (2 mo) instance. Alternatively, it could be because when applied first, the physical disturbance treatment was able to enhance meiofaunal abundance, offsetting the negative nutrient effect. Another explanation for the effect of the order of stressor application on Nematoda may be predation. In plots receiving physical disturbance first, the physical disturbance could have eliminated larger macrofauna or predators allowing prey species to increase in abundance (Bell 1980). Predation generally is important in determining meiofaunal community structure in muddy sediments (Fleeger & Chandler 1983, Chandler & Fleeger 1983, 1984).

4.5. Conclusion

The results of this study support previous calls (Gunderson et al. 2016) to consider not only the identity and magnitude of stressors, but also their temporal dynamics in multiple stressor models. Our manipulative field experiments demonstrate that whether an ecosystem is exposed to multiple stressors simultaneously or asynchronously can influence both the magnitude and direction of impacts. As the way in which stressors interacted was highly dependent on site and the identity of taxa, prediction of stressor impacts also requires knowledge of the environmental tolerances of key species, and their background levels of stress. As the number of stressors impacting the world's ecosystems continues to grow more field-based experiments, conducted under ecologically realistic conditions, are needed to identify how the identity, magnitude, spacing and temporal dynamics of multiple stressor combinations influences their cumulative impact. A predictive understanding of how multiple stressors cumulatively impact ecosystems is essential for effective management that minimizes stressor impact, by controlling their timing, magnitude and/or occurrence.

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This is the supplementary material for

Simultaneous or sequential: the timing and order of multiple stressor applications influences impacts to estuarine meiobenthic communities

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Table S 1. Results of *a posteriori* PERMANOVAs testing for differences between pairs of treatments at each of two study sites. The two stressors, nutrient enrichment (N) and physical disturbance (D) were applied individually or together in multiple stressor treatments. C = control plots, not receiving stressors. H = high magnitudes and L = low magnitudes of the disturbances. Asynchronously applied multiple stressors are denoted by hyphens, while simultaneous multiple stressor treatments are unhyphenated. N =7.

Treatments	Botany Bay		Lane Cove	
	T	P(perm)	T	P(perm)
C, HD-LN	2.030	0.003	1.261	0.168
C, LN-HD	1.106	0.291	1.526	0.078
C, LNHD	1.181	0.231	1.079	0.324
C, HD	0.714	0.736	1.028	0.382
C, LN	1.250	0.236	0.957	0.496
HD-LN, LN-HD	1.321	0.124	0.986	0.404
HD-LN, LNHD	1.619	0.008	1.213	0.178
HD-LN, HD	1.906	0.001	1.385	0.093
HD-LN, LN	1.700	0.009	1.148	0.265
LN-HD, LNHD	1.223	0.193	1.457	0.083
LN-HD, HD	0.781	0.680	1.870	0.037
LN-HD, LN	0.774	0.643	1.068	0.323
LNHD, HD	0.999	0.448	0.949	0.573
LNHD, LN	1.351	0.158	0.707	0.862
HD, LN	1.118	0.287	1.143	0.287

Chapter 5

General Discussion:

This thesis examined how multiple stressors applied independently and together impact communities of poorly studied meiobenthic invertebrates within estuaries of south-eastern Australia. With the number of stressors impacting our ecosystems on the rise, and these stressors rarely occurring on their own, there is need to understand how multiple stressors interact to determine cumulative impacts so that (1) we may predict how new stressors impact ecosystems and (2) we can develop management strategies that are effective in minimising cumulative impacts.

5.1 Single stressor impacts

My study focused on two stressors that are particularly common in estuarine systems: nutrient enrichment and physical disturbance. Previous studies investigating impacts of these stressors on their own have found each to produce significant impacts on ecosystem structure and function (Rossi & Underwood 2002, Morris & Keough 2003, Russell & Connell 2007, Whomersley et al. 2010, Sunback et al. 2010).

Here, mensurative sampling across a gradient in nutrient enrichment (Chapter 2) revealed a relatively weak relationship between nutrient loading and meiofaunal communities at the scale of sites. Although the absence of a strong negative relationship between nutrient enrichment and meiofauna contrasts the results of North American and European studies where enrichment has led to eutrophication and community collapse (Coull & Chandler 1992, Lampadarariou et al. 1997, Schratzberger et al. 2000, Rossi & Underwood 2002, Dalto et al. 2006, Santos et al. 2009, Whomersley et al. 2009, Strain et al. 2014), this result adds to a growing number of studies that suggest that many east Australian estuaries do not exhibit this same response (Bishop et al. 2006, Scanes et al. 2007, York et al. 2012, Kelaher et al. 2013, Nicastro & Bishop 2013, Kenworthy et al. 2016, Nicastro et al. 2017). The divergent response of east Australian estuaries has been explained by the oligotrophic nature of this coastline, which lacks strong upwelling, and has

catchments with weathered, nutrient-poor soils, as well as the relatively recent history of human settlement and modification of this coastline, with nutrient input have not yet reaching the levels of highly modified US and European estuaries (Davidson et al. 1991, Roy et al. 2001, Roughan & Middleton, 2002, Suther et al. 2011). Hence, the present nutrient status of east Australian estuaries may consequently be further from tipping points into eutrophic states. Although the current levels of nutrient loading did not display a significant relationship with meiofauna, this could change, as it is expected that coastal urbanisation will put growing pressure on the east Australian coast, including enhanced sewage inputs (Costanzo et al. 2001). Other Australian estuaries, including Darwin Harbour (Brown et al. 2010) are already exhibiting symptoms of eutrophication, and taking into consideration that climate change can act synergistically with nutrient enrichment to exacerbate impacts (Harley et al. 2006, Darling & Cote 2008), other estuaries over time may also follow suit.

Additionally, the way in which nutrient loading was quantified by this study may also have influenced results. Nutrient loading was calculated at a whole-estuary scale (Roper et al. 2011), which may or may not represent conditions at individual sites. I deliberately chose to sample at sites away from point-source inputs to try and avoid this issue, but nevertheless circulation patterns may lead to nutrient levels at individual sites that deviate from estuary-scale averages. In this study, I did not measure sediment or water nutrient concentrations at my study sites because previous studies have shown that the rapid biological uptake of nutrients leads to weak relationships between loading and environmental nutrient concentrations (Scanes et al. 2007).

In contrast, stronger effects of nutrient enrichment were seen in the patch-scale experiments, which manipulated nutrient levels using fertiliser addition (Chapter 3). The stronger impacts at the patch-scale likely reflect locally higher nutrient concentrations. Nutrients were added to heavily urbanised sites that already had a background of nutrient loading.

On the other hand, impacts of physical disturbance at the patch scale were relatively weak (Chapter 3). This contrasts previous studies that have found

benthos to respond to physical disturbance by reduction in their numbers, both in the instance of large scale disturbances such as bottom trawling (Kaiser & Spencer 1996) and small scale disturbances such as mechanical shell fish harvesting (Ferns et al. 2000), trampling (Dye 2006) or boat wakes (Bishop 2005). For our study, we used raking as the method of physical disturbance, as in some parts of the world this is used as a method of shellfish collection (Kaiser et al. 2001, Mistri et al. 2004) and many other benthic studies have used this as a physical disturbance treatment (Widdicombe & Austen 2001, Austen & Widdicombe 2006, Whomersley et al. 2010, Kenworthy et al. 2016), making it directly comparable to those. In contrast to macrofauna with larger body sizes, meiofauna may be less susceptible to raking as they fit between the tines.

5.2 Multiple stressor impacts

Ongoing urbanisation and human population growth in the coastal zone is increasing the number and diversity of stressors to which estuaries and coastal water bodied are exposed (Roy et al. 2001, Brich et al. 2010, O'Brien et al. 2019). Simultaneously, climate change is superimposing on these systems a range of different stressors, such as rising temperatures, sea level rise, ocean acidification and altered salinity (Halpern et al. 2008). Understanding how these multiple stressors interact to influence ecosystem structure and function is critical to management strategies aimed at maintaining the ecosystem services provided by estuaries.

Though studies on single stressor effects are informative, they present an incomplete picture of stressors impacts, as multiple stressor effects may be additive, synergistic or antagonistic (Clark et al. 1997, Peterson 2001, Lenihan et al. 2003, Crain et al. 2008, Halpern et al. 2007, 2008, Cote et al. 2016, Kenworthy et al. 2016, Piggott et al. 2015, O'Brien et al. 2019). One of the main aspects of my thesis was to disentangle how multiple stressors impact estuarine communities when occurring together. I found that the combined effects of two common estuarine stressors, nutrient enrichment and physical disturbance, were predominantly additive, but in some instances

interactive effects were seen, with impacts varying according to environmental context (Chapter 3, 4).

In natural environments, interactions between stressors are controlled by multiple factors, including the type, intensity and relative timing of each stressor (Crain et al. 2008, Vye et al. 2015, Cote et al. 2016, Gunderson et al. 2016). Most multiple stressor studies investigate the combined effect of stressors added simultaneously (Gunderson et al. 2016). My thesis explored how the timing and sequence of the addition of two stressors influences their cumulative effect (Chapter 4). My study, in demonstrating divergent effects of synchronously and asynchronously applied stressors provides empirical evidence for models (Gunderson et al. 2016) that predict how interactions between stressors vary according to the temporal spacing between these. Specifically, synergistic effects are most likely for simultaneously occurring stressors, while asynchronous stressors are more likely to have additive or antagonistic effects.

5.3 Sources of variation in stressor effects

5.3.1 Spatial scale

This study addressed stressor impacts at two scales: (1) the site scale, using mensurative sampling across an existing gradient (Chapter 2); and (2) the patch scale, using manipulative field experiments (Chapter 3, 4). It found that relationships between nutrient enrichment and meiofauna varied markedly across these two scales, ranging from weak at the large scale, to strong at the small scale.

The differing strength of relationships across the two scales may reflect differences in the sources of nutrient enrichment and or the differing resistance and recovery mechanisms that can be exhibited by communities at the various scales. In estuaries there are multiples sources of nutrient enrichment, which are both natural (e.g upwelling, litter or detritus, storm events, bioturbation of sediments) and anthropogenic (e.g., sewage outfalls, land and agricultural runoff, industrial effluents) in origin (Carpenter et al. 1998, Rothenberger et al. 2009). The bioavailability, and hence impact, of nutrients can differ between sources (Rossi & Underwood 2002, Morris &

Keough 2003, Russell & Connell 2007, Sunback et al. 2010, Whomersley et al. 2009).

At the small scale, nutrient enrichment was through highly controlled fertiliser release (Chapter 3, 4), but at the larger scale (Chapter 2), a variety of point- and non-point source inputs, including agriculture, runoff from urban landscapes, and sewage effluent likely contributed. Whereas following small, patch-scale disturbances, rapid recovery by colonisation from adjacent undisturbed patches may be possible, where the disturbance is larger in scale, recovery may be dependent on settlement and recruitment of organisms from distant sites (Vinebrooke et al. 2004).

5.3.2 Level of biological organisation and taxonomic resolution

This study found that individual taxa varied markedly in their response to stressors. Differential responses of organisms to stressors may reflect their feeding mode, position in sediments, life-history strategy and/or morphology. For example, organisms that feed on organic matter may benefit from nutrient enrichment (Tenore 1988), where others do not. Animals that occupy surface sediments may be more susceptible to mechanical disturbances (Widdicome & Austen 2001), such as raking, than those found deeper, below the directly affected zone. Among surface dwelling organisms, those with an exoskeleton might be more prone to damage from physical disturbance. An advantage of studying ecological effects at community level is that it maximizes the likelihood of detecting effects to sensitive taxa, which are apparent in overall community changes.

A large number of ecotoxicological studies continue to focus on impacts of contaminants to individual, sensitive taxa, often in highly contrived mesocosm settings (review by Przeslawski et al. 2015). On its own, this approach provides a biased picture of stressor impacts to the broader ecosystem, not only because it misrepresents the range of species responses, but also because it fails to consider that interactions among organisms that may dampen or exacerbate impacts of stressors (Lenihan et al. 2003, Vinebrook et al. 2004, Crain et al. 2008). At community level, stressor impacts

may be mitigated through replacement of highly sensitive taxa with more resistant taxa (Vinebrook et al. 2004, Darling et al. 2013). Keystone species or ecological engineers may increase in abundance to mitigate impacts (Lyons et al. 2005).

In addition to the level of biological organisation (e.g. species, communities) at which stressor impacts are considered, the taxonomic resolution of impact assessment might, conceivably, influence the outcome of impact assessments (Vinebrook et al. 2004). In this thesis, stressor impacts were generally assessed at the level of phyla, due to poor taxonomic resources for the meiofauna of the east Australian coast. While species level assessments might fail to detect impacts because they are more prone to being influenced by sources of background variation, phyla level assessments may in some instances fail to detect impacts because in lumping taxa together they miss species or genus-specific responses. Nevertheless, the coarse taxonomic level employed by this study was still able to detect stressor responses. However, it is likely that stronger effects may be seen at finer taxonomic scales (Lenihan et al. 2003, Peterson et al. 2003, Kenworthy et al. 2016).

5.3.4 Background environmental conditions

In this study, divergent effects of stressors were detected in two proximate estuaries, despite being of similar climate, and being situated in the same bioregion. Near-shore coastal zones are complex, both spatially and temporally, and small-scale environmental variability can influence stressor responses (Crain et al. 2008, Whomersley et al. 2010, Bishop & Kelaher 2013, O'Connor & Donohue 2013, Alsterberg et al. 2014, Gladstone-Gallagher et al. 2014, Lyons et al. 2015, Cote et al. 2016). The results of Chapter 2 reiterate the importance of sediment properties in shaping community structure, and hence community response to perturbations (see also Dernie et al. 2003a, Nicastro & Bishop 2013, Kenworthy et al. 2016). Fine sediments, rich in organic matter, can be more sensitive to nutrient enrichment because their background levels of organic matter are closer to tipping points, and because they are typically less well oxygenated than sandier sediments (Thrush et al. 2012). Environmental factors, such as currents which determine delivery of

larvae, and disperse contaminants, can also dictate the speed at which the system recovers (Dernie et al. 2003b).

The background profiling of prevailing stress levels within a system could assist in assessing the impact subsequent additional stressors are likely to have, and hence whether doing nothing or actively managing stressors is the best approach. In instances, where populations and communities are locally adapted to resisting stressors, or a long way from tipping points to alternative or degraded states, active stressor management may have little effect (Loreau 2000, Sanford & Kelly 2011, Bible & Sanford 2016, Peterson et al. 2019). In other instances, where systems are close to tipping points, active management of stressors may be required to avoid ecosystem collapse (Pearson & Rosenberg 1978, Whomersley et al. 2010). An historic perspective of site exposure to stressors may be needed in evaluating likely sensitivity to additional stressors.

5.4 The utility of studying meiofauna

This thesis reinforces the utility of meiofauna in assessing stressor impacts (Montagna 1995, Coul 1999, Giere, 2009, Danovaro et al. 2004, Grego et al. 2009, Zeppilli et al. 2015, 2018, Balsamo et al. 2016, Schratzberger & Ingels 2017). Their small size, relatively low mobility and high abundance means that they can be readily collected from sites of perturbation, to assess impacts of small and large scale disturbances alike (Kennedy & Jacoby 1999). Even when examined at coarse taxonomic resolution, meiofaunal communities display strong effects of environmental change (Warwick 1988, Warwick 1993, Lenihan et al. 2003, Dye 2006). Despite this, many monitoring studies continue to focus on macrofauna instead of meiofauna, due to the more poorly documented taxonomy and biology of the latter.

Meiofauna are of intrinsic value and underpin essential ecosystem functions (Coull 1999), such that is important to understand their response to natural and anthropogenic stressors. Integrated molecular barcoding methods (Schratzberger et al. 2012, Dafforn et al. 2014) may assist in enabling meiofauna to be meaningfully included in biodiversity surveys and stressor

impact assessments, even where their taxonomy is poorly described. Properly designed experiments will provide essential data for conceptually understanding ecosystem responses to multiple stressors.

5.5 Approaches to studying stressor effects

While previous studies have investigated interactive effects of nutrient and physical disturbance on meiofauna (Widdicombe & Austen 2001, Austen & Widdicombe 2006), my study was the first to examine the combined effect of these two stressors on meiofauna in the field (Chapter 3). Whereas the mesocosm experiments detected non-additive effects of the two stressors (Austen & Widdicombe 2006), in my study effects were additive. The differing outcomes of the studies may reflect differences in methods for applying physical and nutrient disturbances (Chapter 3), but also highlight differences in the utility and hence appropriate interpretation of various types of study/experiment.

Among the methods used to investigate stressor impacts are lab and mesocosm experiments, field surveys and experiments and meta-analyses. Perhaps the most common are laboratory and mesocosm studies in which the effects of individual or small groups of stressors on individual or small groups of species are isolated in carefully controlled experiments (Stachowicz et al. 2008, Sundback et al. 2010, Godbold et al. 2011, Wilson et al. 2015). While laboratory and mesocosm studies enable effects of stressors to be isolated from those of confounding factors, and significantly reduce the background spatio-temporal variability above which effects of stressors can be detected, in simplifying ecological food webs and environmental conditions they may paint an unrealistic picture. In some instances, biological interactions (facilitation, predation, competition) may exacerbate or dampen stressor impacts (Lenihan et al. 2003, Bulleri et al. 2014, Gunderson et al. 2016). Background environmental conditions may determine how close biological systems are to tipping points, and whether biological systems are pre-adapted to stressors (Thrush et al. 2012).

Field surveys and experiments have the advantage of incorporating ecological complexity and environmental variability that may mediate stressor effects,

but the inclusion of environmental heterogeneity may make it difficult to isolate stressor effects and detect them above background variability (Dahler & Strong 1996, Crain et al. 2008, Stachowicz et al. 2008, Gunderson et al. 2016). Further while the effects of stressors may be examined at small spatial and temporal scales in field experiments, it is unethical to experimentally introduce stressors at larger spatial or temporal scales. Environmental disasters, or event-driven sampling, where data are available from before and after a stressor being applied may be used to ethically assess stressor impacts over larger temporal or spatial scales (Underwood 1989, Underwood & Chapman 1996, Rossi & Underwood 2002), but afford the researcher little control and are often unreplicated.

The benefit of using meta-analyses is that large volumes of previous studies can be pulled together and summarised, to identify general effects and estimate the effects size of stressors. By looking across studies, meta-analyses can increase statistical power, and hence provide more robust statistical testing of significance. Although Meta-analyses look for generalities of patterns across studies, they may be unable to identify context-specific thresholds and tipping points beyond which stressors have undesirable impacts (Cote et al. 2013, 2016, Stephens et al. 2013, Jackson 2015).

Ideally, studies on multiple stressor impacts will combine carefully controlled mesocosm experiments with large scale field surveys and small scale field experiments to enable robust conclusions to be made.

5.6 Relevance to Management

Australia's coastal ecosystems, and especially its estuaries, have been highly impacted by European settlement and the subsequent population growth, industrialisation and urbanisation (Roy et al. 2001). As the human population continues to grow, and technological innovations continue to revolutionise our lifestyles, new and more stressors continue to be added to these systems at an increasing rate (Halpern et al. 2008). These stressors produce impacts at levels of biological organisation ranging from the molecular to the ecosystem. Understanding the consequences of multiple stressors is consequently more important in management and conservation now than ever

before (Griffen et al. 2016, insensu O'Brien et al. 2019). The results of this study have shown that whether multiple stressors display additive, synergistic or antagonistic effects may vary not only be determined by the identity and magnitude of stressors (Chapter 3), but also environmental context and whether the stressors occur synchronously or asynchronously (Chapter 4).

Whether stressors exhibit additive, antagonistic or synergistic effects will determine whether reductions in individual stressors will be effective in reducing impacts. Where stressor effects are additive, management strategies that remove or reduce one or more of the stressors will be effective, particularly where their combined effect is kept below tipping points (Thrush et al. 2012). Management of interactive multiple stressor effects is, however, more complex. The mitigation of local stressors will be most successful where multiple stressor effects are synergistic (Brown et al. 2014). Where effects are antagonistic, reducing stressors may, to the contrary, exacerbate impacts and the best approach may be to not intervene.

Within dynamic natural environments, the synchronous occurrence of stressors is, presumably, rarer than the asynchronous occurrence. Acknowledging this could change how we manage pressures. Under the scenario in which the first of multiple asynchronously applied stressors places the system into an undesirable or alternative state that cannot be reversed, management strategies should prioritise reducing or eliminating this first stressor (Cote et al. 2016, Thrush et al. 2012). If, by contrast, the first occurring stressor moves the system to a more resilient state, that is less or un-affected by subsequent stressors, the best management strategy may be to not intervene with the initial stressor and, instead, focus on management of subsequently occurring stressors. Hence, successfully predicting and therefore managing multiple stressor impacts is contingent on not only understanding individual stressor inputs, but also the relative temporal dynamics of these.

5.7 Conclusion:

The number and diversity of stressors impacting coastal environments is increasing as a result of the growing human population and climate change. The prediction of multiple stressor interactions, and ecological responses, is very complex, as multiple stressor effects may vary according to their degree of overlap in time and space, which stressor an ecosystem is exposed to first, and the intensity, frequency and scale of the stressors. The assumption by many past studies that multiple stressor act synchronously has biased our understanding of multiple stressor effects. More studies are needed that address how the relative timing of stressors, their overlap in space and time and their magnitudes, frequencies and scales influence ecosystem structure and function, across a range of environmental conditions. Firstly, experimental studies within systems are required, that assess through controlled manipulations how the timing and overlap of multiple stressors influence their combined impact. These may initially be conducted in mesocosms, but ultimately need to be conducted in the field to adequately account for the complexities of the natural world. Secondly, experiments are needed that test how multiple stressor effects vary across environmental gradients, and across ecosystems that vary in biodiversity and trophic structure. Predictions generated by these experiments, and subsequent meta-analyses should be ground-truthed with field surveys of real systems, at ecologically relevant scales. In combination, these approaches may be used to build a conceptual framework for predicting multiple stressor impacts. In this age of the Anthropocene, where ecosystems are exposed to a multitude of stressors, this knowledge is critical for the development of effective and appropriate strategies for managing multiple stressor effects.

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