



Defaunation by deoxygenation: efficacy and divergent responses of estuarine macroinvertebrates

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ABSTRACT: Understanding the influence of macroinvertebrates on ecosystem function often relies on experimental defaunation with methods that remove fauna through minimal sample disturbance. Defaunation is challenging and can lead to confounding effects and/or loss of empirical information when unsuccessful. We evaluated the ability of a deoxygenation treatment to remove macroinvertebrates from sediment cores collected in 2 regions of a microtidal estuary. Only 1 of 16 cores was fully defaunated following 3 deoxygenation cycles. To counteract confounding effects of partial defaunation, we quantified the biomass remaining in each core and used these data as a covariate in statistical models. The unremoved biomass had, in some cases, significant effects on alkalinity fluxes, with positive linear relationships evident, and net phosphate fluxes. The community in the upper estuary that regularly experiences hypoxia exhibited stronger sediment emergence responses (82–100 %). The remaining fauna were spread equally among annelids, molluscs and arthropods in abundance, although arthropods dominated the biomass. In contrast, fewer macroinvertebrates emerged from sediments from the lower estuary (47–89 %), with most of the remaining biomass and abundance being annelids and molluscs. These findings suggest that estuarine taxa have divergent responses to hypoxia and that regional communities are variably prone to eradication of sensitive taxa. Our study shows how the use of defaunation by deoxygenation can create systematic bias, particularly when comparing areas with disparate *in situ* oxygen regimes, and provides a way to quantitatively account for partial defaunation without sacrificing statistical power or using overly destructive methods.

KEY WORDS: Macrofauna removal · Benthic invertebrates · Coastal sediments · Sediment incubations · Behavioural adaptation · Sediment–water interface · Anoxia treatment

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1. INTRODUCTION

Defaunation is a crucial part of the experimental design in biodiversity–ecosystem function studies that determine the risks of anthropogenically driven bio-

diversity loss to ecosystem function (e.g. Emmerson et al. 2001, Norling et al. 2007). The process removes targeted invertebrates from select samples to create an uninhabited set of control samples, allowing faunal effects on ecosystem processes to be assessed.

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However, no standard method is used at present to defaunate sediments (e.g. sieving, freezing or chemical application; Tolhurst et al. 2012), and little is known about the efficacy of defaunation methods and how they might affect the research question(s) being addressed.

There are some recognised trade-offs for each defaunation method. For example, sieving or mixing of sediment followed by freezing are effective defaunation approaches, yet they do so at the expense of the sediment structure and therefore change the distribution of pore-water solutes and organic matter (Porter et al. 2006, Tolhurst et al. 2012). Defaunation methods that destroy sediment structure also alter key microbial communities (e.g. microphytobenthos and nitrifiers) and rates of their metabolic processes (Tolhurst et al. 2012, Bartoli et al. 2020). In comparison, deoxygenation treatments via dinitrogen (N_2) gas purging reduce oxygen (O_2) concentrations in the water column, creating anoxic conditions ($O_2 < 0.2 \text{ mg l}^{-1}$) that cause macroinvertebrates ($\geq 500 \mu\text{m}$ in size) to emerge, allowing their removal (Andersen & Kristensen 1988). This method is advantageous as it maintains the physical structure of the sediment that is a crucial factor in studies assessing biogeochemical effects of invertebrate communities. Deoxygenation treatments of acute exposure to low O_2 conditions can vary in duration from hours (e.g. Andersen & Kristensen 1988, Hansen & Kristensen 1997) to days (e.g. Camillini et al. 2019). Of the few defaunation evaluation studies published, only Stocum & Plante (2006) showed that prolonged deoxygenation treatments (i.e. 12 d) can alter the abundance of bacteria without causing major shifts in bacterial species composition as observed following sieving or freezing.

Defaunation methods rarely remove all of the invertebrates present (Stocum & Plante 2006). In previous applications of deoxygenation treatments, unremoved macroinvertebrates were assumed to have little functional effect and were not accounted for quantitatively (Table S1 in the Supplement at www.int-res.com/articles/suppl/m701p017_supp.pdf). However, the remaining macroinvertebrates may be active, thereby confounding the observed species effects, while the unremoved invertebrate necromass can act as labile carbon, influencing solute fluxes (Hansen & Blackburn 1991, Benbow et al. 2020). Moreover, various benthic macroinvertebrate communities may respond differently to deoxygenation treatments given species-specific physiological and behavioural adaptations to low O_2 conditions (e.g. Riedel et al. 2014), and affinities of certain benthic assemblages to particular sediment conditions in estuaries

(e.g. Rosenberg et al. 2004, Tyler et al. 2009). In this study, we assessed the efficacy of a deoxygenation defaunation treatment on benthic macroinvertebrate communities in sediment from 2 regions of a microtidal estuary that periodically experiences hypoxia (Valesini et al. 2019). The results were used to develop a statistical testing framework to adjust for the impact of partial defaunation on benthic biogeochemistry.

2. MATERIALS AND METHODS

2.1. Study background

Sampling was conducted in the microtidal Peel-Harvey Estuary located 90 km south of Perth, Western Australia, during spring (September–October 2018) after most of the annual winter rainfall had occurred (see Valesini et al. 2019 for site information). Sediment cores (9.5 cm internal diameter, 50 cm length, $N = 32$) of good ($n = 8$) and fair ($n = 8$) sediment condition, and of good ($n = 6$) and poor ($n = 10$) sediment condition, were collected from the lower (Harvey, $n = 16$) and upper (Murray River, $n = 16$) estuary, respectively. Sediment condition was determined using a rapid assessment protocol developed for the estuary based on scores related to sediment texture, colour and odour (Hallett et al. 2019). Good sediments are usually highly oxygenated, coarse to fine sands, while poor sediments are organic-rich silts and clays with low O_2 content.

To assess the effects of macroinvertebrate communities on ecosystem processes, half of the cores were defaunated to create control cores without macroinvertebrates (see Section 2.2). Both defaunated and faunated cores were then uncapped (with a $250 \mu\text{m}$ mesh placed on faunated cores to prevent faunal movement) and pre-incubated for 12 h in a 150 l incubator, allowing hydrological conditions to stabilise. All cores were then incubated with sealed tops for 12 h, during which a Hach HQ30d LDO probe was used to measure dissolved O_2 concentrations, and surface water samples were collected 5 times to determine concentrations of dissolved inorganic carbon (DIC), alkalinity, phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrate and nitrite (NO_x), dissolved organic nitrogen (DON) and N_2 gas. Dark, light and net fluxes ($\mu\text{mol m}^{-2} \text{ h}^{-1}$) across the sediment–water interface were calculated for each variable using linear regressions as a function of solute concentrations over time, sediment surface area and volume of overlying water, adjusting for water sample collection.

For more details on water sample processing and flux calculation, see Cronin-O'Reilly et al. (2022, this volume).

2.2. Defaunation

Defaunation by deoxygenation based on the method of Andersen & Kristensen (1988) was used, as it enables faunal removal whilst maintaining the sediment structure. The method was repeated 3 times to target stress-tolerant estuarine macroinvertebrate species. A preliminary trial revealed that some species (e.g. *Capitella capitata*) survived and remained buried in the sediment after a single deoxygenation.

Each deoxygenation cycle consisted of 4 steps (Fig. 1). Firstly, the overlying water in cores was purged with N₂ gas for 2 h to reduce O₂ concentrations. Secondly, cores were capped with no headspace for 12 to 20 h to maintain low O₂ conditions and allow benthic macroinvertebrates time to surface in search of available O₂. The final capping time was set at 12 h to allow for a sufficient pre-incubation period. Thirdly, the cap was removed, and overlying water and top sediment layer (<5 mm) were siphoned through a 500 µm sieve to remove macroinvertebrates, which included a small amount of sediment (<10 g, ~0.7 % over entire defaunation process). Removal of surfaced invertebrates was necessary to prevent the addition of labile carbon (i.e. invertebrate necromass) to the sediment–water interface, which can drive ecosystem processes (Benbow et al. 2020). Siphoned water, including the finer sediment that passed through the sieve, was placed back into each core while trying to avoid sediment disturbance. The above siphoning procedure was also performed on faunated cores to replicate the same disturbance to these cores, yet no sieve fraction was

removed. Fourthly, the replaced surface water was aerated temporarily (<2 h) to establish oxic conditions for surficial aerobic microorganisms (e.g. nitrifiers) that may have been disturbed during low O₂ conditions. A Hach HQ30d LDO probe was used to measure O₂ concentrations in select cores during each deoxygenation cycle.

2.3. Post-defaunation analysis

Both faunated and defaunated cores were fully sieved post-incubation using a 500 µm sieve. The residue from each core and the sieve fractions removed during defaunation were fixed using a 4 % formalin solution and subsequently transferred to 70 % ethanol. Benthic macroinvertebrates were extracted, identified and counted, with taxon biomass measured using blotted wet weights (±0.1 mg). The proportions of total abundance and biomass removed and remaining in each defaunated core were quantified after each deoxygenation cycle to evaluate defaunation efficacy. Taxon biomasses were averaged across replicate good, fair and poor sediments for each deoxygenation cycle and for what remained in the cores.

The following analyses were performed using PRIMER v7 (Clarke & Gorley 2015) and PERMANOVA+ (Anderson et al. 2008). Shade plots were created from the averaged taxon biomasses, with taxa constrained using index of association group-average cluster dendrograms and significantly ($p \leq 0.05$) distinct taxon aggregates determined by a similarity profile test (Clarke & Gorley 2015). To test the extent to which partial defaunation impacted the measured solute fluxes and to account for any such effects (Cronin-O'Reilly et al. 2022), the unremoved invertebrate biomass (g) from defaunated cores was

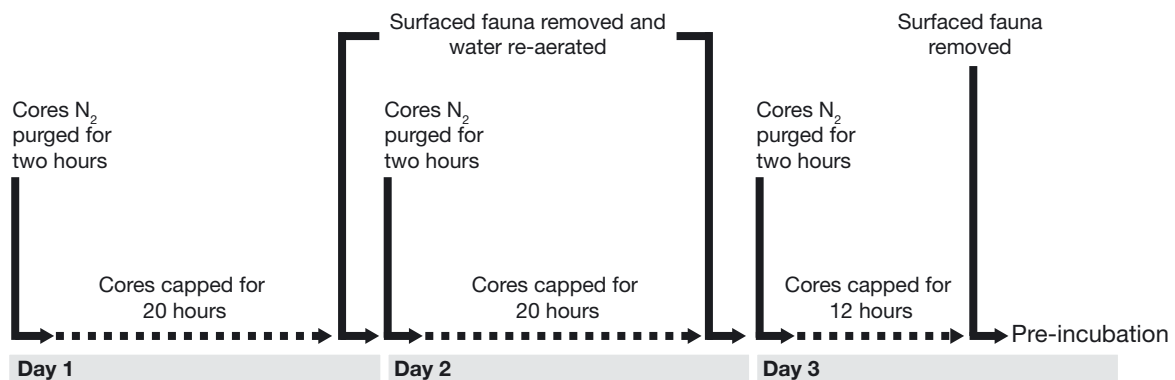


Fig. 1. Process of the defaunation by deoxygenation method, showing the main steps taken and their duration

used to quantify a covariate that could be added to permutational analysis of variance (PERMANOVA; Anderson 2001) models, which assessed the effects of sediment condition and the community on solute fluxes. Biomass (blotted wet weight, g) was used to characterise the covariate, in contrast to abundance, as invertebrate biomass is a leading predictor of macroinvertebrate functional effects (e.g. Emerson et al. 2001), recognising that this biomass would have included shells of small bivalves (e.g. *Arthritica semen*). Remaining invertebrate necromass may account for a potential addition of labile carbon to the cores, which can drive solute fluxes (Benbow et al. 2020). When a covariate is included in PERMANOVA, the factors must be added sequentially (Type 1 SS) as repeated tests with the order-of-fit of factors rotated. Where a significant ($p \leq 0.05$) covariate effect on a flux occurred, linear regressions assessed the relationship between the 2 variables. For community composition analyses (shade plots with clusters), biomass was fourth-root transformed yet remained untransformed when included as a covariate in PERMANOVA models to prevent reducing covariate variability that may be attributed to moderate flux variations.

3. RESULTS

3.1. Defaunation efficacy

After N_2 purging and capping, O_2 concentrations in the overlying water of deoxygenated cores ranged from 0.1 to 2.7 mg l⁻¹ (average: 1.1 mg l⁻¹, $n = 11$). Of the 16 cores subjected to defaunation, only 1 (Poor 1) from the upper estuary had all of its macroinvertebrates removed. Otherwise, there were no obvious differences in method efficacy between sediments of varying condition (Fig. 2). Defaunation was more effective in cores from the upper estuary, with 82–100% and 67–100% of the total abundance and biomass removed, respectively, whereas 47–89% and 25–95%, respectively, were removed from cores from the lower estuary. Irrespective of sediment condition, consecutive deoxygenation cycles resulted in continued biomass removal from upper estuary cores, whereas varying proportions were initially removed from lower estuary cores, with limited consecutive removal (Fig. 2). Shade plots of taxa consecutively removed showed a trend in cores from the lower estuary, with arthropods removed first (e.g. *Grandidierella propodentata*) followed by molluscs (e.g. *Spisula*

trigonella), with annelids remaining (e.g. *Prionospio cirrifer*; Fig. 3a). In upper estuary cores, taxa from various phyla were removed (e.g. *Dipolydora socialis*, *G. propodentata*) and remained (e.g. *C. capitata*, Chironominae spp., Synopiidae aff. sp. 1; Fig. 3b). For lower estuary cores, most remaining biomass belonged to annelids (81%) and molluscs (19%), while little arthropod and nematode biomass remained (<1%). The same trends were reflected in the number of individuals remaining (69, 25, 4 and 2%, respectively). Remaining biomass in upper estuary cores was dominated by arthropods (49%), while the remaining individuals were almost equally spread among the arthropods (33%), molluscs (29%) and annelids (38%). Taxon biomasses and abundances (totals and proportions) that remained in defaunated cores are provided in Table S2.

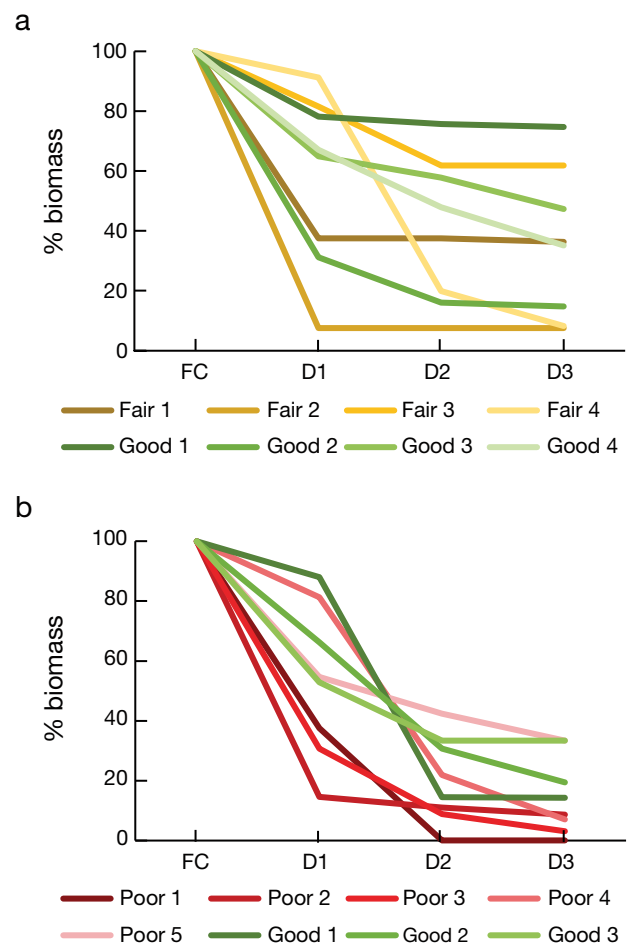


Fig. 2. Community biomass (%) remaining after consecutive deoxygenation cycles (D1–D3) from the full community (FC) in each replicate core (1–5) of sediment conditions from the (a) lower and (b) upper Peel-Harvey Estuary, Western Australia

3.2. Impact of defaunation efficacy on biogeochemistry

Of the 24 different solute fluxes tested (Table S3), there were significant covariate (unremoved biomass) effects on light ($p = 0.027$ – 0.034) and net ($p = 0.034$ – 0.036) alkalinity fluxes in lower estuary cores, and on net PO_4^{3-} fluxes in upper estuary cores ($p = 0.027$ – 0.035 ; Table 1). The covariate had a significant positive relationship with light ($R^2 = 0.30$, $p = 0.03$) and net ($R^2 = 0.33$, $p = 0.02$) alkalinity fluxes (Fig. S1).

4. DISCUSSION

We assessed the efficacy of a defaunation by deoxygenation treatment (modified version of Andersen & Kristensen 1988) and its impact on benthic biogeochemistry. The defaunation method did not completely remove macroinvertebrates from sediment cores, with the unremoved biomass significantly influencing alkalinity and PO_4^{3-} fluxes, indicating the confounding effect that partial defaunation may have on fluxes of some solutes.

The defaunation method induced hypoxia rather than the anoxia that was previously assumed despite an increased N_2 purging duration, with O_2 concentrations averaging 1.1 mg l^{-1} . Previous studies employing this method did not report O_2 levels, so it is difficult to say how representative our findings are. It is possible that measured values here were affected by the fact that we did not ensure an air-tight seal around the measurement probe but rather placed the probe (\varnothing : 14 mm) into a sampling port (\varnothing : 15 mm) in the lid of the cores, so the amount of O_2 within the cores post treatment may have been overestimated.

The surprisingly variable success of defaunation of samples from different regions of the estuary suggests divergent deoxygenation responses of benthic taxa. Deoxygenation resulting in anoxia or hypoxia is a frequent and persistent environmental disturbance in microtidal estuaries of south-western Australia, including the upper reaches of the Peel-Harvey Estu-

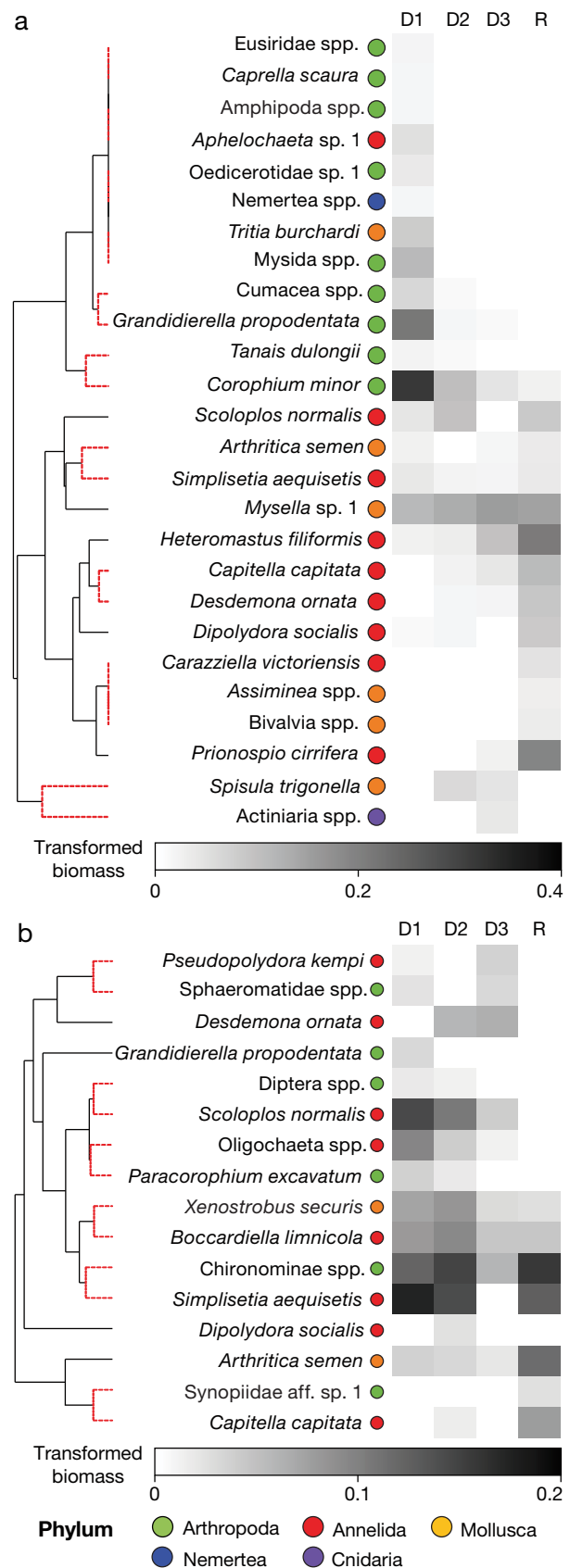


Fig. 3. Average taxon biomass (fourth-root transformed) removed following deoxygenation cycles (D1–D3) and the biomass of remaining taxa (R) across cores from the (a) lower and (b) upper Peel-Harvey Estuary. Grey scale illustrates variation in biomass from highest (black) to lowest (i.e. absent, white). Benthic taxa are coded by phylum and clustered into significantly distinct (black lines) and non-distinct (red dashed lines) taxon aggregates

Table 1. Results for 2-way sediment condition \times community PERMANOVA tests of differing orders-of-fit on solute fluxes that had significant covariate (i.e. unremoved biomass) effects in the lower and upper Peel-Harvey Estuary, which include the mean squares (MS), pseudo- F ratio, significance level (p) and components of variation (COV) for each test. **Bold** font indicates statistically significant results ($p \leq 0.05$)

Estuary region	Flux	Factor	df	MS	Pseudo- F	p	COV
Lower	Light alkalinity	<u>Order-of-fit 1</u>					
		Covariate	1	1 593 500	7.133	0.027	292.63
		Sediment condition	1	988 540	4.425	0.049	355.39
		Community	1	359 620	1.610	0.240	168.85
		Condition \times Community	1	7326	0.033	0.853	–341.48
		Residual	11	223 410			472.66
		<u>Order-of-fit 2</u>					
		Covariate	1	1 593 500	7.133	0.034	292.63
		Community	1	33 494	0.150	0.706	–184.81
		Sediment condition	1	1 314 700	5.885	0.033	457.85
		Condition \times Community	1	7326	0.033	0.856	–341.48
		Residual	11	223 410			472.66
	Net alkalinity	<u>Order-of-fit 1</u>					
		Covariate	1	113 530	6.037	0.036	76.94
		Sediment condition	1	401	0.021	0.903	–55.12
		Community	1	250	0.013	0.916	–62.32
		Condition \times Community	1	23 819	1.267	0.276	52.01
		Residual	11	18 807			137.14
		<u>Order-of-fit 2</u>					
		Covariate	1	113 530	6.037	0.034	76.94
		Community	1	51	0.003	0.951	–58.08
		Sediment condition	1	601	0.032	0.876	–59.14
		Condition \times Community	1	23 819	1.267	0.279	52.01
		Residual	11	18 807			137.14
Upper	Net PO ₄ ^{3–}	<u>Order-of-fit 1</u>					
		Covariate	1	0.70	5.809	0.027	0.20
		Sediment condition	1	0.61	5.073	0.033	0.32
		Community	1	1.26	10.48	0.006	0.51
		Condition \times Community	1	0.47	3.865	0.116	0.65
		Residual	10	0.12			0.35
		<u>Order-of-fit 2</u>					
		Covariate	1	0.70	5.809	0.035	0.20
		Community	1	0.56	4.624	0.031	0.29
		Sediment condition	1	1.32	10.928	0.009	0.53
		Condition \times Community	1	0.47	3.865	0.113	0.65
		Residual	10	0.12			0.35

ary (Tweedley et al. 2016a, Valesini et al. 2019), and in eutrophic estuaries globally (Howarth et al. 2011). *In situ*, emergence of macroinvertebrates from the sediment and relocation to oxygenated areas is a recognised behavioural adaptation to hypoxia (Riedel et al. 2014). Here, the proportions of macroinvertebrates removed from cores following sediment emergence does not equate directly to survival as some may have surfaced, tried to leave the sealed core and died. *In situ*, a proportion of these invertebrates may have been able to relocate and survive. There was continued macroinvertebrate emergence and removal from cores from the upper estuary where hypoxia

regularly occurs (Valesini et al. 2019), whereas macroinvertebrates in the lower estuary typically did not emerge after the initial deoxygenation treatment cycle preventing their removal. Perhaps low O₂ stress recognition by the community from the lower estuary is lacking, with little behavioural adaptation despite periodic development of anoxic sediment conditions in this region (Lukatelich & McComb 1989, Hallett et al. 2019). Alternatively, these species were more physiologically adapted to hypoxia so that they could endure burial for the duration of low O₂ conditions (Riedel et al. 2014). It is also possible that some macrofauna were kept alive from the temporary (<2 h) re-

aeration between deoxygenation cycles, which may have influenced their endurance to remain buried. As such, our findings more likely reflect the responses of invertebrates to short (<1 d), periodic durations of low O₂ stress rather than those sustained under prolonged hypoxia. Consideration should be given to avoid temporarily re-aerating cores during consecutive deoxygenation treatments. Overall, the non-random nature of defaunation efficacy between estuarine regions indicates that systematic bias can unintentionally be created in studies that use this method, particularly those comparing ecosystem function in areas of varying deoxygenation susceptibility.

The divergent community response to deoxygenation between regions was also reflected in the species composition of benthic macroinvertebrates consecutively removed. In the lower Peel-Harvey Estuary, arthropods (mainly crustaceans) were removed first, followed by molluscs and annelids, which comprised most of the taxa remaining in the sediment. This aligns with the general understanding of O₂ stress tolerances among benthic phyla (Warwick & Clarke 1993), with stress-sensitive arthropods emerging and dying first. This is congruent with the prolonged hypoxia effects observed in the nearby Swan-Canning Estuary, where most crustaceans were eradicated first, while some molluscs and annelids persevered (Tweedley et al. 2016a). In contrast, the mixture of benthic taxa that emerged from the upper Peel-Harvey Estuary sediments, with remaining individuals equally spread among arthropods, molluscs and annelids, may reflect selection of macroinvertebrate species to regional stressors. This stress-tolerant community remained largely unaltered by deoxygenation, while the community in the lower estuary appears prone to eradication of some benthic groups. However, because previous studies did not report either surface water O₂ levels following N₂ purges or the recovery of invertebrates following deoxygenation treatments, it is impossible to assess whether our findings represent a unique adaptation of local communities to low O₂ conditions or a ubiquitous artifact of this defaunation method. The observed differences between regions and taxa do suggest that the efficacy of the deoxygenation treatment depends on site history and community composition. This should be considered in future deoxygenation defaunation studies, which would benefit from having a standard set of reporting parameters (e.g. O₂ concentrations measured or biomass recovered post experiment) to quantify the efficacy of defaunation and enable comparisons among studies.

The overall effect of partial defaunation on ecosystem function may also be considered low, having affected only 3 of the 24 different types of solute fluxes investigated. This may suggest that the inefficiency of defaunation techniques, like the treatment applied here, may be largely negligible. However, it must be noted that the target of this evaluation is an underdeveloped, pioneering community that lacks larger bioturbating species (Tweedley et al. 2016b), potentially downplaying the issue. Future methods testing is recommended to determine the effects of having larger macroinvertebrates remain in sediment cores to substantiate the pervasiveness of these effects, or lack thereof. Currently, the differential impact of defaunation observed here highlights the importance of post-incubation analysis at the species level. This study provides a template for (1) the use of a deoxygenation treatment for defaunation with minimal physical disturbance, (2) quantification of method efficacy, and (3) accounting for confounding effects that partial defaunation may have on future work, emphasising the need to quantify method efficacy.

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