

Response of benthic assemblages to multiple stressors: comparative effects of nutrient enrichment and physical disturbance

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ABSTRACT: Stressors to ecological communities often overlap in time and space and may have additive, synergistic or antagonistic effects. Nutrient enrichment and physical disturbance are 2 commonly co-occurring stressors to estuarine ecosystems, but their combined effects have mainly been investigated in mesocosm experiments of unknown relevance to field scenarios. Here, the interacting effects of these 2 stressors were examined at 2 field locations (Botany Bay and Lane Cove, New South Wales, Australia) using a fully orthogonal manipulative experiment. All possible combinations of zero, low and high intensities of nutrient enrichment and physical disturbance on macrofaunal and microphytobenthic communities were examined. Effects of stressors were generally site-specific and additive, differing in terms of magnitude of effects, although some idiosyncratic interactive effects were demonstrated for selected species. Where effects of stressors were observed, nutrient enrichment generally increased microphytobenthic biomass and altered the macrofaunal community structure while physical disturbance produced limited impacts. The divergent results of this and previous mesocosm experiments, which found primarily interactive effects of the stressors, highlights the importance of undertaking field experiments that offer a greater element of realism. Furthermore, this study, in finding differing responses to stressors at the 2 sites, highlights the importance of environmental context in mediating effects.

KEY WORDS: Multiple stressors · Context dependence · Nutrient enrichment · Disturbance · Macrobenthos · Microphytobenthos · Field experiments

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INTRODUCTION

How organisms respond to naturally occurring and anthropogenic stressors has been a subject that has dominated the marine ecological literature for many decades (Dayton 1971, Connell 1978, Paine & Levin 1981, Menge & Sutherland 1987, Hall 1994, Crain et al. 2008). Early studies considered the effects of stressors independently from one another and on individual species, yet stressors to natural ecological sys-

tems rarely occur singularly (Crain et al. 2008, Halpern et al. 2008), and species seldom occur alone. Effects of multiple stressors cannot easily be predicted from the effects of singular stressors because, when combined, they are rarely neutral but have additive, subtractive or synergistic effects (Crain et al. 2008, Darling & Côté 2008, Bijma et al. 2013). Yet many studies continue to examine the effects of stressors individually and on single species or small subgroups of taxa (Crain et al. 2008). As coastal develop-

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ment and climate change continue to increase the frequency and intensity of stressors (Halpern et al. 2008, Bijma et al. 2013), there is a growing need to understand the combined effects of stressors, so as to develop appropriate policy and management strategies to manage, and perhaps minimise, their impact.

Of the studies that have considered the effects of multiple stressors on organisms, many have been conducted in the controlled environment of experimental mesocosms, with species isolated from their habitats and communities (Godbold et al. 2011, Hicks et al. 2011). Although such studies allow the effects of stressors to be examined in the absence of other confounding influences, the extent to which results can be applied to natural systems is unclear because they fail to take into consideration the role of the environmental and biotic context in mediating stressor impacts. For example, organisms can make use of microhabitats to minimise exposure to stressors (Jones & Boulding 1999) and, under natural conditions, ecological interactions such as competition, facilitation and predation may dampen or exacerbate stressor impacts (Christensen et al. 2006, Hicks et al. 2011). *In situ* mesocosms have been used effectively to add an element of realism to assessments of stressor impacts (e.g. Christensen et al. 2006). Nevertheless, while studies such as these offer interesting insights and explore important relationships between changes in ecosystem function and biodiversity under scenarios of environmental change, they do not represent natural conditions, and field studies are still needed as part of the experimental framework (for a review of the topic see Crain et al. 2008, Przeslawski et al. 2015 and references therein).

Estuarine environments are among the most heavily impacted in the world, with >40% of the world's human population living within 100 km of the coast. Among the numerous stressors affecting these systems on a daily basis, nutrient enrichment and physical disturbances of sediments are often singled out as the most severe (Gray 1997). Leaching of nutrients from the terrestrial environment, atmospheric deposition and decomposition are natural sources of nutrient loading in these environments, but these can become exacerbated by human influences (Nixon 1995). For example, the discharge of sewage into aquatic environments, as well as run-off of nutrient-rich fertilisers, cleaning products and animal wastes from the land can enhance delivery of nitrogen and phosphorus to estuarine systems (Nixon 1995, Vitousek et al. 1997a). Where nitrogen and/or phosphorus is a limiting resource, addition of these nutrients has the potential to greatly stimulate primary productiv-

ity (Vitousek et al. 1997a, Cloern 2001). Excess nutrients have been linked to eutrophication events worldwide, whereby algal blooms are facilitated, ultimately leading to hypoxic and anoxic conditions through over-stimulation of oxygen-consuming microbial decomposition (Vitousek et al. 1997b).

High human population densities around estuaries also facilitate many small-scale disturbances to estuarine sediments through recreation, e.g. boat wake, anchorage, propeller scarring, coastal walking (Bishop 2005, Rossi et al. 2007), and as a consequence of livelihood exploitation, e.g. bait digging, fishing, dredging (Wynberg & Branch 1994, Somerfield et al. 1995, Brown & Wilson 1997). These widely studied physical impacts can lead to the disturbance and redistribution of sediments (Hall 1994), causing damage to sediment dwelling organisms or burrows (Hall & Harding 1997). These physical disturbances, therefore, have the potential to alter the distribution, abundance and diversity of macrofaunal assemblages (Fraterrigo & Rusak 2008). At a larger scale, natural events such as storms also cause physical disturbances to sediments, making the bed more susceptible to erosion and further stimulating damaging scour impacts (Yeo & Risk 1979). While sediment grain size can mediate the effect of disturbance on communities (Lindegarh & Hoskin 2001), grain size itself can also become altered by intense disturbance effects (Bishop 2005), thereby facilitating a change in community structure.

The aim of this study was to explore how 2 stressors, nutrient enrichment and physical disturbance, interact under natural field conditions. Nutrient enrichment and physical disturbances often overlap in time and space. For example, nutrient enrichment influences whole catchments, within which physical disturbances may also occur at smaller scales. Additionally, storm events that flush nutrient pollution into estuarine and coastal areas may also cause physical disturbance (Harris 2014). Despite this, most studies that have examined effects of these stressors on sediment communities have done so independently, and those that have examined interacting effects have done so in small-scale mesocosm experiments (see Widdicombe & Austen 2001, Austen & Widdicombe 2006). According to Huston's (1979) dynamic equilibrium model, which posits that larger disturbances will be required to disrupt competitive dominance when productivity is high, the 2 stressors are predicted to display non-additive effects. Mesocosm experiments provide support for this hypothesis (Widdicombe & Austen 2001, Austen & Widdicombe 2006). In buckets, experimental manipulations of

nutrient enrichment (via addition of dried and powdered *Ascophyllum*) and physical disturbance (via surface raking) revealed that diversity was lower than expected when low frequencies of physical disturbance were applied in combination with high levels of organic enrichment or vice versa (Widdicombe & Austen 2001, Austen & Widdicombe 2006). Diversity was higher than expected when both disturbance and enrichment were either high or low (Widdicombe & Austen 2001). Despite these results, it is unknown whether these non-additive effects also occur in field systems in which environmental conditions may buffer effects and recruitment of fauna is possible. Here, manipulative field experiments were conducted in 2 estuaries of eastern Australia to test the hypotheses that (1) the 2 stressors would display non-additive effects on the microphytobenthos (MPB) and macrofaunal communities that are not easily predicted from their independent effects, and (2) the nature of interactions would vary across field sites that differ in biotic and abiotic conditions.

MATERIALS AND METHODS

Study site

Field experiments were conducted between May and September 2012 at 2 locations within Sydney, New South Wales, Australia: Tambourine Bay within Lane Cove River (33° 49' 39" S, 151° 09' 38" E) and Woolooware Bay within Botany Bay (34° 01' 11" S, 151° 07' 46" E). Sites were unvegetated intertidal mud flats adjacent to mangrove habitat. Each was within 15 km of the estuary mouth, was situated in a highly urbanised catchment and had sandy-mud sediments. Sites were away from storm-water drains, which transport nutrients into estuaries. Although the sites may be subject to minimal trampling and the occasional boat run-aground, neither was subject to back-ground raking or substantial bait-digging prior to experimental intervention. At an intertidal elevation of ~0.5 m above mean low water springs, 70 square plots (0.5 × 0.5 m) were randomly established at each site and marked by a single post in the upper left corner of the plot. Each plot was separated by approximately 3 m.

Experimental approaches

The interactive effect of stressors was determined using a fully orthogonal experimental design with 2 factors: nutrient enrichment and physical distur-

bance. Each factor had 3 levels of intensity: zero, low or high. At each site 7 plots were randomly assigned to each of the resulting 9 experimental treatments. The remaining 7 plots per site were procedural controls for the method of nutrient enrichment (see below).

Physical disturbance was manipulated by raking sediments (50 cm wide rake) to a depth of 2–4 cm. Raking is one of the most commonly used methods for physically disturbing sediments (e.g. Cowie et al. 2000, Whomersley et al. 2010), and mimics cockle hand raking (Kaiser et al. 2001, Mistri et al. 2004). For plots assigned to the low or high physical disturbance, an area of approximately 1 m², centering on but extending beyond the experimental plot, was raked in a cross-hatched pattern. The low treatment consisted of 2 strokes of the rake, perpendicular to one another. This level of disturbance has previously been shown to illicit a response from intertidal communities (Whomersley et al. 2010). The high treatment consisted of 6 strokes, each stroke perpendicular to the previous. This disturbance was applied at the start of experiment and then monthly over a 4 mo period.

Nutrient plots were enriched using Scotts Osmocote Pro, 8–9 mo coated fertiliser pellets (N:P:K ratio of 16:4.8:8.3). These pellets, which gradually release nutrients, provide a controlled method of testing the effects of chronic nutrient enrichment in soft sediment benthic environments without need for replenishment (Worm et al. 2000). Plots were given 0 g (zero), 500 g (low) or 1000 g (high) of fertiliser. The high nutrient treatment was based on the level of nutrient loading that might be experienced in the vicinity of the discharge point of a moderately sized sewage treatment plant (Morris & Keough 2002, 2003a, O'Brien et al. 2010), and the low treatment was set at half of this. For each of the plots assigned to the high or low nutrient treatment, nutrients were dispensed via 5 bags per plot, made from nylon pantyhose, among which the fertiliser was evenly distributed. These bags were spread evenly within each plot and were buried approximately 2 to 4 cm below the surface of the sediment. The nylon provided a fine permeable membrane through which nutrients could leach out into the plots. A procedural control tested for any experimental artefacts associated with burying the fertiliser; for this treatment, nylon bags contained sediment in place of fertiliser and were deployed in the same manner as the nutrient treatments. Nylon bags containing slow-release fertiliser or sediment (for the procedural control) were added to plots at the start of the experiment.

Sampling

Prior to initial manipulation of stressors in May 2015, the chlorophyll and organic content of a subset ($n = 25$) of plots at each site was sampled to determine background conditions. Additionally, the spectral reflectance of the sediment and the macrofaunal community were sampled in each plot immediately prior to the manipulation of stressors. Sediment and MPB variables were subsequently sampled monthly, for 4 mo between June and September, and macrofauna were sampled after 2 (July) and 4 (September) mo. Sampling of chlorophyll *a* (chl *a*) content and spectral reflectance were together used as proxies for the biomass of MPB (Tolhurst et al. 2005, Kromkamp et al. 2006). Sampling was conducted immediately prior to each re-application of the physical disturbance treatment. Sampling at Lane Cove was not possible in the first month following initial disturbance due to large amounts of overlying water caused by a sizable low pressure system. The location of sample collection within each plot at each sampling time was noted to avoid repeated collection of samples from the same area.

The sediment organic content and chl *a* concentration were assessed through the contact coring method (Ford & Honeywill 2002) whereby the top 2 mm of the sediment surface was flash-frozen with liquid nitrogen and then freeze-dried. A single randomly positioned 2463 mm² core was collected from each plot at each sampling time. Over a period of 48 h, photosynthetic pigments were extracted from a 200 mg subsample of sediment from each core using 1.5 ml of 90 % acetone under dark conditions within a –80°C freezer. Samples were agitated after 24 h for 10 s by using a vortex mixer. The chl *a* concentration of the acetone solution was determined spectrophotometrically using the method of Jeffrey & Humphrey (1975), and the chl *a* content of sediment was calculated per unit area (mg m⁻²). A second 2 g subsample of sediment was taken from each contact core to determine the organic content through loss-on-ignition (450°C for 4 h).

An Ocean Optics USB2000 spectroradiometer was used to measure the spectral reflectance of the sediment surface *in situ*. Reflectance values (*R*) were made in the visible (675 nm) and infra-red (750 nm) parts of the spectra, and were used to calculate the normalised difference vegetation index (NDVI), a measure of the photosynthetically related biomass based upon reflectance of chl *a* in the sediment (Kromkamp et al. 2006):

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

Three measurements were collected per plot, allowing an average NDVI to be calculated. Reflectance measurements were discarded where there was interference from surface water. Nevertheless, at each sampling time, measurements from at least $n = 3$ plots per treatment could be included in the analyses.

One large core (10 cm diameter, 10 cm depth) was collected from each plot to quantify macrofaunal biodiversity at each of the sampling times indicated above. Sediment cores were taken from between nylon bags, so as not to disrupt the dispensing of nutrients. Sediment core samples were sieved through a 500 µm diameter mesh, and material retained on the sieve was fixed in 10% buffered formalin. Retained material was subsequently transferred into 70% ethanol and examined under a dissecting microscope to separate fauna from other remaining material. Fauna were identified to species level or morphospecies where this was not possible.

Statistics

Permutational analyses of variance (PERMANOVA: Anderson 2001, Anderson et al. 2008) were used to analyse univariate and multivariate data. Although initially developed for multivariate application, PERMANOVA can also be run on univariate data (Anderson et al. 2008) and has the advantage over ANOVA that it does not have assumptions regarding the underlying distribution of the data and can be used on any distance matrix (Anderson et al. 2008). Multivariate analyses, using Bray-Curtis dissimilarities, were run on macrofaunal composition data. Macrofaunal composition data were square-root transformed prior to PERMANOVA in order to down-weight the effect of species dominance and ordinated using 2-dimensional non-metric multidimensional scaling (nMDS). Univariate tests, using Euclidean distance matrices, were run on each of sediment organic content, chl *a* content, NDVI, Shannon's diversity of macrofauna, total abundance of macrofauna, species richness of macrofauna and the abundance of macrofaunal species that were key discriminators of multivariate differences among treatments.

First, for macrofauna and NDVI variables, 3-way analyses on time 0 data, collected prior to application of perturbations, and including the factors nutrient enrichment (fixed; 3 levels: zero [0N], low [LN], high [HN]), physical disturbance (fixed; 3 levels: zero [0D], low [LD], high [HD]) and site (random; 2 levels)

tested for any pre-existing differences in variables of interest among plots and between sites that coincided with their treatment assignment. Due to only a subset of contact core samples taken at time 0, we were unable to run a complete analysis testing differences between treatments for chl *a* and organic content; however, a 1-way analysis was conducted testing for pre-existing differences between sites. Second, 4-way analyses, with the factors time (4 levels for most variables [1, 2, 3, 4 mo after perturbation], and 2 levels for macrofauna [2, 4 mo]), site (2 levels, random), treatment (2 levels: undisturbed, disturbance control) and plot (random; nested within site and treatment) tested for experimental artefacts of the method of nutrient application. Third, 5-factor analyses with the factors site (random, 2 levels), nutrient enrichment (fixed; 3 levels), physical disturbance (fixed; 3 levels), plot (random, 7 levels, nested within site \times nutrients \times disturbance) and time (random, 2 or 4 levels) tested for interacting effects of the 2 stressors across both sites and all time periods. The inclusion of plot as a nested factor enabled time to be treated as a repeated measure. Following these analyses, pairwise post hoc tests were conducted to identify significant differences between factors. Where the number of possible permutations was lower than 100, Monte Carlo (MC) testing was run to generate p-values from constructed asymptotic permutation distributions for the pseudo-*F* statistic. All analyses were conducted using the PRIMER v6 statistical program with the PERMANOVA+ addition (Clarke & Gorley 2006, Anderson et al. 2008).

The macrofaunal multivariate analysis revealed large spatial and temporal differences in communities resulting in strong interactions between month and site. These prevented the factors of interest (nutrient enrichment and physical disturbance) from being appropriately tested and so separate tests were run for each month and site using a reduced model including only the factors nutrient enrichment and physical disturbance. Key discriminating species, contributing to multivariate differences in macrofaunal community structure, were identified by SIMPER analysis and were defined as those having a dissimilarity/standard deviation ratio >1 between nutrient or physical disturbance treatments. Of these, species that were present at both sites were analysed using the complete 5-factor model, with abundances fourth-root transformed. Discriminating species that were unique to a site were analysed under the reduced 2-factor model. Permutational multivariate dispersions (PERMDISP) were analysed alongside the multivariate PERMANOVAs to assess the extent

to which any treatment effects were driven by differences in dispersion between treatments and, unless otherwise indicated, were non-significant.

RESULTS

MPB component

Prior to experimental manipulations, there were no differences in organic content or chl *a* content of sediments between the 2 sites, although NDVI was significantly greater at Lane Cove than Botany Bay (main effect of site PERMANOVA: $p < 0.05$; see Figs. S1 & S2, Table S1 in the Supplement at www.int-res.com/articles/suppl/m562p037_supp.pdf). Across the 2 sites, there were no pre-existing differences in NDVI among plots that corresponded to treatment assignments (PERMANOVA $p > 0.05$, Table S2 in the Supplement; insufficient plots were sampled to address this question for organic content and chl *a*). At none of the sampling times following application of stressors were there experimental artefacts of the method of fertiliser burial on any of the 3 variables, i.e. organic content, chl *a* or NDVI (PERMANOVA $p > 0.05$; Table S3 in the Supplement).

Organic content did not respond to the addition of stressors at either site (Table 1). By contrast, site-specific effects of stressors on the 2 measures of MPB biomass, chl *a* concentration and NDVI were observed (Table 1). Neither variable displayed interactive effects between the 2 stressors, so additive effects could be interpreted. Chl *a* concentration displayed a significant interaction between physical disturbance, site and month (Table 1, Fig. 1A,B), whereas there was no effect of physical disturbance on NDVI at either site (Table 1). In Botany Bay (Fig. 1A), although a slight negative impact of high physical disturbance on chl *a* was observed in August (Month 3), prior to September (Month 4) the effects of disturbance were non-significant (*a posteriori* pairwise comparisons $p > 0.05$). In September, plots receiving the high physical disturbance treatment had a significantly lower chl *a* concentration than those not receiving physical disturbance ($t = 1.99$, $p(\text{perm}) = 0.048$). There were no differences between the undisturbed and low disturbance treatments ($t = 1.59$, $p(\text{perm}) = 0.113$), or the low and high disturbance treatments ($t = 0.14$, $p(\text{perm}) = 0.898$). At Lane Cove (Fig. 1B), lower levels of chlorophyll were observed in plots receiving high than low physical disturbance in July (Month 2; $t = 3.42$, $p(\text{perm}) = 0.002$), but in August these 2 treatments did not sig-

Table 1. PERMANOVAs examining the interacting effects of nutrients (N), disturbance (D), month (M) and site (S) on organic content, chlorophyll *a* concentration and normalised difference vegetation index (NDVI) in Botany Bay and Lane Cove estuaries. Plot (P) was nested within $N \times D \times S$. Nutrient enrichment and physical disturbance factors contained 3 levels of applied stress (zero, low and high). The 3 months (July, August, September) during which both sites were sampled are included in the analysis. **Bold** type indicates a significant effect at $p < 0.05$; $n = 3-7$ for all treatment levels

Factor	Organic content			Chlorophyll <i>a</i>			NDVI		
	df	Pseudo- <i>F</i>	p(perm)	df	Pseudo- <i>F</i>	p(perm)	df	Pseudo- <i>F</i>	p(perm)
N	2	1.68	0.255	2	3.95	0.070	2	1.22	0.406
D	2	3.47	0.076	2	2.49	0.143	2	0.34	0.916
M	2	1.60	0.371	2	0.84	0.492	3	0.58	0.693
S	1	7.62	0.002	1	0.12	0.747	1	2.59	0.116
$N \times D$	4	0.54	0.859	4	1.10	0.422	4	0.75	0.696
$N \times M$	4	0.42	0.821	4	27.40	0.002	6	1.45	0.336
$N \times S$	2	0.54	0.828	2	1.25	0.282	2	3.42	0.039
$D \times M$	4	0.58	0.730	4	0.08	0.981	6	0.79	0.615
$D \times S$	2	0.42	0.916	2	1.11	0.323	2	2.33	0.104
$M \times S$	2	2.37	0.078	2	9.88	0.001	3	25.91	0.001
$N \times D \times M$	8	1.24	0.363	8	1.80	0.221	12	0.53	0.855
$N \times D \times S$	4	1.46	0.166	4	0.47	0.945	4	1.23	0.262
$N \times M \times S$	4	0.78	0.572	4	0.05	0.998	6	1.54	0.161
$D \times M \times S$	4	2.10	0.054	4	3.05	0.015	6	1.55	0.174
$P(N \times D \times S)$	107	1.83	0.002	112	1.56	0.005	110	1.93	0.001
$N \times D \times M \times S$	8	1.30	0.230	8	0.83	0.577	12	1.69	0.083
Residual	179			187			301		

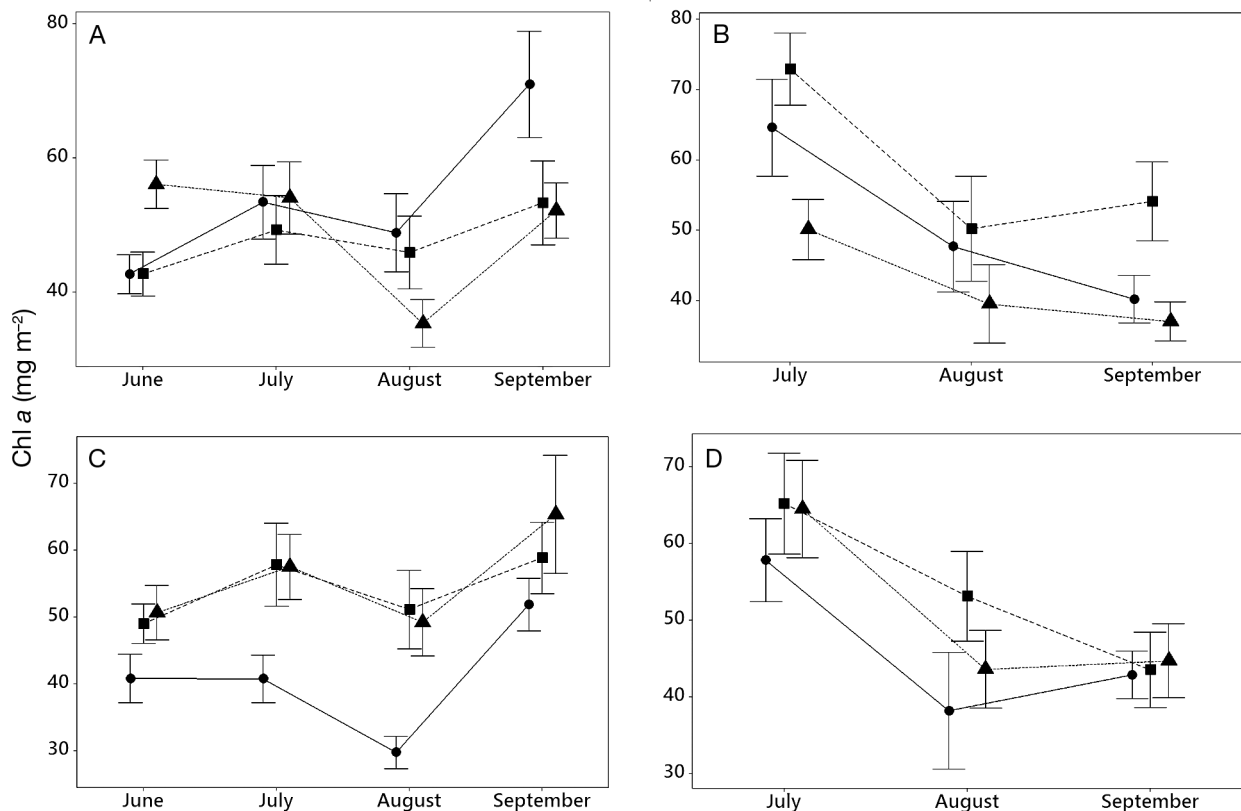


Fig. 1. Mean (\pm SE) chlorophyll *a* concentration of surface sediments in experimental plots. Differences among disturbance treatments through time at (A) Botany Bay and (B) Lane Cove. Differences among nutrient treatments through time at (C) Botany Bay and (D) Lane Cove. Sampling within Lane Cove could not be done in June due to large amounts of overlying water. For all graphs, 0 treatment = circles, solid line; low treatment = squares, long-dashed line; high treatment = triangles, short-dashed line; $n = 3-7$ for all treatment levels

nificantly differ, and there were no significant differences between other pairs of treatments in either July or August (*a posteriori* pairwise comparisons: $p > 0.05$). In September, low disturbance resulted in significantly greater concentrations of chl *a* than in the undisturbed ($t = 2.34$, $p(\text{perm}) = 0.022$) or high disturbance treatment ($t = 2.97$, $p(\text{perm}) = 0.004$), but there was no difference between the high disturbance and undisturbed treatments ($t = 0.71$, $p(\text{perm}) = 0.497$).

Despite chl *a* displaying a significant interaction between nutrient addition and month (N \times M interaction; Table 1) and a weak trend for a greater concentration of chl *a* in plots receiving a low or high nutrient addition than no nutrients (Fig. 1C,D), in none of the months were significant differences among nutrient treatments found (*a posteriori* pairwise comparisons: $p > 0.05$). By contrast, NDVI displayed a significant interaction between nutrient enrichment and site (Table 1), with effects of nutrient enrichment apparent in Botany Bay only (Fig. 2). In Botany Bay, plots receiving either a low ($t = 2.16$, $p(\text{perm}) = 0.032$) or high nutrient addition ($t = 2.75$, $p(\text{perm}) = 0.011$) had a significantly greater NDVI than unenriched plots. There were no differences between the low and the high treatment ($t = 0.63$, $p(\text{perm}) = 0.557$).

Macrofauna

Analysis of macrofaunal community structure prior to manipulations revealed that multivariate commu-

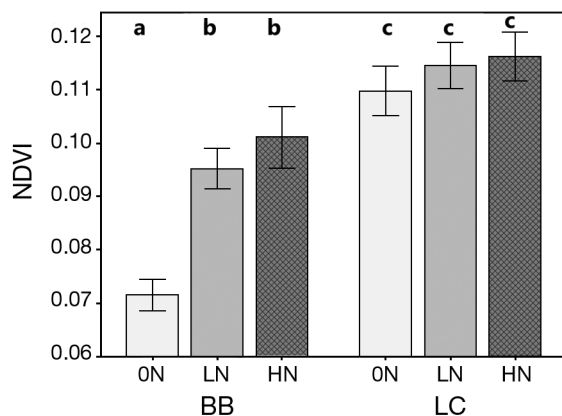


Fig. 2. Mean (\pm SE) normalised difference vegetation index (NDVI) in plots at Botany Bay (BB) and Lane Cove (LC) following 4 mo of continued nutrient enrichment at 3 levels (0N: zero, LN: low, HN: high). Data were averaged across all months and physical disturbance treatments as there was no month \times nutrient or disturbance \times nutrient enrichment interaction; $n = 3$ –7 for all treatment levels. Letters indicate statistically significant differences between treatments (PERMANOVA post hoc tests)

nity composition differed significantly between the sites (pseudo- $F_{1,108} = 31.63$, $p(\text{perm}) = 0.001$), with a greater abundance of invertebrates per plot at Lane Cove than Botany Bay (pseudo- $F_{1,108} = 3.70$, $p(\text{perm}) = 0.031$). Sixty-five species were identified, of which 28 were common between the 2 sites, 19 species were unique to Botany Bay, and 18 were unique to Lane Cove. Shannon diversity and total plot species richness did not significantly differ between the 2 sites (PERMANOVA $p > 0.05$; Table S2). At neither of the sites were there pre-existing differences among plots that corresponded to treatment assignments (Table S2). Furthermore, at none of the sampling times were there experimental artefacts associated with the method of nutrient addition on macrofauna (PERMANOVA $p > 0.05$; Table S3).

In the analysis comparing stressor impacts to macrofauna between the 2 sites, and across all sampling times, there were no effects of nutrient enrichment or physical disturbance on community composition or biodiversity indices; however, there was a significant interaction between month and site (PERMANOVA $p(\text{perm}) < 0.05$). When sites and times were considered separately, stressor effects on macrofauna could not be distinguished in July (PERMANOVA, $p > 0.05$), and in September, following 4 mo of continued exposure to stressors, there was no interacting effect of nutrients and physical disturbance on any of the measures of macrofaunal community structure, at either site, allowing interpretation of main effects (Table 2). Whereas the September analysis revealed no effect of physical disturbance on any of the measures of macrofaunal community structure, nutrient enrichment affected multivariate community composition at Botany Bay, and at Lane Cove it had a weak, though non-significant effect on this variable (Table 2, Fig. 3). At Botany Bay, the highly enriched treatment was significantly different from the unenriched treatment only ($t = 1.63$, $p(\text{perm}) = 0.031$), with no differences between the low and the highly enriched treatments ($t = 1.36$, $p(\text{perm}) = 0.077$) or between the low enrichment and unenriched treatments ($t = 1.04$, $p(\text{perm}) = 0.386$). Additionally at Botany Bay, the multivariate dispersions were significantly greater in the enriched plots in comparison to the controls (PERMANOVA, *a posteriori* tests, $0N < LN = HN$; PERMDISP: $F_{2,58} = 8.94$, $p(\text{perm}) = 0.002$). There were no treatment effects on Shannon diversity for either Botany Bay or Lane Cove (Table 2). However, for Lane Cove, there was a treatment effect of nutrient enrichment on the total macrofaunal abundance whereby increasing enrichment caused a decrease

Table 2. Two-way PERMANOVAs examining the interacting effects of nutrients (N) and disturbance (D) on the macrofaunal community at sites within Botany Bay and Lane Cove estuaries. Nutrient enrichment and physical disturbance factors contained 3 levels of applied stress (zero, low or high) over 4 mo (M). **Bold** type indicates a significant effect at $p < 0.05$; $n = 5-7$ for all treatment levels

	df	Species richness		Abundance		Shannon diversity		Multivariate	
		Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)
Botany Bay									
N	2	0.67	0.562	0.51	0.618	1.20	0.311	1.92	0.035
D	2	0.36	0.702	0.19	0.846	0.64	0.523	1.31	0.181
N × D	4	0.48	0.767	0.52	0.766	0.55	0.699	0.98	0.476
Residual	52								
Lane Cove									
N	2	1.22	0.270	3.66	0.027	0.47	0.604	1.48	0.059
D	2	0.34	0.727	0.70	0.493	0.59	0.548	0.68	0.882
N × D	4	0.46	0.750	0.50	0.714	1.91	0.108	0.85	0.863
Residual	52								

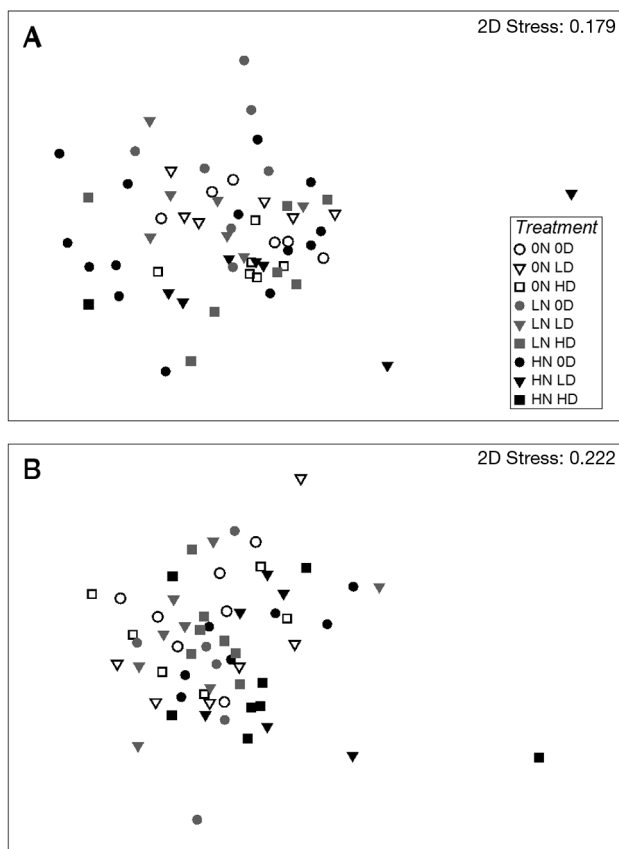


Fig. 3. Two-dimensional non-metric multidimensional scaling configuration displaying macrofaunal composition at (A) Botany Bay and (B) Lane Cove following 4 mo of zero (0) low (L) and high (H) nutrient enrichment (N) and physical disturbance (D). Points represent individual plots. Data are square-root transformed and matrix is based upon Bray Curtis similarity; $n = 5-7$

in total abundance (Table 2; PERMANOVA, *a posteriori* tests, $0N > LN > HN$). Analysis of dispersions also revealed that increased nutrient enrichment significantly decreased the dispersion of abundances at Lane Cove with no differences in dispersion of either low or highly enriched treatment (PERMDISP: $F_{2,58} = 7.06$, $p(\text{perm}) = 0.027$).

SIMPER analysis identified 6 discriminating species contributing most to dissimilarity between nutrient treatments in Botany Bay, 4 of which coincided with those found in Lane Cove. These were *Mysella* sp., *Mediomastus australiensis*, *Prionospio* sp. and *Nephtys australiensis*. These species were among the most abundant species found at each site. The additional discriminating species at Botany Bay were *Salinator fragilis* and *Owenia australis*. Lane Cove had a total of 12 discriminating species which also included 2 species of *Gammarus* amphipods, Oedicerotidae sp. amphipods, *Scoloplos* sp., *Laternula* sp., *Platynereis uniseris*, *Australonereis ehlersi* and large nematodes.

In repeated-measures analyses that included both sites, *M. australiensis* did not display any significant effects of nutrient enrichment or physical disturbance at either of the sites or at any of the sampling times (Table 3). Nutrient enrichment caused a significant alteration of the abundance of *Prionospio* sp. at Botany Bay only (nutrient enrichment × site interaction; Table 3; *a posteriori* pairwise comparisons: $p < 0.05$; Fig. 4). Pairwise comparisons revealed that at Botany Bay, the high nutrient enrichment plots had significantly lower *Prionospio* sp. abundances than the unenriched plots ($t = 2.42$, $p(\text{perm}) = 0.016$), whereas there were no significant differences between the unenriched and low enrichment plots, or the low and high enrichment plots ($p > 0.05$).

Table 3. PERMANOVA examining the interacting effects of nutrients (N), disturbance (D), month (M) and site (S) on the key discriminating species common to both sites within Botany Bay and Lane Cove estuaries. Plot (P) was nested within $N \times D \times S$. Nutrient enrichment and physical disturbance factors contained 3 levels of applied stress (zero, low or high), with sampling after 2 and 4 mo (i.e. July, September). **Bold** type indicates a significant effect at $p < 0.05$; $n = 5-7$ for all treatment levels

Factor	df	<i>Mediomastus australiensis</i>		<i>Prionospio</i> sp.		<i>Nephtys australiensis</i>		<i>Mysella</i> sp.	
		Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)	Pseudo- <i>F</i>	p(perm)
N	2	0.69	0.629	0.56	0.697	0.46	0.758	1.17	0.509
D	2	0.78	0.586	0.66	0.631	1.62	0.324	0.17	0.949
M	1	4.22	0.326	0.96	0.629	0.11	0.506	35.18	0.343
S	1	13.98	0.001	2.90	0.086	14.03	0.001	7.15	0.010
$N \times D$	4	0.21	0.977	1.87	0.197	4.66	0.026	2.87	0.087
$N \times M$	2	1.04	0.497	8.67	0.104	1.90	0.355	483.73	0.003
$N \times S$	2	0.68	0.530	2.64	0.045	2.13	0.138	1.15	0.310
$D \times M$	2	2.73	0.264	1.45	0.375	0.79	0.565	6.32	0.132
$D \times S$	2	0.80	0.466	1.11	0.397	0.48	0.637	0.69	0.512
$M \times S$	1	4.73	0.037	8.06	0.007	11.36	0.002	0.58	0.455
$N \times D \times M$	4	4.22	0.092	0.92	0.516	0.27	0.893	6.81	0.045
$N \times D \times S$	4	0.92	0.544	0.81	0.681	0.68	0.772	0.60	0.867
$N \times M \times S$	2	1.69	0.193	0.25	0.769	0.20	0.833	0.01	0.989
$D \times M \times S$	2	0.47	0.639	0.89	0.429	1.25	0.299	0.07	0.933
$P(N \times D \times S)$	110	1.46	0.033	1.12	0.295	0.97	0.570	3.08	0.001
$N \times D \times M \times S$	4	0.79	0.538	1.30	0.290	0.74	0.564	0.10	0.983
Residual	101								

Across both sites and all sampling times, there was a significant interaction between nutrient enrichment and physical disturbance for the species *N. australiensis* (Table 3, Fig. 4). Among plots that received low nutrient enrichment, abundances of *N. australiensis* were significantly greater in plots that also received low physical disturbance than those that were not disturbed ($t = 16.64$, $p(\text{perm}) = 0.013$), with no significant difference between plots receiving high or low physical disturbance ($t = 0.40$, $p(\text{perm}) = 0.829$) and between plots receiving high or no physical disturbance ($t = 1.94$, $p(\text{perm}) = 0.223$). By contrast, among plots receiving high or zero nutrient enrichment, there was no significant effect of physical disturbance on *N. australiensis* abundance (*a posteriori* pairwise comparisons: $p > 0.05$). There was no significant effect of nutrient enrichment within any of the 3 levels of physical disturbance (*a posteriori* pairwise comparisons: $p > 0.05$).

Mysella sp. displayed a 3-way interaction between nutrient enrichment, physical disturbance and month, with no differences between sites (Table 3, Fig. 4). Among unenriched plots, there was no significant effect of physical disturbance in either of the months (*a posteriori* pairwise comparisons: $p > 0.05$). Among plots subjected to low nutrient enrichment, abundances were greater in plots receiving high than no physical disturbance in each of the months (July: $t = 21.30$, $p(\text{MC}) = 0.037$; September: $t = 19.32$, $p(\text{MC}) = 0.031$) and in July, there was also a significant differ-

ence between the high and low disturbance treatment (HD > LD; $t = 22.42$, $p(\text{MC}) = 0.037$) but all other pairwise comparisons were non-significant ($p > 0.05$). Among plots receiving high nutrient enrichment, plots receiving high physical disturbance had greater abundances of *Mysella* sp. than undisturbed plots in July ($t = 31.87$, $p(\text{MC}) = 0.017$), whereas in September, highly disturbed plots contained fewer individuals than those that were undisturbed ($t = 12.33$, $p(\text{MC}) = 0.049$), with no other pairwise comparisons between levels of physical disturbance significant for either of the sampling times ($p > 0.05$). Examining the interaction the other way around, in July, plots receiving no physical disturbance had lower abundances of *Mysella* sp. when they received low as compared to no nutrient enrichment ($t = 51.61$, $p(\text{MC}) = 0.013$), but other pairwise contrasts revealed no other significant differences (*a posteriori* pairwise comparisons: $p > 0.05$). In September, both the low and high nutrient treatments had lower abundances of *Mysella* sp. in comparison to the unenriched treatment (low: $t = 17.51$, $p(\text{MC}) = 0.33$; high: $t = 41.83$, $p(\text{MC}) = 0.017$) but did not significantly differ from one another ($t = 0.04$, $p(\text{MC}) = 0.977$). There was no effect of nutrient enrichment on *Mysella* sp. among plots receiving low or high physical disturbance (*a posteriori* pairwise comparisons: $p > 0.05$).

Few of the species that were key discriminating taxa at only 1 of the 2 sites displayed significant responses to stressors (Table S4). At Lane Cove,

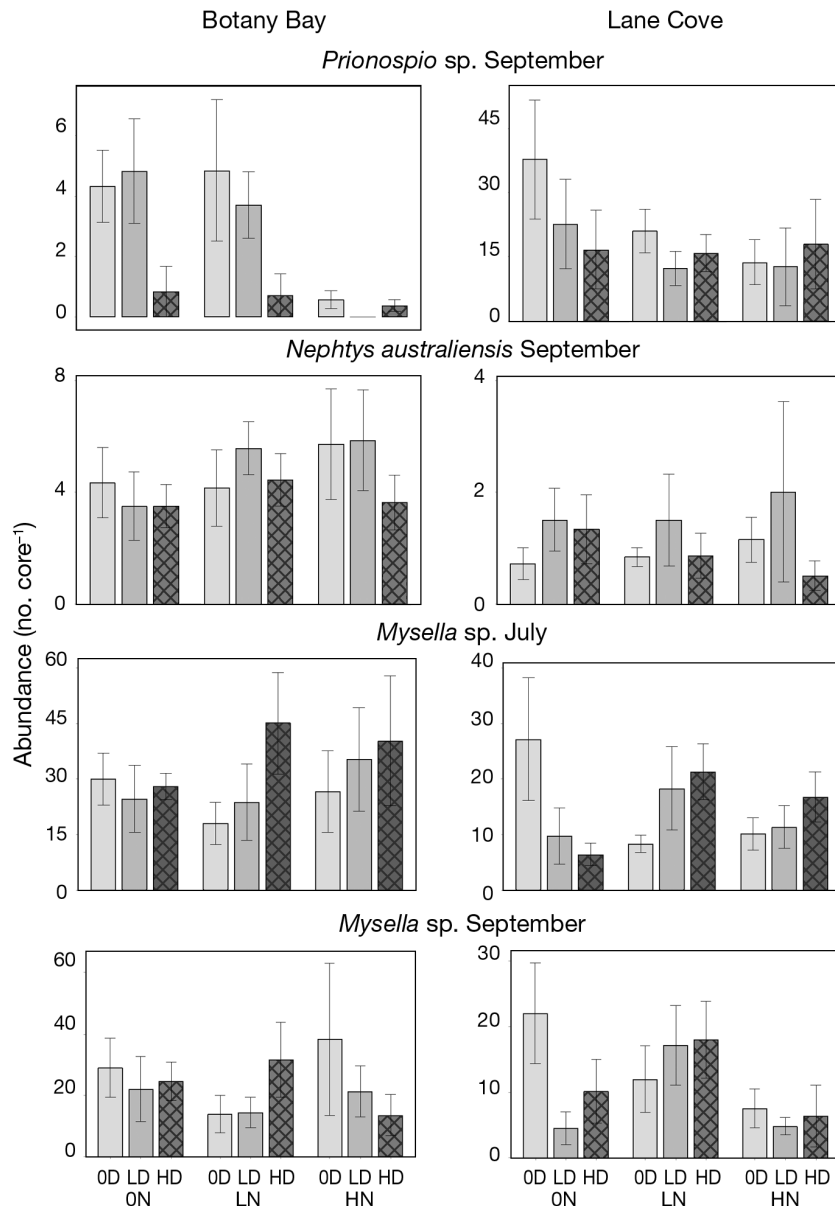


Fig. 4. Mean (\pm SE) abundance of common macrofaunal species in experimental plots at Botany Bay and Lane Cove, 2 (July) or 4 (September) mo after commencement of stressor application. Plots were randomly assigned to 1 of 3 levels of nutrient enrichment (0N = zero, LN = low, HN = high) and 1 of 3 levels of physical disturbance (0D = zero, LD = low, HD = high), N = 5–7

Scoloplos sp. displayed a significant interaction between nutrient enrichment and physical disturbance (pseudo- $F_{2,52} = 2.91$, $p(\text{perm}) = 0.039$; Fig. 5A). Among plots subjected to zero physical disturbance, plots receiving low ($t = 2.23$, $p(\text{perm}) = 0.034$) and high ($t = 2.13$, $p(\text{perm}) = 0.050$) nutrient contained fewer *Scoloplos* sp. than those receiving zero enrichment, with no differences between the low and high nutrient treatments. By contrast, among plots receiv-

ing low or high physical disturbance, there was no effect of nutrient enrichment (PERMANOVA *a posteriori* tests: $p > 0.05$). When the interaction was examined the other way around, among plots subjected to zero nutrient enrichment, high physical disturbance resulted in significantly fewer *Scoloplos* sp. as compared to undisturbed controls ($t = 2.46$, $p(\text{perm}) = 0.030$), whereas there were no differences between plots receiving low and no disturbance ($t = 2.38$, $p(\text{perm}) = 0.064$) or low and high disturbance ($t = 0.12$, $p(\text{perm}) = 0.99$). Among plots receiving low or high nutrient enrichment, there was no significant effect of disturbance on *Scoloplos* sp. (PERMANOVA *a posteriori* tests: $p > 0.05$). At Lane Cove, nematodes displayed a significant main effect of nutrient enrichment only (pseudo- $F_{2,52} = 3.25$, $p(\text{perm}) = 0.045$; Fig. 5B) whereby abundance decreased with increasing nutrient enrichment (PERMANOVA *a posteriori* tests: $p < 0.05$).

DISCUSSION

The limited number of mesocosm studies focussing on the interaction between physical disturbance and nutrient enrichment have highlighted the potential for interactions between these 2 stressors (Widdicombe & Austen 2001, Austen & Widdicombe 2006). Here we sought to expand upon this earlier work by examining the interaction between nutrient enrichment and physical disturbance under larger-scale field conditions that offer an element of ecological realism. To our knowledge, it represents the first field-based study to examine the multiple effects of these 2 stressors in an intertidal sedimentary habitat (but see Rossi & Underwood 2002 for a discussion on the impacts of organic matter burial that result both from physical disturbance of sediments and nutrient enrichment during decay). We found site-specific effects of nutrient enrichment and physical disturbance, with limited interactions between stressors. The study thereby demonstrates the role environ-

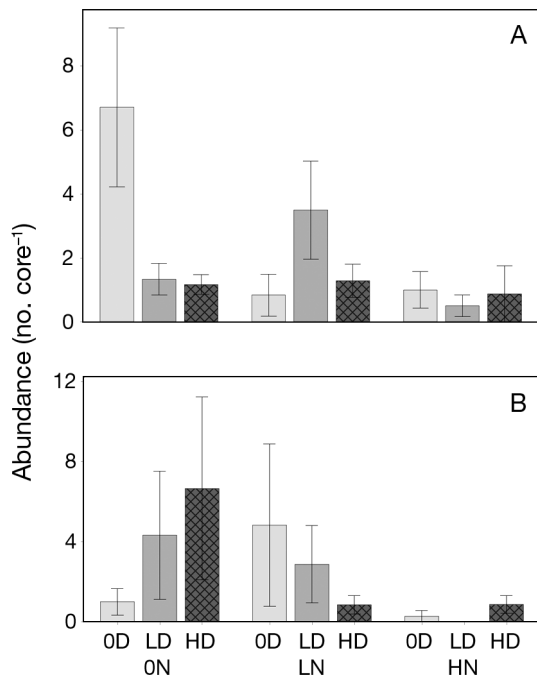


Fig. 5. Mean (\pm SE) abundance of (A) *Scoloplos* sp. and (B) nematodes in experimental plots at Lane Cove following 4 mo of nutrient enrichment (3 levels: 0N = zero, LN = low, HN = high) and physical disturbance (0D = zero, LD = low, HD = high). $n = 5-7$

mental context plays on mediating the impact of multiple stressors, and raises the possibility that communities may be more resilient to increased stress than can be predicted from laboratory studies alone.

Of the 2 stressors examined in this study, nutrient enrichment generally elicited stronger ecological responses than physical disturbance. Eastern Australian estuaries are, in general, oligotrophic and phosphorus-limited (Bishop et al. 2006, Scanes et al. 2007, Kelaher et al. 2013). In nutrient-limited systems such as these, nutrient enrichment typically stimulates bottom-up responses of the MPB (O'Brien et al. 2010, Pascal et al. 2013). This growth may then lead to cascading positive effects on higher trophic levels (York et al. 2012). Nevertheless, because excess primary production can also result in deterioration of sediment conditions through over-stimulation of oxygen-consuming bacteria, the effects of nutrient enrichment on macrofaunal abundance and richness can range from positive (Morris & Keough 2003a,b) to negative (Fitch & Crowe 2010, Botter-Carvalho et al. 2014), depending on the level of enrichment and environmental conditions (Pearson & Rosenberg 1978).

In the present study, an effect of nutrient enrichment on MPB was identified at both study sites

(although effects were stronger in Botany Bay), and the effect was positive, in agreement with mesocosm studies (e.g. Sundbäck et al. 2010). While nutrient additions acted to alter macrofaunal community structure at each of the sites, effects were weaker than expected, with few species significantly affected by stressors, and the nature of effects variable between these. It has been hypothesised that estuarine communities have an inherent ability to resist stress at levels that would push other aquatic systems over a threshold, and which are above and beyond that which they are subjected to daily (e.g. salinity fluctuations, tides, and emersion periods; see Elliott & Whitfield 2011). Within nature, biotic interactions may reduce the impacts of nutrient enrichment. The limited effects of nutrient enrichment observed in this study indicate there could be a mix of top down and bottom up effects impacting the community. Although in nutrient-limited systems, nutrient enrichment facilitates autotrophic growth, this effect can become masked by top-down processes where increased productivity is matched by increased grazing pressure (Pascal et al. 2013). Alternatively, the weak effects of nutrient enrichment observed in the present study may be a function of other abiotic and biotic characteristics of the sites, an aspect that would be controlled for in closed systems. Sedimentary grain size and starting community composition can play an important role in interpreting how communities will respond to stressors (Lindegarth & Hoskin 2001, Rossi & Underwood, 2002, Whomersley et al. 2010). The relatively weak effects of nutrient enrichment detected in our study are consistent with larger-scale surveys that indicate that nutrient enrichment is potentially a weak driver of change in benthic community composition in comparison to environmental characteristics, such as grain size (Nicastro & Bishop 2013). Alternatively, the weak effects of nutrient enrichment seen in our study may reflect the small scale of the manipulations. Larger-scale disturbances alter system dynamics, ultimately impacting recovery and community resilience (Ellis et al. 2000).

Both MPB and macrofauna displayed a limited to neutral response to physical disturbance, despite predictions, based on previous field studies, of negative effects of this stressor from destruction of sediment structure, burrows and damage to softer-bodied species (Hall 1994, Brown & Wilson 1997, Hall & Harding 1997, Dernie et al. 2003, Rossi et al. 2007). Community or species-specific responses to physical disturbance have been previously noted to vary according to environmental context (Whomer-

sley et al. 2010) and previous mesocosm experiments have found that, in line with predictions of the dynamic equilibrium hypothesis (Huston 1979), the effects of physical disturbance vary across productivity gradients caused by nutrient enrichment (Widdicombe & Austen 2001, Austen & Widdicombe 2006). Consistent with the dynamic equilibrium hypothesis, a number of macrofaunal species in this study displayed responses to physical disturbance that varied according to the prevailing levels of nutrient enrichment. Overall, however, the number of species exhibiting such a non-additive response was few, and nutrient enrichment was the dominant stressor impacting community composition in these systems.

While small-scale disturbances such as raking and trampling sediments have been shown to negatively impact sediment communities in other studies (Rossi et al. 2007, Whomersley et al. 2010), the spatial scale and frequency of raking may simply not have been great enough to elicit a strong response in the communities examined here. In this experiment, plots were less than 1 m in diameter, allowing species to rapidly recolonise from outside affected areas (see Lee et al. 2011). This small scale of physical disturbance might be analogous to that caused by clam raking, boats running up on intertidal sediments or propeller scarring. However, for disturbances such as storms, that cause physical disturbance at the scale of the entire site, movement of species between affected areas would not be possible. Treatment effects at this level are likely to facilitate the selection for more resilient organisms that are able to tolerate stressors (Sanford & Kelly 2011). The relatively low monthly frequency of raking may also have contributed to the weak to absent responses of taxa to physical disturbance. The frequency of disturbance is an important factor determining the magnitude of impact (Connell 1978, White & Pickett 1985), with infrequent disturbances providing opportunity for organisms to recolonise in between, particularly if the spatial scale of the disturbance is small, but frequent disturbances preventing recovery before the next perturbation. Nevertheless, in Northern Europe, a single raking event covering an area of 36 m² that perturbed large benthic species had long-term ecological impacts that lasted over a year (Kaiser et al. 2001). Impacts are therefore likely to be dependent on the size and scale of the raking event, as well as the species present. Although the physical disturbance imposed by repeated sampling of sediments may have complicated results by leading to small-scale

disturbance across all experimental plots, irrespective of disturbance treatment (Lindegarth & Underwood 2002), we were careful to sample different areas on each sampling date.

Within the literature, there are many examples of context-dependent effects of manipulations, even across geographically proximate and/or environmentally similar locations (e.g. Whomersley et al. 2010, Bishop & Kelaher 2013, Gladstone-Gallagher et al. 2014). In our study, the differing response between the 2 sites to environmental perturbations may be a direct effect of environmental differences between sites, or an indirect effect arising from differences in their community composition. Although the 2 sites did not differ in background sediment organic content or chlorophyll concentration and were selected to be of similar grain size, other environmental variables are likely to have differed. Botany Bay is a large industrial area with a history of contamination, whereas Lane Cove is surrounded by bushland and is used for recreational purposes such as hiking, boating and fishing. Habitat characteristics such as grain size, organic content and water content mediate the speed of benthic recovery from perturbations (Dernie et al. 2003). Additionally, under natural conditions, habitat heterogeneity can buffer against treatment effects (Godbold et al. 2011) or lead to substantial background variation, against which treatment effects are hard to detect (Bulling et al. 2008). Community structure may mediate stressor impacts by dictating the functional capability of the ecosystem, and the ways in which species may interact to buffer the effects of stressors (Bulling et al. 2008, Godbold & Solan 2009).

Additionally, differences between the sites in background stressors may have contributed to the differing sensitivity of their communities to the experimental perturbations. Background stress can mediate how a system will respond to further stress via 2 main mechanisms. First, prevailing stress may select for organisms that have enhanced resistance to local stressors and so are able to withstand further disturbance (Sanford & Kelly 2011). Second, prevailing stressors can determine how far away a system is from a tipping-point beyond which the application of additional stress pushes the system over a threshold value, resulting in a system collapse (Pearson & Rosenberg 1978, Whomersley et al. 2010). How far away systems are from tipping points is related to the conditions to which fauna are adapted and the trade-offs they have made either through evolutionary or local adaptations (Sanford & Kelly 2011, Botero et al. 2015). In dynamic environments subjected to multi-

ple stressors such as estuaries, it would be expected that communities are dominated by species that are resilient to perturbation (Sanford & Kelly 2011). Only where stressors exceed the threshold of resilience is there system collapse or change to another stable state.

Although previous mesocosm studies have identified interactions between nutrient enrichment and physical disturbance (e.g. Widdicombe & Austen 2001), our study demonstrates that the results of such studies cannot necessarily be used to infer responses of benthic communities to stressors in the field. While some studies have found agreement between the outcomes of field and mesocosm experiments (e.g. Sundbäck et al. 2010), mesocosm studies can never completely replicate the natural environment and conclusions about the way in which multiple stressors interact can differ between these approaches (Crain et al. 2008, Alsterberg et al. 2014, Przeslawski et al. 2015). For example, Cowie et al. (2000) observed a negative effect of physical disturbance on the macrofauna in both experimental mesocosms and *in situ* treatments; however, the strength of the decline of species was greater in mesocosm experiments. Adverse effects are likely to be amplified in mesocosm studies due to the stress imposed on organisms as a consequence of handling and containment in confined conditions, and because processes such as recruitment that act to weaken impacts in field scenarios cannot always occur (see Cowie et al. 2000). The heterogeneity of environments such as mudflats and the patchiness of species can contribute to the differing responses observed between laboratory and mesocosm studies (Crain et al. 2008), or even between different field sites (Norkko et al. 2010). While mesocosms provide a controlled environment, there is growing evidence that environmental context (such as prevailing nutrient status) is often the determinant factor governing how systems will respond to stress (Sundbäck et al. 2007, Bishop & Kelaher 2013, O'Connor & Donohue 2013). Thus it follows that, while mesocosm and laboratory experiments are often a quick and simple tool in understanding the consequences of change, these experiments should be utilised to formulate theories to be tested empirically (see Benton et al. 2007), and field experiments must be used to determine true impacts of stressors under naturally occurring conditions. Furthermore, the implications of multiple stressor impacts are well known to vary depending upon the experimental protocol (laboratory/mesocosm; see Crain et al. 2008 and references therein) or the responses measured (Alsterberg et al. 2014). The

differing method of nutrient enrichment between our study, which applied fertiliser to plots, and the previous mesocosm studies that enriched sediments via application of dried and ground-up *Ascophyllum* (Widdicombe & Austen 2001, Austen & Widdicombe 2006) may have contributed to differences in the conclusions of the respective studies. Similarly, the differing frequency of physical disturbance between our study, in which plots were raked monthly, and the mesocosm experiments, in which plots were raked daily to monthly (Widdicombe & Austen 2001, Austen & Widdicombe 2006), may also have led to differences.

CONCLUSIONS

This study analysed the combined impact of selected multiple stressors, i.e. nutrient enrichment and physical disturbance, in a field setting. While both stressors have been previously identified to be detrimental to communities and in mesocosm experiments produce non-additive effects, the combined impacts observed in this study were weak, mainly additive (at least at the chosen levels of stress), and mediated by environmental context. The differing results of this *in situ* study, compared to those of previous mesocosm experiments, as well as the site-specificity of effects, which can only be determined using *in situ* studies, highlight the important role that abiotic and biotic features of the environment play in mediating stressor effects. Furthermore, the resilience of benthic communities will determine their response to multiple stressors. The minor responses observed within this study highlight that benthic sedimentary communities are more resilient to increased stress than previous studies would indicate. Studies are now needed that examine which aspects of the environment or community are most important in mediating stressor impacts.

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