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Density-dependent responses of the brittlestar *Amphiura filiformis* to moderate hypoxia and consequences for nutrient fluxes

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ABSTRACT: Within coastal marine habitats, intense nutrient cycling and near-seabed primary production rates are strongly influenced by the transport and transformation of materials within the sediment and across the sediment–water interface. Through processes such as bioturbation and bio-irrigation, benthic infauna play a significant role in mediating this transport, and modify many chemical and physical reactions. However, coastal ecosystems are experiencing growing impacts from a number of environmental stresses, one of which is reduced levels of dissolved oxygen (DO), known as hypoxia. Hypoxic events in coastal areas are predicted to increase as global warming and human-induced eutrophication intensify, with predicted consequences for infaunal community diversity and ecosystem function. Using a mesocosm experiment, we investigated the effects of short-term, sub-lethal hypoxia (14 d, 3.59 mg O_2 l^{-1}) and organism density (500, 900, 1300, 1700 and 2100 ind. m^{-2}) on the bioturbation activity of the brittlestar *Amphiura filiformis.* Nutrient fluxes were measured as an important contribution to ecosystem function. Hypoxia resulted in reduced brittlestar activity (in terms of sediment surface bioturbation), increased efflux of ammonium and silicate and an increase in the ratio of NH_4^+:NO_x when brittlestar densities were high. No significant effects of hypoxia were detected on brittlestar burrow depth. Our results illustrate that population density plays a crucial role in exacerbating the effects of hypoxia, possibly due to greater biological oxygen demands and increased waste products as organism density increases. Consequently, during moderate reductions in DO, densely populated communities may actually be more vulnerable to hypoxic stress and exhibit greater shifts in ecosystem function than sparsely populated communities.

KEY WORDS: Low oxygen · Bioturbation · Invertebrate ecology · Benthic biogeochemistry · Population dynamics · Climate change · Eutrophication · Ecosystem processes

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INTRODUCTION

Anthropogenic activities are having wide-spread impacts on habitats and ecosystems, resulting in global declines in biodiversity (Butchart et al. 2010). Biodiversity loss, at both global and local scales, raises concerns that ecosystem functioning and the provision of goods and services cannot be maintained (Solan et al. 2004a, Riedel et al. 2014). One growing environmental stress affecting coastal ecosystems is a significant increase in the occurrence of low dissolved oxygen (DO) conditions, i.e. hypoxia. Hypoxia is now widely recognised as one of the key environmental stressors, and is predicted to increase in coastal areas

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as global warming and human-induced eutrophication intensify (Diaz & Rosenberg 2008, Vaquer-Sunyer & Duarte 2008, Howarth et al. 2011). Traditionally, conditions are defined as hypoxic when DO levels fall below 2.0 mg O_2 l^{-1}, as this threshold refers to the oxygen level below which fisheries collapse (Vaquer-Sunyer & Duarte 2008) and there is significant disturbance to benthic communities through organism mortality, extinction and migration (Diaz & Rosenberg 2008). However, ample experimental evidence exists to suggest that this DO threshold between normoxic conditions and hypoxia may be set too low for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel & Childress 2013). An organism's response to reduced DO is species-specific, and initially manifests through changes in that organism's behaviour and physiology, with migratory behaviour (for those that can) or mortality being the end-point (Grieshaber et al. 1994). Moderate reductions in DO to levels still far above the 'classic' threshold of 2.0 mg O_2 l^{-1} have been shown to affect organism growth, reproduction, locomotion, behaviour and feeding (summarised in Gray et al. 2002). These impacts at the organism level will likely also affect important processes that contribute to ecosystem functioning, yet the impact of reduced DO (i.e. at a level above 2.0 mg O_2 l^{-1}) on near-shore marine and estuarine communities and the processes they support is not well understood (Froehlich et al. 2015).

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

Benthic infauna are responsible for the biogenic mixing of the sediment, a process known as bioturbation, which directly or indirectly affects sediment matrices (Shull 2009, Kristensen et al. 2012). Through the creation of pits, mounds and burrows, sediment ingestion and excretion, as well as the bio-irrigation of subsurface burrows, benthic infauna play a significant role in mediating the rate and depth of many chemical and physical reactions. This ultimately drives carbon and nitrogen cycling, establishes O_2, pH and redox gradients, determines sediment porosity and permeability and sets microbial activity rates and diversity (Herbert 1999, Shull 2009, Laverock et al. 2010, Bertics et al. 2013).

The response of any individual infaunal organism to hypoxia is highly variable and dependent on the severity and duration of the hypoxic event (Spicer 2016). In addition, species-specific traits such as O_2 tolerance, mobility and the behavioural or physiological adaptations that different species express can lead to a variety of impacts on community structure and diversity (Rosenberg et al. 1991, 2001, Vaquer-Sunyer & Duarte 2008). Ultimately, severe and prolonged hypoxia can lead to extreme responses in benthic communities, reducing biodiversity through forced migration, increased vulnerability to predation, reduction of suitable habitats and excessive physiological stress leading to mortality (Rosenberg 2001, Rabalais et al. 2002). However, before these extreme reactions are observed, responses to hypoxia are often initially expressed through changes in organism physiology and behaviour (Grieshaber et al. 1994). Documented changes include reduced growth in oyster larvae and juveniles (Baker & Mann 1992), delayed embryonic development in gastropods (Chan et al. 2008) and reduced metabolic rates and oocyte growth in brittlestars (Calder-Potts et al. 2015). Behavioural responses, that alter biogenic activity, include elongated bivalve siphons, abandonment of burrows and reduced burrowing depths and activity of infauna (Sturdivant et al. 2012). Importantly, behavioural data may provide a link between individual response and population change, especially if the behaviour alters the structure and function of the community (Boyd et al. 2002).

Ecosystem engineers are defined as species that modify, maintain and create habitats and, through their actions, modulate the availability of resources to other species (Lawton 1994, O'Reilly et al. 2006). One such species, the brittlestar *Amphiura filiformis* (Müller, 1776), is an active and well-studied bioturbator (Solan & Kennedy 2002, Solan et al. 2004a, O'Reilly et al. 2006, Queirós et al. 2013, 2015). *A. filiformis* is primarily a suspension feeder that remains buried below the sediment surface and protrudes one or more arms into the water column. It actively undulates its arms and pumps its disc for respiratory gas exchange, burrow ventilation and irrigation, in addition to collection and expulsion of food and waste (Vopel et al. 2003, Calder-Potts et al. 2015). *A. filiformis* is also a dominant species in many coastal

and shelf areas of the NE Atlantic, and its effects on sediment properties may explain its structuring effect in infauna communities (Queirós et al. 2006).

The effects of traditionally defined hypoxia on the biology of *A. filiformis* are relatively well documented. Hypoxic exposure reduces *A. filiformis* disc diameter growth (Hylland et al. 1996), reduces arm regeneration rates and delays spawning (Nilsson & Sk ld 1996, Nilsson 1999), reduces metabolic rates, reduces oocyte growth and delays reproductive development (Calder-Potts et al. 2015). However, research that examines the links between the biological, physiological and behavioural consequences of more moderate reductions in DO and potential ecosystem effects are limited.

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

Consequently, we conducted a mesocosm experiment in which *A. filiformis* were exposed to 14 d of moderate hypoxia in order to address the following questions: (1) Does exposure to moderate hypoxia affect *A. filiformis* behaviour, measured in terms of bioturbation activity? (2) Do any changes in *A. filiformis* behaviour affect nutrient fluxes in the sediment, as a proxy for the ability to maintain ecosystem function? (3) What role does population density play in maintaining ecosystem function? (4) If density is a significant factor, do populations with a higher density of individuals display greater resilience to hypoxic stress than populations with lower densities, possibly as a consequence of greater bioturbation activities and thus increased porewater exchange? Bioturbation activity was measured using 2-dimensional (2D) imaging and particle tracing methods (Mahaut & Graf 1987, Gilbert et al. 2003, Solan et al. 2004b). Tracer data were then used to quantify 2 different parameters: maximum bioturbation depth and percentage of the sediment surface reworked. Nutrient flux data were collected in triplicate from each experimental aquarium.

MATERIALS AND METHODS

The data presented here were generated from the mesocosm experiment documented in Calder-Potts et al. (2015); consequently, the methods presented here summarise information relating to sediment and animal collection procedures, experimental setup and monitoring and seawater O_2 manipulation methods. For full details relating to the experimental setup refer to Calder-Potts et al. (2015). Analytical methods for bioturbation and nutrient flux measurement are not covered in Calder-Potts et al. (2015) and are therefore described in detail below.

Sediment collection

On 25 May 2012, sediment was collected at a water depth of ~10 m from an area of 'very fine sand' with an overlaying surface layer of 'clay/silt' in Cawsand Bay, Plymouth, UK (50° 21.998' N, 4° 7.961' W), using a 0.1 m^2 US-NL box-corer. Once retrieved, the surface layers of sediment (top 10 to 15 cm) were placed into bags and transported to the Plymouth Marine Laboratory (PML) mesocosm facility where sediment was sieved (2 mm) in filtered seawater (10 µm diam. Hydrex filters). A total of 50 experimental glass aquaria (L × W × H = 20 × 5 × 30 cm) were filled with the sieved sediment to a depth of 19 ± 1 cm, leaving 11 cm of overlying water. Each aquarium was connected to a flow-through seawater system that delivered aerated, twice-filtered (10 and 1 µm diam. Hydrex filters) seawater from a 450 l header tank via a peristaltic pump (323E; Watson Marlow) set at a rate of 20 ± 0.5 ml min^{-1}. One water inlet pipe was connected to each aquarium 1.5 ± 0.5 cm above the sediment surface, which did not cause sediment re-suspension. Each aquarium was completely filled with seawater, resulting in the outflow of water being a steady overflow that was caught by an exterior holding tank and drained away. The average water volume held within each aquarium was 1100 cm^3, resulting in an approximate complete water renewal rate every 55 min. Water flow rates across the sediment surface were not measured, but did not cause any visible disturbance to the sediment surface. Aquaria were kept under these conditions for a further 21 d, to allow the sediment to settle and for biogeochemical processes and gradients to re-establish. Aquaria containing sediment that showed any visual signs of bioturbation during this time were removed from the experiment.

Brittlestar collection

Individuals of *Amphiura filiformis* were collected (12 to 14 June 2012) from the same site as the sediment. Specimens were carefully sorted by hand to avoid damage (such as arm loss) and gently washed with fresh seawater. Only individuals with a disc diameter >4 mm (based on the size at which adults reach sexual maturity; O'Connor et al. 1983) plus 5 intact arms were placed into containers (vol. = 250 ml, 3 ind. container^{-1}) containing freshly collected seawater and transported to PML within 3 h of collection. There were no mortalities recorded during the experimental period. On each sampling day (T0, T6, T10 or T14), brittlestars were recovered from the sampled aquaria to supply material for physiological and histological analyses as detailed in Calder-Potts et al. (2015).

Experimental design and setup

Of the 50 sediment aquaria prepared, 42 were selected for use in the experiment. Aquaria were haphazardly assigned to 1 of 2 O_2 levels (normoxia: 8.09 ± 0.06 mg l^{-1} or hypoxia: 3.59 ± 0.04 mg l^{-1}) and 1 of 6 organism density levels (0, 5, 9, 13, 17 or 21 ind. aquaria^{-1}, equating to 0, 500, 900, 1300, 1700 and 2100 ind. m^{-2} respectively). All brittlestars were introduced to the aquaria 5 d prior to time point T0 for a 5 d settling period under normoxic conditions. Time point T0 marked the start of the experiment and the beginning of hypoxic exposure. Six aquaria (one from each density treatment), previously haphazardly selected due to the addition of luminophore tracers 5 d prior, were removed and sampled to create 'pre-exposure T0' data. After 6, 10 and 14 d (hereafter known as T6, T10 and T14 time intervals), a further 6 normoxic aquaria and 6 hypoxic aquaria, again including all density levels, had completed their bioturbation and nutrient sampling regimes (as detailed below) and were removed from the experiment to allow for further analysis of brittlestar biology as detailed in Calder-Potts et al. (2015).

Seawater manipulations

DO levels were reduced using a computerised control system (Walchem Webmaster Series), which regulated the addition of O_2-free nitrogen gas to large header tanks (450 l) in order to purge the water of oxygen. Modified water from these header tanks was then supplied to the experimental aquaria via a peristaltic pump. The seawater within the header tanks and the experimental aquaria were monitored daily for DO, temperature, salinity and pH using a multiprobe (9828; Hanna Instruments). Within the normoxic experimental aquaria the mean (±95% CI) DO of seawater was recorded as 8.09 ± 0.06, and the seawater in the hypoxic aquaria DO level was 3.59 ± 0.04. Experimental seawater conditions are documented in full in Calder-Potts et al. (2015). Ample experimental evidence exists to challenge the traditional hypoxic level of 2.0 mg O_2 l^{-1} as being insufficient to detect the onset of hypoxia impacts for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel & Childress 2013). Consequently, in this experiment a higher threshold of DO was used to examine if any alterations in behaviour and functionality may occur.

Due to the large differences in brittlestar density within the aquaria, higher brittlestar density treatments did have a slightly lower DO caused by greater levels of organism respiration. However, the differences were comparatively small and unlikely to have caused significant impacts to brittlestars. Within the normoxic aquaria, mean (±95% CI) seawater DO within the lowest density treatment (5 ind. aquaria^{-1}) was 8.22 ± 0.12 mg O_2 l^{-1}, whilst in the highest density treatment (21 ind. aquaria^{-1}), DO was 7.92 ± 0.11 mg O_2 l^{-1}. This is a difference in the means of 0.3 mg O_2 l^{-1}. Within the hypoxic aquaria, average DO within the lowest density treatment (5 ind. aquaria^{-1}) was 3.78 ± 0.04 mg O_2 l^{-1}, whilst in the highest density treatment (21 ind. aquaria^{-1}), DO was 3.51 ± 0.08 mg O_2 l^{-1}. This is a difference in the means of 0.27 mg O_2 l^{-1}.

Acquisition of bioturbation data

Image capture

Bioturbation data were acquired using a luminophore tracer technique (Mahaut & Graf 1987) and 2D imaging under UV light to monitor the movement of luminophores over time. Luminophore particles are naturally occurring quartz material coated with a fluorescent dye. The luminophores (Partrac Ltd.) used were chosen to match the sediment granulometry of the collection site (Cawsand) and had a median grain size of 60 µm. Luminophores (0.2 g cm^{-2} = 20 g aquaria^{-1}) were added to the experimental aquaria 5 d in advance of their allocated sampling day (T0, T6, T10 or T14), resulting in a staggered addition of luminophores across the experimental period. Lumi-

nophores were added to each aquarium by evenly pouring them into the overlying water. Settlement of luminophores took approximately 1 h, during which time water circulation to the aquaria was ceased.

Each aquarium was then photographed once every 24 ± 1 h for a total of 6 d (6 images aquarium^{-1}). To do this, aquaria were individually removed from the experimental system and carefully placed at one end of a custom-made black box which housed at the other end (and at a fixed focal distance from the aquarium) a digital SLR camera (Canon EOS 1000D, 10.1 MP). Within the box the aquaria were illuminated by a 8 W UV light (see Schiffers et al. 2011, their supporting material Fig. S1). A custom-made frame was fixed in the camera box that held the aquaria in the exact same position each time a photograph was taken. The camera was set for an exposure of 10 s, f = 5.6, ISO = 200 (pixel size: 0.00004 cm^2) and was controlled remotely via a PC using the software GB Timelapse, v.3.6.1 (Granite Bay Software). The UV light within the photo box was necessary for luminophore excitation, and produced enough light to distinguish the sediment–water profile. Images were captured in RGB format and saved using a JPEG compression (size: 3888 × 2592 pixels). After each photograph session, aquaria were returned to the experimental system and re-connected to their respective flow-through water treatment. The 6th and final photograph for each aquaria occurred on a sampling day (T0, T6, T10 or T14).

Image preparation and data extraction

Using ImageJ v.1.4.3 software, all photographs were cropped to a size of 2996 × 2200 pixels, which removed the edges of the glass aquaria. Onto each image, the water–sediment interface was drawn manually. This line represented the initial reference used to calculate luminophore penetration depths. Luminophore positions in each image were quantified using custom-made, semi-automated algorithms for R v.2.15.1 (R Development Core Team 2012, Queirós et al. 2015) and Image J v.1.4.3 modified from Queirós (2010). The algorithm acts as an automated standardised method for image segmentation (threshold analysis), which accounts for potential changes in the apparent brightness of luminophore pixels as particle mixing occurs during the aquarium incubations. In summary, each image was transformed to a binary matrix, where luminophore pixels were assigned the value of 1 and sediment pixels a value of 0. Image data were automatically compiled as a count of luminophores per pixel layer (i.e. depth) within each image, with sediment depth calculated relative to the linearised sediment–water interface. Luminophores per pixel layer were then summed, creating a row total which was used to re-construct vertical profiles of luminophores within the sediment from each photograph, in addition to profile sequences for the set of 6 images.

Quantifying bioturbation

The luminophore tracer profiles extracted from each image were used to estimate 2 aspects of bioturbation. Firstly, MLD was used as a proxy for maximum bioturbation depth, and estimated by determining the deepest image pixel row containing at least 5 luminophore pixels. Secondly, bioturbation activity was estimated by calculating the proportion of sediment surface reworked (SSR), measured as 100% minus the percentage of tracer left in the surficial layers (the first 1 cm of sediment) at the end of each time point, i.e. from the 6th and final image (Maire et al. 2006).

Nutrient analysis

Nutrient samples were taken from each aquarium on their designated sampling day (T6, T10 or T14). Within each aquarium, water overlying the sediment and water from the inflow pipe connected to the header tanks were sampled separately, both in triplicate. Each individual sample (50 ml) was filtered through a 47 mm ø GF/F filter and stored in an acid-washed Nalgene bottle. In total, 150 ml (3 × 50 ml samples) of water was collected for analysis from each aquarium and a further 150 ml was collected from each water inflow pipe. This created 3 paired samples which were used to calculate nutrient fluxes within each aquarium. The samples collected from the overlying water within each aquarium were all carefully taken at the same height above the sediment surface (1 ± 0.5 cm), but at 3 different points across the length of the aquarium (5, 10 and 15 cm). Samples were stored and frozen at −20°C until analysed using a segmented flow nutrient auto-analyser (AAIII; SEAL Analytical). Standard methods were used to determine ammonium (NH_4^+), nitrate NO_3^-, nitrite NO_2^-, silicate (SiO_4^{4-}) and phosphate (PO_4^{3-}) concentrations (Brewer & Riley 1965, Grasshoff 1976, Mantoura & Woodward 1983, Kirkwood 1989). Nutrient fluxes were calculated using

Eq. (1) (from Widdicombe & Needham 2007). Fluxes across the sediment–water interface provide an estimation of the net change of nutrient *x* within the experimental aquaria, and give an indication of the alterations in biogeochemical cycling caused by a reduction in dissolved oxygen concentrations and also by changes in brittlestar activities and abundance.

$$F_x = ((C_i - C_o) \times Q)/A \quad (1)$$

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

Statistical analyses

Statistical analyses were carried out using the software package Minitab v.17.0. The Shapiro-Wilk test for normality and Levene's test for homogeneity of variance were completed on each parameter measured. When necessary, a square root or $\log_{10}$ + 1 transformation was applied. NH_4^+ flux data were the exception and could only be normalised using a 'sine' transformation. Each parameter was analysed using a general linear model (GLM) analysis of variance (ANOVA), with 'water treatment' (normoxic or hypoxic), 'brittlestar density' (0, 5, 9, 13, 17 and 21 ind. $aquaria^{-1}$), and 'experimental time' (0, 6, 10 and 14 d) as the factors. Prior to analyses of nutrient flux data within the experimental aquaria, nutrient measurements originating from the header tanks were tested for 'tank effects'. Header tank nutrient data could not be normalised using any transformation and was analysed using the non-parametric Mann-Whitney *U* rank sum test.

Treatments containing no *A. filiformis* (i.e. a brittlestar density of zero) were excluded from analyses on maximum luminophore depths (MLD) and % SSR because, as expected, luminophores were not disturbed or bioturbated within these treatments. By excluding the zero density treatment, MLD and % SSR relationships with brittlestar density were not artificially strengthened or skewed due to the addition of a zero activity data point because no brittlestars were present. The zero brittlestar density treatments were included in the nutrient flux analyses because they provide insight into background nutrient cycling rates in the absence of *A. filiformis*.

RESULTS

Bioturbation activity

MLD. The mean (±95% CI) MLD measured across all aquaria (excluding the zero density treatment) was 7.99 ± 0.57 cm. Analyses revealed no significant effects of the experimental parameters on MLD (Table 1a).

Percent SSR. In both the normoxic and hypoxic water treatments, % SSR was significantly greater as brittlestar density increased (Fig. 1, Table 1b). There was also a significant effect of experimental time, whereby on average, in both water treatments, less sediment surface was reworked the longer the brittlestars remained in the experimental system (Table 1b). For example, the average % SSR across both water treatments and all density treatments at T0 was 41.67%, which decreased to 30.56% at T6, 28.02% at T10 and 22.49% at T14. In addition, the effect of brittlestar density on % SSR varied significantly according to the exposure to different oxygen regimes, as indicated by the presence of a significant interaction effect between water treatment and brittlestar density (Table 1b). For example, the largest

Table 1. General linear model ANOVA for (a) maximum luminophore depths (MLD) and (b) percentage of sediment surface reworked (% SSR). Adj. SS: adjusted sum of squares; Adj. MS: adjusted mean squares. **Bold**: significant at $p < 0.05$

Source	df	Adj. SS	Adj. MS	*F*	p
(a) MLD					
Water treatment	1	0.00	0.00	0.00	0.998
Density	4	14.36	3.59	1.37	0.277
Time	3	11.74	3.91	1.49	0.245
Water treatment × density	4	19.98	5.00	1.90	0.145
Error	22	57.71	2.62		
Total	34	110.38			
(b) % SSR					
Water treatment	1	16.8	16.83	0.16	0.692
Density	4	7578.8	1894.71	18.09	**<0.001**
Time	3	1228.8	409.59	3.91	**0.022**
Water treatment × density	4	1347.7	336.93	3.22	**0.032**
Error	22	2304.5	104.75		
Total	34	13034			

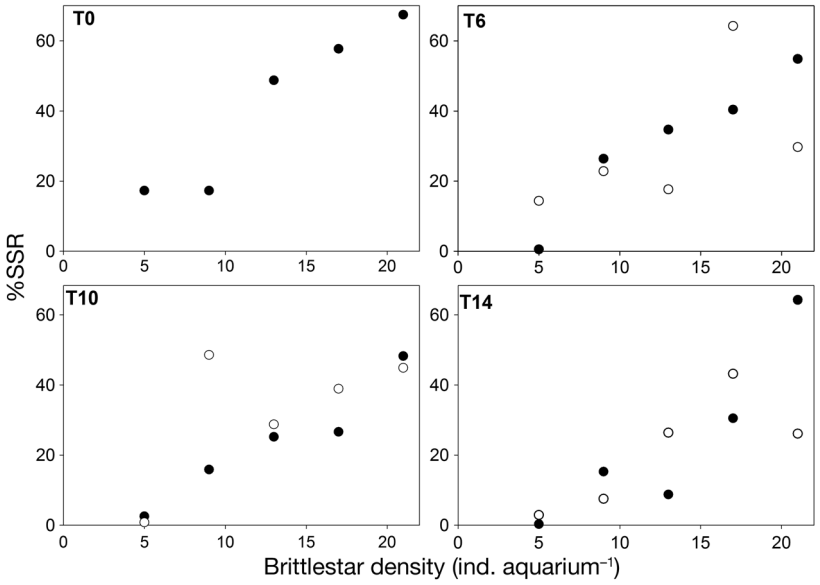

Fig. 1. Percentage of sediment surface reworked (% SSR) (top 1 cm only) against brittlestar density at time points T0, T6, T10 and T14. Points represent individual aquaria. (●) normoxia; (○) hypoxia

differences in % SSR between the normoxic and hypoxic aquaria occurred in the highest brittlestar density treatment (21 ind. aquaria^{-1}) at T6 and T14. At T6 within the normoxic aquaria % SSR = 54.80%, whilst in the hypoxic aquaria % SSR = 29.68%. At T14 within the normoxic aquaria % SSR = 64.16%, whilst in the hypoxic aquaria % SSR = 26.10%. There were no significant effects of water treatment in isolation and no interaction effects between water treatment and time (Table 1b).

Nutrients

Header tank effects. Analyses of nutrient measurements from the header tanks revealed that there were significant differences in nitrate and phosphate nutrient concentrations between the normoxic and hypoxic header tanks, despite the tanks receiving seawater from the same source (Table 2). Consequently, header tank nitrate and phosphate data were examined in greater detail.

Header tank nitrate. The differences in nitrate concentrations between the 2 header tanks started at T10 and increased with experimental time, with the largest differences occurring at T14. Nitrate concentration within the normoxic header tank at T10 was 6.46 ± 0.080 µM and the corresponding hypoxic nitrate concentration was 5.91 ± 0.059 µM, a decrease of 8.5% (Mann-Whitney U = 1.00, t = 494.00, $p < 0.001$, n = 18). At T14, nitrate concentration within the normoxic header tank had increased to 7.14 ± 0.12 µM, whilst nitrate concentrations in the hypoxic header was 4.71 ± 0.24 µM, a decrease of 34.0% compared to the normoxic tank (Mann-Whitney U = 0.00, t = 153.00, $p < 0.001$, n = 18.00).

Header tank phosphate. PO_4^{3-} concentrations between the normoxic and hypoxic header tanks remained closely matched until T14. At T14, PO_4^{3-} concentration in the normoxic header tank was 0.27 ± 0.015 µM, whilst concentrations in the hypoxic header tank were significantly lower at 0.17 ± 0.032 µM, a decrease of 37.0% (Mann-Whitney U = 35.00, t = 460.00, $p < 0.001$, n = 18).

Nitrate and nitrite fluxes in experimental aquaria. To investigate the effects of hypoxia, brittlestar density and time within the experimental aquaria, combined nitrate and nitrite measurements (hereafter known as NO_x) were examined. During the experiment, NO_x influx (from the overlying water into the sediment) predominantly occurred in both the normoxic and hypoxic aquaria (Fig. 2a–c). Analyses revealed that no significant effects were detected between water treatments, brittlestar density and experimental time; however, there was a significant interaction effect between water treatment and experimental time (Table 3a). This was due to the slight increase in NO_x flux into the sediment within the normoxic aquaria after 14 d experimental exposure (Fig. 2c).

NH_4^+ fluxes in experimental aquaria. In aquaria containing no brittlestars there were minimal amounts of NH_4^+ flux, but efflux of NH_4^+ consistently occurred in aquaria that contained brittlestars, irrespective of the different O_2 regimes (Fig. 2d–f). Analyses revealed that water treatment, brittlestar density, experimental time and their interactions did not significantly affect NH_4^+ flux (Table 3b). However, Fig. 2f indicates that NH_4^+ efflux at T14 had increased in the aquaria exposed to hypoxic seawater that contained brittlestar densities of 13, 17 and 21 ind. aquaria^{-1}. A subsequent GLM ANOVA was conducted on T14 NH_4^+ flux data from the high brittlestar density treatments (13, 17 and 21 ind. aquaria^{-1}). At T14, within the high brittlestar density treatments NH_4^+ efflux was significantly greater within the hypoxic aquaria compared to the normoxic aquaria (Fig. 2f, Table 3c).

Table 2. Mann-Whitney *U* rank sum test on header tank nutrient concentrations (µM). N = 54; MWU: Mann-Whitney *U* statistic. **Bold p-values** indicate significance at p < 0.05

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

Ratios of NH_4^+:NO_x. Concentrations (µM) of NH_4^+ and NO_x in the experimental aquaria are presented as ratios [NH_4^+:NO_x] (Fig. 2g–i). Comparing ratio concentrations of NH_4^+ and NO_x better conveys which sedimentary processes, such as nitrification, ammonification and denitrification, are favoured within the experimental aquaria (Fig. 2g–i). A higher ratio value indicates greater NH_4^+ concentrations, favouring processes that produce NH_4^+ such as nitrate ammonification, or processes that have decreased nitrate and ni-

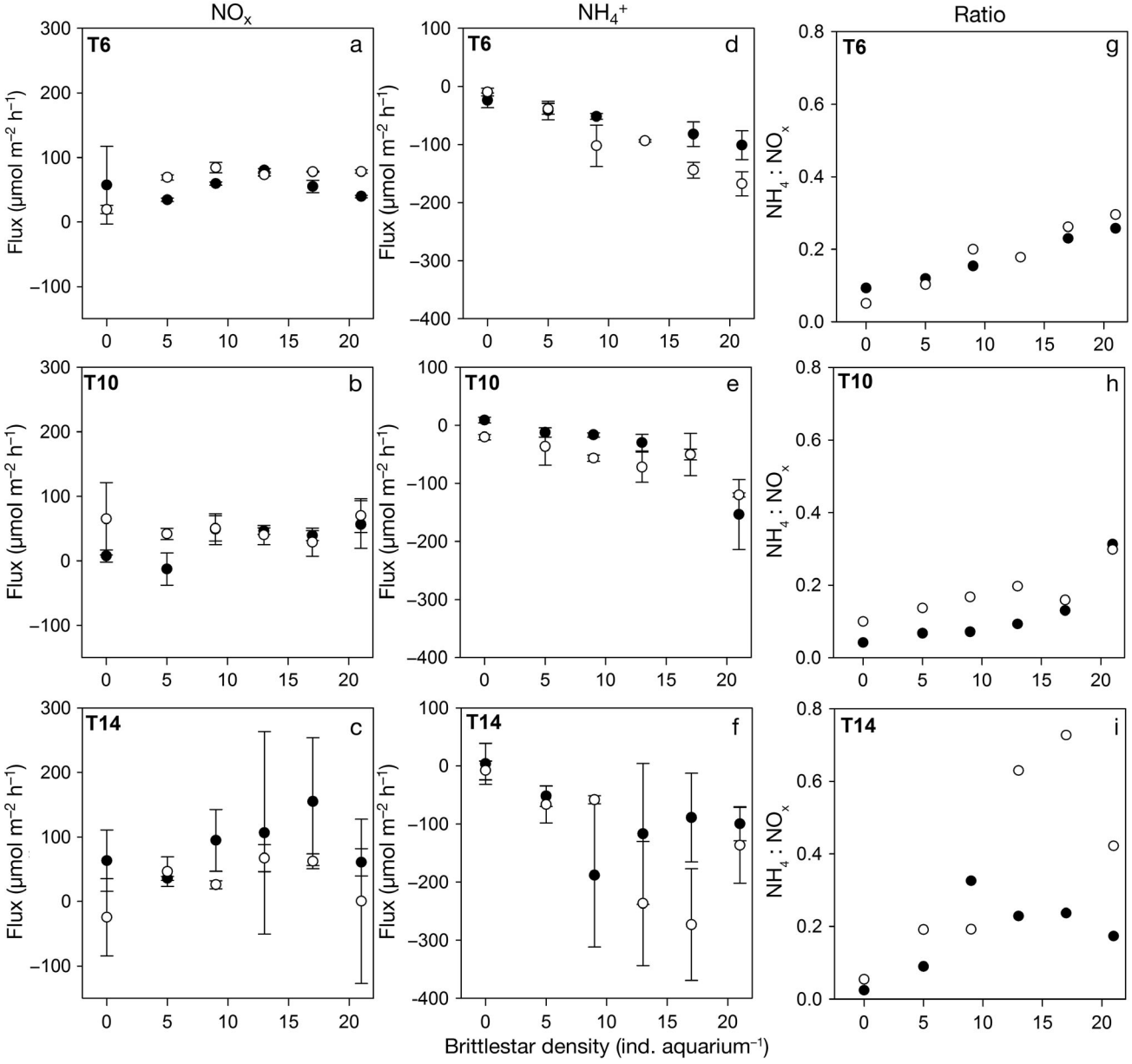

Fig. 2. (a–c) NO_x flux, (d–f) ammonium (NH_4^+) flux and (g–i) NH_4^+:NO_x ratios in experimental aquaria at time points T6, T10 and T14. Data for NO_x and NH_4^+ fluxes are means ± 95% confidence intervals; NH_4^+:NO_x ratio data calculated from mean concentrations. For NO_x and NH_4^+ flux, positive results represent nutrient influx, whilst negative results represent nutrient efflux. (●) Normoxia; (○) hypoxia

trite such as denitrification. A low ratio value indicates greater NO_x concentrations, and favours processes that produce nitrite and nitrate and decrease NH_4^+, such as nitrification. Water treatment, brittlestar density and experimental time all had a significant effect on the NH_4^+:NO_x ratios, with the normoxic aquaria displaying lower NH_4^+:NO_x ratios compared to the hypoxic aquaria, and differences increasing over the experimental time period (Table 3d). At T6, normoxic NH_4^+:NO_x ratios were an average of 6.07% lower than the hypoxic aquaria ratios. At T10, this difference increased to 32.38% and at T14, the normoxic NH_4^+:NO_x ratios were an average of 51.35% lower than the hypoxic aquaria. At T6 and T10, NH_4^+:NO_x ratios increased steadily with brittlestar density (Fig. 2g,h). At T14, the normoxic NH_4^+:NO_x ratios peaked at brittlestar density 9, and slightly decreased and plateaued at the higher brittlestar density treatments (Fig. 2i). Within the hypoxic treatment at T14, NH_4^+:NO_x ratios remained similar to normoxic levels but only in the low density treatments (0 to 9 ind. aquaria^{-1}). In the high density treatments (13 to 21 ind. aquaria^{-1}), NH_4^+:NO_x ratios increased (Fig. 2i). The interactions between water treatment and brittlestar density, and water treatment and experimental time had no significant effect on NH_4^+:NO_x ratio data (Table 3d).

PO_4^{3-} flux. PO_4^{3-} influx primarily occurred throughout the experimental period, but a certain degree of variability was observed within the data, with some points indicating PO_4^{3-} efflux (Fig. 3a–c). There were no significant effects of any experimental parameter on PO_4^{3-} flux (Table 3e).

SiO_4^{4-} flux. At T6, SiO_4^{4-} efflux consistently occurred in brittlestar density treatments of 9 ind. aquaria^{-1} or greater, irrespective of water treatment. After T6, variability in the SiO_4^{4-} flux increased, resulting in some data points representing SiO_4^{4-} efflux and others representing SiO_4^{4-} influx (Fig. 3d–f).

Table 3. General linear model ANOVA for (a) NO_x flux; (b) ammonium (NH_4^+) flux (complete data set); (c) NH_4^+ flux at T14 within the high brittlestar density treatments (13, 17 and 21 ind. aquaria^{-1}); (d) NH_4^+:NO_x ratios; (e) phosphate (PO_4^{3-}) flux; (f) silicate (SiO_4^{4-}) flux (complete data set); and (g) SiO_4^{4-} flux at T14 within the high brittlestar density treatments (13, 17 and 21 ind. aquaria^{-1}). Adj. SS: adjusted sum of squares; Adj. MS: adjusted mean squares (MS). **Bold p-values** indicate significance at $p < 0.05$

Source of variation	df	Adj. SS	Adj. MS	*F*	p
(a) NO_x flux					
Water treatment	1	640.30	640.30	1.00	0.329
Density	5	8160.30	1632.10	2.55	0.061
Time	2	2956.30	1478.10	2.31	0.125
Water treatment × density	5	3617.20	723.40	1.13	0.377
Water treatment × time	2	10386.10	5193.10	8.11	**0.003**
Error	20	12805.90	640.30		
Total	35	38566.10			
(b) NH_4^+ flux					
Water treatment	1	0.12	0.12	0.23	0.640
Density	5	3.16	0.63	1.14	0.372
Time	2	0.15	0.07	0.13	0.875
Water treatment × density	5	2.53	0.51	0.92	0.490
Water treatment × time	2	0.82	0.41	0.74	0.489
Error	19	10.49	0.55		
Total	34	17.16			
(c) NH^{4+} flux (T14, high density treatments)					
Water treatment	1	58240.00	58240.00	9.18	**0.009**
Density	2	14844.00	7422.00	1.17	0.339
Error	14	88774.00	6341.00		
Total	17	161859.00			
(d) NH^{4+}:NO_x ratios					
Water treatment	1	0.06	0.06	5.97	**0.024**
Density	5	0.39	0.08	7.53	**<0.001**
Time	2	0.08	0.04	3.87	**0.039**
Water treatment × density	5	0.02	0.00	0.38	0.859
Water treatment × time	2	0.04	0.02	1.87	0.182
Error	19	0.20	0.01		
Total	34	0.80			
(e) PO_4^{3-} flux					
Water treatment	1	16.75	16.75	0.73	0.402
Density	5	74.66	14.93	0.65	0.663
Time	2	39.76	19.88	0.87	0.434
Water treatment × density	5	50.79	10.16	0.44	0.812
Water treatment × time	2	0.13	0.06	0.00	0.997
Error	20	457.30	22.87		
Total	35	639.40			
(f) SiO_4^{4-} flux					
Water treatment	1	427.60	427.60	0.61	0.445
Density	5	17675.20	3535.00	5.02	**0.004**
Time	2	3106.10	1553.00	2.21	0.136
Water treatment × density	5	1288.40	257.70	0.37	0.866
Water treatment × time	2	10017.10	5008.60	7.11	**0.005**
Error	20	14082.80	704.10		
Total	35	46597.10			
(g) SiO_4^{4-} flux (T14, high density treatments)					
Water treatment	1	41393.90	413 93.90	17.30	**0.001**
Density (high)	2	146.80	73.40	0.03	0.970
Error	14	33507.40	2393.40		
Total	17	75048.10			

Analyses using all of the SiO_4^{4-} data revealed that brittlestar density significantly affected SiO_4^{4-} flux (Table 3f). Fig. 3 indicates that higher density treatments increased the efflux of SiO_4^{4-}. There was also an interaction effect between water treatment and experimental time, whereby, similarly to NH_4^+, SiO_4^{4-} efflux within hypoxia at T14 in the high brittlestar density treatments increased (Table 3f). Further analyses focusing on SiO_4^{4-} flux at T14 within the high brittlestar treatments (13, 17 and 21 ind. aquaria^{-1}) revealed that SiO_4^{4-} efflux was significantly greater in aquaria exposed to hypoxia compared to the corresponding normoxic treatment (Fig. 3f, Table 3g).

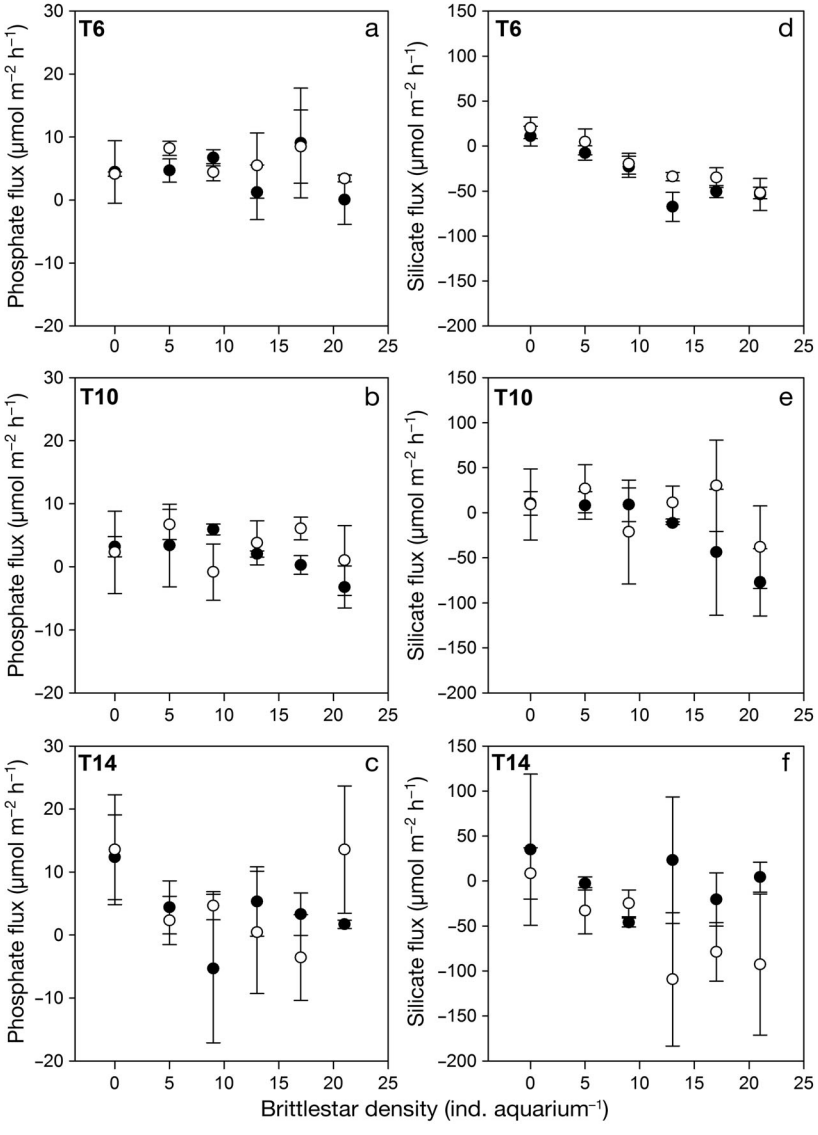

Fig. 3. Mean (±95% CI) (a–c) phosphate (PO_4^{3-}) flux and (d–f) silicate (SiO_4^{4-}) flux in experimental aquaria at time points T6, T10 and T14. Positive results represent nutrient influx, whilst negative results represent nutrient efflux. (●) normoxia; (○) hypoxia

DISCUSSION

Exposure of *Amphiura filiformis* to moderate hypoxia for 14 d significantly increased NH_4^+ and SiO_4^{4-} efflux, and caused an increase in NH_4^+:NO_x ratios when brittlestar densities were high (>1300 ind. m^{-2}). Additionally, there were idiosyncratic alterations in brittlestar activity (in terms of sediment surface reworked) with significant interaction between the water treatments and brittlestar density. There are several possible explanations for these results: the impact of moderate hypoxia on individual *A. filiformis* may have been so small that it only became detectable at high densities, and/or there was an interaction between high-density aggregations and low dissolved O_2 that exacerbated the effects of hypoxia. We were unable to identify which scenario was most likely to have initiated the observed changes in brittlestar activity and behaviour, but either way, our results demonstrate a potential impact on this species from a mild level of hypoxia when living in dense aggregations.

In earlier work based on the same experiment, Calder-Potts et al. (2015) found that prolonged hypoxia (>14 d) resulted in reduced respiration rates and hindered female oocyte growth and development, but brittlestar density had no effect on the physiological parameters measured. They concluded that during hypoxia *A. filiformis* may strategically allocate its energy into locomotory arm movements to increase burrow irrigation rates and prevent the build-up of toxins. This conclusion is supported by the results presented here, with brittlestars in the high density and longest incubation treatments potentially increasing burrow irrigation rates, explaining in part the rise in NH_4^+ and SiO_2 efflux and alterations in sediment surface bioturbation patterns. This also demonstrates that individuals of *A. filiformis* have considerable tolerance to short-term hypoxia, probably due to their life-mode, natural habitat

and potential exposure to diel-cycling in changing DO conditions.

Bioturbation of *A. filiformis* under normoxic conditions

Under normoxic conditions, there was a consistently positive relationship between brittlestar density and % SSR. At all densities, brittlestars appeared to continue with routine burrow maintenance, with visibly excavated mounds, feeding arms protruding and all individuals buried within the sediment. Therefore it is reasonable to assume that each individual (all were similar in size) may have equally contributed to surface sediment bioturbation activities, producing the observed additive relationship with density. Previous measurements of % SSR by *A. filiformis* from the same location, measured at natural field densities (214.28 ind. m^{-2}) over the same incubation time, ranged between 1 and 27% (Queirós et al. 2015). This is comparable to the % SSR measurements for our lowest experimental densities of 500 to 1300 ind. m^{-2}, with a mean of 5 to 27% SSR respectively. However, when calculated per individual, our results showed lower sediment surface mixing than found by Queirós et al. (2015). Several factors could explain these differences, including confinement within aquaria, deployment into mesocosm conditions, the use of sieved homogenised sediment and/or an increase in brittlestar densities compared to the field location. We recognise that there is a considerable difference between the natural field density of brittlestars (214.28 ind. m^{-2}, reported in Queirós et al. 2015) and even the lowest aquarium densities (500 ind. m^{-2}) used in this experiment. However, the current experiment was also designed to allow for robust examination of the biological effects of hypoxia on *A. filiformis* (results reported in Calder-Potts et al. 2015), and 500 ind. m^{-2} (equating to 5 ind. $aquaria^{-1}$) was the lowest manipulated density treatment we could use during this experiment. Although this raises questions about the effects of increasing population density when brought into the laboratory and the ecological relevance of this experiment to the specific *A. filiformis* population at Cawsands, Plymouth, the range of brittlestar densities used here are comparable to other *A. filiformis* populations found across Europe (e.g. O'Connor et al. 1983, Sköld et al. 1994, Rosenberg et al. 1997, Gilbert et al. 2003, Solan et al. 2004b). Additionally, in order to ascertain how population density may affect brittlestar functionality or resilience, the experiment needed to contain a range of brittlestar densities above (or indeed below) natural field densities. Thus the densities used here allowed for the controlled testing of hypotheses within a carefully monitored mesocosm environment, and provide valuable data that are widely applicable to *A. filiformis* in general, rather than to the specific population used within this study.

In acknowledging this discrepancy in densities, we also need to consider the potential differences in mesocosm experiments compared to natural environments. For example, previous laboratory experiments have shown that once *A. filiformis* buries itself, it can remain within the burrow cavity for weeks or even months if conditions are favourable (Woodley 1975). Other experiments have shown that *A. filiformis* can exhibit density-dependent migration, moving both within and on top of the sediment to less populated areas, given the space to do so (Rosenberg et al. 1997). Observations of a natural population have shown that *A. filiformis* individuals can distribute themselves in alternating patterns of disc chamber placements such that they are one shallow, one deep; ranging from depths of 2.0 to 6.5 cm (O'Reilly et al. 2006). Clearly it is difficult to pinpoint the exact effects of being confined within aquaria, but it is likely that experimental procedures limit migratory movements within sediments, which could affect optimal dispersal patterns. Despite this, we would still expect bioturbation activities for burrow maintenance, irrigation and feeding to be maintained. During this experiment, food availability was comparable to levels within the local environment (see Calder-Potts et al. 2015), water velocity within aquaria was low, as per the collection site (Uncles & Torres 2013) and conditions between the normoxic and hypoxic aquaria (except DO levels) were comparable, all of which strengthen our confidence in the results.

Experimental time significantly affected % SSR, with decreased surface mixing in both water treatments as the experiment progressed. This is likely due to the time the brittlestars spent within aquaria prior to luminophore addition; although T0 brittlestars were allowed a 6 h period before luminophores were added, they were both added to the aquaria on the same day. For T6, T10 and T14 bioturbation measurements, brittlestars had been within the aquaria for 6, 10 and 14 d respectively prior to the addition of luminophores. Therefore, the effect of time is likely to be related to differences between the initial burrow formation activities and long-term burrow maintenance activities rather than an experimental effect of being contained within aquaria.

Bioturbation of *A. filiformis* under hypoxic conditions

When exposed to hypoxia, there was a breakdown in the relationship between % SSR and brittlestar density seen in normoxic conditions. For example, at T6 the highest rates of % SSR occurred in the second highest brittlestar density treatment, while at T10 the highest rates of % SSR occurred in the second lowest density treatment. At T14, within the highest brittlestar density treatment % SSR was around 38% less in the hypoxic treatment compared to the equivalent density in normoxic conditions.

Within the hypoxic treatment, brittlestars did remain buried within the sediment for the majority of the experiment, but occasionally individuals were observed on the sediment surface. Although the experiment was not monitored during nighttime, it is possible that brittlestars within the hypoxic treatment left their burrows and spent time on the sediment surface in search of more favourable conditions, as has been observed in other fauna experiencing hypoxia (Sturdivant et al. 2012). Differences in sediment surface exploration, in addition to increased bio-irrigation rates, may have moved and mixed the sediment in different ways compared to the normoxic treatment. This small shift in behaviour from routine burrow maintenance, as observed under normoxic conditions, to possible extended periods of burrow irrigation and surface exploration due to hypoxic exposure, may represent the early stages of moderate hypoxic impacts, and could underlay the differences in surface sediment bioturbation patterns.

The luminophore imaging technique provided no evidence that moderate hypoxia affected the maximum burrow depths of *A. filiformis*. This is somewhat surprising given that previous studies (e.g. Sturdivant et al. 2012) have shown a relationship between hypoxia and burrowing depth. However, if brittlestar (or disc chamber) placement had moved closer to the sediment surface during the current hypoxic exposure, it is possible that remnant burrows, which were formed prior to hypoxic exposure, were still present and some tracer particles could have found their way into these now unoccupied burrow structures. Although some brittlestars were occasionally spotted on the sediment surface for brief periods of time, it is also possible that the hypoxic treatment level used here was not severe enough to reduce burrow depths.

Cycling of NO_x and NH_4^+ during normoxia

The majority of recycled nitrogen released from the sediments to the overlying water is in the form of NH_4^+, which is generally regenerated from the decomposition and deamination of organic matter. It then passes from the sediments to the overlying water via diffusion or advection (bio-irrigation), where it can be assimilated by phytoplankton (Kemp et al. 1990). Before it escapes the sediments, and when oxygen is present, a portion of this NH_4^+ is oxidised to NO_3^- (nitrate), a process known as nitrification. NO_3^- can then be used by denitrifying bacteria (Kemp et al. 1990).

In our experiment, NO_x influx and NH_4^+ efflux were persistent features, and our data agrees with previous studies using sediments collected from nearby sites within Plymouth Sound. These previous studies documented sediments acting as a source of NH_4^+ and a sink for NO_x (Wood et al. 2009, Murray et al. 2013), and suggested that rates of nitrification were insufficient to totally support levels of denitrification with the sediment. Formal statistical analysis suggested that under normoxic conditions, brittlestar density had no significant effect on the sediment uptake of NO_x or the release of NH_4^+. Other mesocosm studies using similar sediment type and densities of *A. filiformis* also found that brittlestar density had no significant effects on NO_x or NH_4^+ fluxes under control conditions (Wood et al. 2009, Murray et al. 2013). However, in the current study an increase in the ratio of NH_4^+ to NO_x in the overlying water indicated that there was a significant shift in the balance between these nutrients at higher brittlestar densities. The balance between NH_4^+ and NO_x is set by a number of interdependent biogeochemical processes occurring both in the sediment and the overlying water. Small changes in these individual processes may be not be statistically significant but when combined in an integrative measure, such as the NH_4^+:NO_x ratio, significant impacts may become detectable. These impacts may also build up over time, making differences more apparent towards the end of the exposure experiment, as was seen in the current study. If *A. filiformis* either had no impact on any N-cycling processes or an equal impact on all N-cycling processes, the NH_4^+:NO_x ratio would be expected to remain constant. In the current study this ratio increased with brittlestar density, suggesting the presence and activities of *A. filiformis* was favouring processes that produced NH_4^+ (e.g. excretion of metabolic NH_4^+ by the brittlestars or from microbes) and/or removed NO_x (e.g. denitrification)

over those processes that oxidised NH_4^+ and produced NO_x (e.g. nitrification and, to some extent anammox). This does not mean that *A. filiformis* activities only stimulated NH_4^+ production and NO_x oxidation — it is likely that it also stimulated NH_4^+ oxidation, but to a lesser extent. It is reasonable to expect NH_4^+ production to increase with brittlestar density as excretion products rise, and for bacterial mineralisation of organic matter to intensify as burrow structures increase in numbers and surface area (Papaspyrou et al. 2005). Bacterial abundance and activity can be 10-fold higher in burrow walls compared to the surrounding environment, aiding other sedimentary processes such as nitrification and denitrification (Papaspyrou et al. 2005, Laverock et al. 2010). During the final sampling time point (T14), the positive linear relationship between the NH_4^+:NO_x ratio and brittlestar densities broke down at the highest *A. filiformis* densities and the sediment uptake of NO_x was reduced. This suggests that *A. filiformis* was actually stimulating NH_4^+ oxidation processes, such as nitrification, but not generally at a rate sufficient to totally keep pace with the increase in NH_4^+. However, at the higher brittlestar densities something reduced the sediment uptake of NO_x, reduced the release of $NH_4^+$and therefore lowered the NH_4^+:NO_x ratio. This suggests that in large aggregations of *A. filiformis* the balance shifts back towards processes that produce NO_x away from processes that produce $NH_4^+$and consume NO_x. It is generally accepted that the most important role of bioturbation in stimulating remineralisation reactions is the introduction of oxygen into subsurface sediments (Kristensen & Kostka 2005), but perhaps this is only the case above certain densities of bioturbators, and in low density areas the main impact of bioturbation could be to increase NH_4^+ supply and stimulate denitrification. Future studies which employ targeted sampling of specific N-cycling processes, coupled with microbial functional group analysis, would be of great value in testing this possibility.

Cycling of NO_x and NH_4^+ during hypoxia

For the first 2 sampling points (T6, T10), there was no difference between the normoxic and hypoxia treatments in terms of the effects of *A. filiformis* density on NH_4^+ release, NO_x uptake or the NH_4^+:NO_x ratio. At the final sampling point (T14), however, high brittlestar densities (≥1300 ind. m^{-2}) produced a NH_4^+ efflux in hypoxia treatments which was significantly greater than in normoxic conditions. With little change occurring in the NO_x uptake rates, this increase in NH_4^+ release also drove a large increase in the NH_4^+:NO_x ratio. This result is supported by Villnäs et al. (2012) who reported that an increase in the duration of hypoxic exposure significantly increased the efflux of NH_4^+. Whilst it is difficult to separate out how hypoxia, bioturbation, brittlestar excretion and bacterial remineralization independently affect NH_4^+ fluxes, results from Villnäs et al. (2012) also highlighted the importance of considering benthic abundance and biomass when studying N-cycling in sediments. Calder-Potts et al. (2015) showed that hypoxic exposure resulted in a decrease in oxygen uptake rates by brittlestars, indicating that metabolic activity had decreased. Therefore, it is possible that the observed increases in NH_4^+ within the high-density treatments were due to excretion processes linked to increased brittlestar biomass and through bio-irrigation of burrow structures, which enhanced the advection of NH_4^+ into the overlying water.

Additionally, as was generally seen under normoxic conditions, microbial processes responsible for NH_4^+ removal (i.e. nitrification) again appeared to be unable to keep pace with processes of anaerobic NH_4^+ generation, especially at high brittlestar density treatments, and thereby could not maintain the balance that was observed under normoxic conditions. However, our data also suggest that under hypoxic conditions there was little evidence for enhanced stimulation of nitrification in the densest aggregations of brittlestars, contrary to the situation observed in the normoxic treatments. Although at the very highest brittlestar density there was some evidence that this nitrification stimulation was beginning to occur. Consequently, moderate hypoxia may have indirectly changed sedimentary microbial processes and nutrient cycling by altering the behaviour of bioturbating organisms.

Cycling of PO_4^{3-} and SiO_4^{4-} during normoxia

Under oxygenated conditions and in oxidised areas such as burrow walls, PO_4^{3-} sorption onto insoluble iron–manganese compounds can readily occur, resulting in PO_4^{3-} influx into the sediment. The capacity of this process is determined by the supply of Fe(III) in the sediment, with macrofaunal activities increasing the amount of oxidised surface area available for PO_4^{3-} accumulation (Karlson et al. 2007). During our experiment, PO_4^{3-} primarily fluxed into the sediment, with experimental parameters having no effect. Previous laboratory experiments using *A.*

filiformis and sediment from Plymouth Sound have reported contradictory results. Wood et al. (2009) found brittlestar density significantly increased sediment uptake of PO_4^{3-}, whilst Murray et al. (2013) found no significant effects on PO_4^{3-} flux when *A. filiformis* was present compared to aquaria with no macrofauna. We suggest that given the high degree of variability within the PO_4^{3-} flux results, statistically significant outcomes were unlikely.

SiO_4^{4-} fluxes are thought to be a balance between oxic precipitations into the sediment and excretion of SiO_4^{4-}-rich waste from infauna and diatom decomposition. Infaunal bioturbation activities contribute to nutrient fluxes through promotion of an oxidised environment within the sediment adjacent to burrows, within which compound oxidation may occur. In this experiment, the majority of aquaria exhibited SiO_4^{4-} efflux, representing SiO_4^{4-} regeneration into the water column; but at T10, some measurements indicated influx of SiO_4^{4-}, possibly explained by microalgal uptake or adsorption processes at the sediment–water interface (Bartoli et al. 2009). We found that brittlestar density significantly increased SiO_4^{4-} efflux, possibly due to increased mobilisation of SiO_4^{4-} from porewaters (Bartoli et al. 2009). Previous mesocosm experiments failed to detect a significant effect of *A. filiformis* on SiO_4^{4-} flux (Wood et al. 2009), although this could be due to discrepancies in the amount of organic matter and the degradation of benthic diatoms within sediments (Villnäs et al. 2012) between the different studies.

Cycling of PO_4^{3-} and SiO_4^{4-} during hypoxia

In hypoxic conditions, iron-bound PO_4^{3-} is generally released into the porewater as Fe(III) and is reduced to Fe(II), causing efflux of PO_4^{3-} (Belias et al. 2007). However, in our experiment, PO_4^{3-} generally fluxed into the sediment, with water treatment having no effect. During our experiment, oxygen was limited (i.e. hypoxic) but not unavailable (i.e. anoxic). It may be reasonable to assume that with some oxygen still present in the hypoxic treatment, PO_4^{3-} adsorption onto ferric iron still occurred. However, Villnäs et al. (2012) did not observe an increase in PO_4^{3-} efflux from sediments exposed to hypoxia, and concluded that this was likely due to the low content of PO_4^{3-} in the sediment. This may also be true for our experiment, and could mask any potential effects of hypoxia and brittlestars. Unfortunately, no analyses of dissolved and particulate PO_4^{3-} in our sediments were carried out.

Similarly, in the case with NH_4^+, 14 d exposure to hypoxia at high brittlestar densities (1300 to 2100 ind. m^{-2}) resulted in increased SiO_4^{4-} efflux compared to the normoxic aquaria. In support of our results, previous studies have also documented a rise in SiO_4^{4-} efflux during prolonged hypoxia (Villnäs et al. 2012). It is likely that a combination of bioturbation and bioirrigation activities, degradation of benthic diatoms and release of SiO_4^{4-} from surfaces of hydrated oxides of iron due to reduced oxic precipitation into the sediments contributed to the SiO_4^{4-} efflux results observed here (Villnäs et al. 2012).

Experimental limitations

Changes in header tank nutrient concentrations

Reduced concentrations of NO_3^- and PO_4^{3-} occurred within the hypoxic header tank after 10 d (NO_3^-) and 14 d (PO_4^{3-}) experimental exposure, despite both header tanks receiving filtered seawater from the same source. Unfortunately, at the time of experimentation, samples to test for microbial growth within the header tanks and aquaria were not taken. Despite these alterations, nutrient flux measurements can be interpreted with confidence, as they were calculated using differences between the corresponding header tank and aquaria; thus, whilst it may not be ideal to have differences in absolute values between the normoxic and hypoxic header tanks, it is not critical to the comparisons of nutrient fluxes. For example, the reduction in PO_4^{3-} concentrations within the hypoxic header tank at T14 did not cause any significant effects to the aquaria flux results, and the high levels of variability in PO_4^{3-} flux data occurred within both the normoxic and hypoxic treatments. Additionally, NO_3^- levels within the hypoxic header tank, the hypoxic NO_x flux and NH_4^+:NO_x ratio values can be evaluated with confidence for several reasons. (1) There was no difference in NO_x fluxes between the hypoxic and normoxic experimental aquaria at T10 (Fig. 2b), indicating that the sedimentary processes occurring within the experimental aquaria were not significantly affected by the differences in header tank concentrations. (2) The differences in NO_x fluxes at T14 (Fig. 2c) were caused by an increase in NO_x flux in the normoxic aquaria, not a reduction in NO_x within the hypoxic aquaria compared to previous time points. (3) The similarity in NH_4^+:NO_x ratios from T6 to T10 in both water treatments indicates that the processes occurring within each experimental aquarium were comparable and similar, despite the reduc-

tion in NO_3^- concentrations in the hypoxic header tank at T10.

Experimental design

Each experimental aquarium received filtered seawater from either a hypoxic or normoxic header tank. Sharing water supply from a header tank has its limitations, and this setup could be considered as pseudoreplication, with the concern that something could have happened to either the normoxic or hypoxic header tank, which would have influenced the results independently from the treatment effect. However, in the current study this is highly unlikely, as seawater parameters were monitored daily and remained consistent, and all equipment used was well 'seasoned' and had been used successfully in many previous experiments. Finally, all experimental aquaria were kept covered with a black tarpaulin sheet, minimising any photosynthetic activities of microphytobenthos.

Ecological effects and conclusions

Moderate hypoxia will not cause an immediate loss in biodiversity and species richness compared to severe hypoxic and anoxic events, but it may initiate changes in organism physiology and behaviour that have the potential to alter ecosystem function. We have demonstrated how population density plays an important role in determining the impacts of hypoxia; dense patches of *A. filiformis* may exhibit larger changes in behaviour and shifts in ecosystem function compared to sparse patches, as competition for oxygen and resources heighten and O_2 diffusion into the sediment reduces. The duration of a hypoxic event will also be important in determining the individual and community effects, as different species have varying thresholds and sensitivities to decreased O_2 concentrations. In the present study, and in previous work (Calder-Potts et al. 2015), *A. filiformis* exhibited an initial tolerance to hypoxia, with significant effects only occurring after 14 d exposure. The results from Calder-Potts et al. (2015) were consistent with the view that *A. filiformis* is an 'oxyconformer', reducing its metabolic rate with declining pO_2. However, when oxygen is still available, 'oxyconformers' can be behavioural 'oxyregulators', attempting to maintain constant levels of oxygen in their burrows or body fluids through compensatory adjustments in ventilatory efforts, such as burrow irrigation (Pörtner 2010). This concept supports our conclusions that after prolonged hypoxic conditions, *A. filiformis* may have increased burrow irrigation rates in an attempt to maintain oxygen levels within the burrow, and to avoid the build-up of toxins. This subtle change in brittlestar behaviour under hypoxic conditions altered sediment surface bioturbation patterns, and increased the efflux of NH_4^+, possibly reducing nitrification rates. In areas where persistent hypoxia and reduced O_2 diffusion into the sediments occur, inhibition of nitrification and the subsequent decrease in denitrification could result in a build-up of nitrogen. This build-up would further the unpredictable eutrophication phenomena (Huesemann et al. 2002), which would inhibit an area's ability to recover and rehabilitate, and cause a loss of biodiversity and functionality.

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LITERATURE CITED

Baker SM, Mann R (1992) Effects of hypoxia and anoxia on larval settlement, juvenile growth, and juvenile survival of the oyster *Crassostrea virginica.* Biol Bull (Woods Hole) 182:265–269

Bartoli M, Longhi D, Nizzoli D, Como S, Magni P, Viaroli P (2009) Short term effects of hypoxia and bioturbation on solute fluxes, denitrification and buffering capacity in a shallow dystrophic pond. J Exp Mar Biol Ecol 381: 105–113

Belias C, Dassenakis M, Scoullos M (2007) Study of the N, P and Si fluxes between fish farm sediment and seawater. Results of simulation experiments employing a benthic chamber under various redox conditions. Mar Chem 103:266–275

Bertics VJ, Löscher CR, Salonen I, Dale AW, Gier J, Schmitz RA, Treude T (2013) Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea. Biogeosciences 10:1243–1258

Boyd WA, Brewer SK, Williams PL (2002) Altered behaviour of invertebrates living in polluted environments. In: Dell'Omo G (ed) Behavioural ecotoxicology. John Wiley & Sons, Chichester, p 293–336

Boyle RA, Dahl TW, Dale AW, Shields-Zhou GA and others (2014) Stabilization of the coupled oxygen and phosphorus cycles by the evolution of bioturbation. Nat Geosci 7: 671–676

Brewer PG, Riley JP (1965) The automatic determination of nitrate in seawater. Deep-Sea Res 12:765–772

Butchart SHM, Walpole M, Collen B, van Strien A and others (2010) Global biodiversity: indicators of recent declines. Science 328:1164–1168

Calder-Potts R, Spicer JI, Calosi P, Findlay HS, Widdicombe S (2015) A mesocosm study investigating the effects of hypoxia and population density on respiration and reproductive biology in the brittlestar, *Amphiura filiformis*. Mar Ecol Prog Ser 534:135–147

Canfield DE, Farquhar J (2009) Animal evolution, bioturbation, and the sulfate concentration of the oceans. Proc Natl Acad Sci USA 106:8123–8127

Chan H, Xu W, Shin P, Cheung S (2008) Prolonged exposure to low dissolved oxygen affects early development and swimming behaviour in the gastropod *Nassarius festivus* (Nassariidae). Mar Biol 153:735–743

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321:926–929

Froehlich HE, Hennessey SM, Essington TE, Beaudreau AH, Levin PS (2015) Spatial and temporal variation in nearshore macrofauna community structure in a seasonally hypoxic estuary. Mar Ecol Prog Ser 520:67–83

Gattuso JP, Frankignoulle M, Wollast R (1998) Carbon and carbonate metabolism in coastal aquatic ecosystems. Annu Rev Ecol Syst 29:405–434

Gilbert F, Hulth S, Stromberg N, Ringdahl K, Poggiale JC (2003) 2-D optical quantification of particle reworking activities in marine surface sediments. J Exp Mar Biol Ecol 285-286:251–263

Grasshoff K (1976) Methods of seawater analysis. Verlag Chemie, Weinheim

Gray JS, Wu RSS, Or YY (2002) Effects of hypoxia and organic enrichment on the coastal marine environment. Mar Ecol Prog Ser 238:249–279

Grieshaber MK, Hardewig I, Kreutzer U, Pörtner HO (1994) Physiological and metabolic responses to hypoxia in invertebrates. Rev Physiol Biochem Pharmacol 125: 43–147

Herbert RA (1999) Nitrogen cycling in coastal marine ecosystems. FEMS Microbiol Rev 23:563–590

Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, Marino R, Billen G (2011) Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. Front Ecol Environ 9: 18–26

Huesemann MH, Skillman AD, Crecelius EA (2002) The inhibition of marine nitrification by ocean disposal of carbon dioxide. Mar Pollut Bull 44:142–148

Hylland K, Sköld M, Gunnarsson JS, Skei J (1996) Interactions between eutrophication and contaminants. IV. Effects on sediment-dwelling organisms. Mar Pollut Bull 33:90–99

Karlson K, Bonsdorff E, Rosenberg R (2007) The impact of benthic macrofauna for nutrient fluxes from Baltic Sea sediments. Ambio 36:161–167

Kemp WM, Sampou P, Caffrey J, Mayer M (1990) Ammonium recycling versus denitrification in Chesapeake Bay sediments. Limnol Oceanogr 35:1545–1563

Kirkwood DS (1989) Simultaneous determination of selected nutrients in seawater. ICES CM 1989/C:29. ICES, Copenhagen

Kristensen E, Kostka JE (2005) Macrofaunal burrows and irrigation in marine sediment: microbiological and biogeochemical interactions. In: Kristensen E, Haese R, Kostka JE (eds) Interactions between macro- and microorganisms in marine sediments. American Geophysical Union, Washington, DC, p 125–157

Kristensen E, Penha-Lopes G, Delefosse M, Valdemarsen T, Quintana CO, Banta GT (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Mar Ecol Prog Ser 446:285–302

Laverock B, Smith CJ, Tait K, Osborn MA, Widdicombe S, Gilbert JA (2010) Bioturbating shrimp alter the structure and diversity of bacterial communities in coastal marine sediments. ISME J 4:1531–1544

Lawton JH (1994) What do species do in ecosystems? Oikos 71:367–374

Liu KK, Atkinson L, Quiñones R, Talaue-McManus L (2010) Carbon and nutrient fluxes in continental margins: a global synthesis. Springer, Berlin

Mahaut M, Graf G (1987) A luminophore tracer technique for bioturbation studies. Oceanol Acta 10:323–328

Maire O, Duchêne JC, Rosenberg R, de Mendonça JB Jr, Grémare A (2006) Effects of food availability on sediment reworking in *Abra ovata* and *A. nitida*. Mar Ecol Prog Ser 319:135–153

Mantoura RFC, Woodward EMS (1983) Optimization of the indophenol blue method for the automated determination of ammonia in estuarine waters. Estuar Coast Shelf Sci 17:219–224

Murray F, Widdicombe S, McNeill CL, Solan M (2013) Consequences of a simulated rapid ocean acidification event for benthic ecosystem processes and functions. Mar Pollut Bull 73:435–442

Nilsson HC (1999) Effects of hypoxia and organic enrichment on growth of the brittle stars *Amphiura filiformis* (O.F. Müller) and *Amphiura chiajei* Forbes. J Exp Mar Biol Ecol 237:11–30

Nilsson HC, Sköld M (1996) Arm regeneration and spawning in the brittle star *Amphiura filiformis* (O.F. Müller) during hypoxia. J Exp Mar Biol Ecol 199:193–206

O'Connor B, Bowmer T, Grehan A (1983) Long-term assessment of the population dynamics of *Amphiura filiformis* (Echinodermata: Ophiuroidea) in Galway Bay (west coast of Ireland). Mar Biol 75:279–286

O'Reilly R, Kennedy R, Patterson A (2006) Destruction of conspecific bioturbation structures by *Amphiura filiformis* (Ophiuroidea): evidence from luminophore tracers and *in situ* time-lapse sediment-profile imagery. Mar Ecol Prog Ser 315:99–111

Papaspyrou S, Gregersen T, Cox RP, Thessalou-Legaki M, Kristensen E (2005) Sediment properties and bacterial community in burrows of the ghost shrimp *Pestarella tyrrhena* (Decapoda:Thalassinidea). Aquat Microb Ecol 38:181–190

Pörtner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881–893

Queirós AM (2010) Ecosystem engineers in diversity and process relationships. PhD dissertation, Bangor University, Menai Bridge

Queirós AM, Hiddink JG, Kaiser MJ, Hinz H (2006) Effects of chronic bottom trawling disturbance on benthic biomass, production and size spectra in different habitats. J Exp Mar Biol Ecol 335:91–103

Queirós AM, Birchenough SNR, Bremner J, Godbold JA and others (2013) A bioturbation classification of European marine infaunal invertebrates. Ecol Evol 3:3958–3985

Queirós AM, Stephens N, Cook R, Ravaglioli C and others (2015) Can benthic community structure be used to predict the process of bioturbation in real ecosystems? Prog Oceanogr 137:559–569

R Development Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rabalais NN, Turner RE, Wiseman WJ (2002) Gulf of Mexico hypoxia, a.k.a 'The Dead Zone'. Annu Rev Ecol Syst 33: 235–263

Riedel B, Pados T, Pretterebner K, Schiemer L and others (2014) Effect of hypoxia and anoxia on invertebrate behaviour: ecological perspectives from species to community level. Biogeosciences 11:1491–1518

Rosenberg R (2001) Marine benthic faunal successional stages and related sedimentary activity. Sci Mar 65: 107–119

Rosenberg R, Hellman B, Johansson B (1991) Hypoxic tolerance of marine benthic fauna. Mar Ecol Prog Ser 79: 127–131

Rosenberg R, Nilsson HC, Hollertz K, Hellman B (1997) Density-dependent migration in an *Amphiura filiformis* (Amphiuridae, Echinodermata) infaunal population. Mar Ecol Prog Ser 159:121–131

Rosenberg R, Nilsson HC, Diaz RJ (2001) Response of benthic fauna and changing sediment redox profiles over a hypoxic gradient. Estuar Coast Shelf Sci 53: 343–350

Schiffers K, Teal LR, Travis JMJ, Solan M (2011) An open source simulation model for soil and sediment bioturbation. PLOS ONE 6:e28028

Seibel BA, Childress JJ (2013) The real limits to marine life: a further critique of the respiration index. Biogeosciences 10:2815–2819

Shull DH (2009) Bioturbation. In: Steele JH, Turekian KK, Thorpe SA (eds) Encyclopedia of ocean sciences, 2nd edn. Academic Press, San Diego, CA, p 395–400

Sköld M, Loo LO, Rosenberg R (1994) Production, dynamics and demography of an *Amphiura filiformis* population. Mar Ecol Prog Ser 103:81–90

Solan M, Kennedy R (2002) Observation and quantification of *in situ* animal-sediment relations using time-lapse sediment profile imagery (t-SPI). Mar Ecol Prog Ser 228: 179–191

Solan M, Cardinale BJ, Downing AL, Englehardt KAM, Ruesink JL, Srivastava DS (2004a) Extinction and ecosystem function in the marine benthos. Science 306: 1177–1180

Solan M, Wigham BD, Hudson IR, Kennedy R and others (2004b) *In situ* quantification of bioturbation using time-lapse fluorescent sediment profile imaging (f-SPI), luminophore tracers and model simulation. Mar Ecol Prog Ser 271:1–12

Spicer JI (2016) Respiratory responses of marine animals to environmental hypoxia. In: Solan M, Whiteley NM (eds) Stressors in the marine environment. Oxford University Press, Oxford, p 25–33

Sturdivant SK, Diaz RJ, Cutter GR (2012) Bioturbation in a declining oxygen environment, *in situ* observations from Wormcam. PLOS ONE 7:e34539

Uncles RJ, Torres R (2013) Estimating dispersion and flushing time-scales in a coastal zone: application to the Plymouth area. Ocean Coast Manage 72:3–12

Vaquer-Sunyer R, Duarte CM (2008) Thresholds of hypoxia for marine biodiversity. Proc Natl Acad Sci USA 105: 15452–15457

Villnäs A, Norkko J, Lukkari K, Hewitt J, Norkko A (2012) Consequences of increasing hypoxic disturbance on benthic communities and ecosystem functioning. PLOS ONE 7:e44920

Vopel K, Thistle D, Rosenberg R (2003) Effect of the brittle star *Amphiura filiformis* (Amphiuridae, Echinodermata) on oxygen flux into the sediment. Limnol Oceanogr 48: 2034–2045

Widdicombe S, Needham HR (2007) Impact of CO_2-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. Mar Ecol Prog Ser 341:111–122

Wood HL, Widdicombe S, Spicer JI (2009) The influence of hypercapnia and the infaunal brittlestar *Amphiura filiformis* on sediment nutrient flux: Will ocean acidification affect nutrient exchange? Biogeosciences 6:2015–2024

Woodley JD (1975) The behaviour of some amphiurid brittle stars. J Exp Mar Biol Ecol 18:29–46

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