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

Running page head: Effects of hypoxia on brittlestars

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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 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 indiv. 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 illustrated 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.

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 occurrence of low dissolved oxygen conditions, i.e. hypoxia. Hypoxia is now widely recognised as one of the key environmental stressors and is predicted to increase in coastal areas as global warming and human-induced eutrophication intensify (Diaz & Rosenberg 2008, Vaquer-Sunyer & Duarte 2008, Howarth et al. 2011). Traditionally, conditions are defined as hypoxic when dissolved oxygen 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 and 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 nearshore 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 less than 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. 2011). Through the creation of pits, mounds and burrows, sediment ingestion and excretion, as well as the bio-irrigation of subsurface burrows, benthic infauna play a significant role in mediating the rate and depth of many chemical and physical reactions. This ultimately drives carbon and nitrogen cycling, establishes 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, Rosenberg et al. 2001, Vaquer-Sunyer & Duarte 2008). Ultimately, severe and prolonged hypoxia can lead to extreme responses in benthic communities, reducing biodiversity through

100 forced migration, increased vulnerability to predation, reduction of suitable habitats
101 and excessive physiological stress leading to mortality (Rosenberg 2001, Rabalais et
102 al. 2002). However, before these extreme reactions are observed, organism
103 responses to hypoxia are often initially expressed through changes in organism
104 physiology and behaviour (Grieshaber et al. 1994). Documented changes include
105 reduced growth in oyster larvae and juveniles (Baker & Mann 1992), delayed
106 embryonic development in gastropods (Chan et al. 2008) and reduced metabolic
107 rates and oocyte growth in brittlestars (Calder-Potts et al. 2015). Behavioural
108 responses include elongated bivalve siphons, abandonment of burrows and reduced
109 burrowing depths and activity of infauna (Sturdivant et al. 2012). Importantly,
110 behavioural data may provide a link between individual response and population
111 change, especially if the behaviour alters the structure and function of the community
112 (Boyd et al. 2002).
113 Ecosystem engineers are defined as species that modify, maintain and create
114 habitats and, through their actions, modulate the availability of resources to other
115 species (Lawton 1994, O'Reilly et al. 2006). One such species, the brittlestar
116 *Amphiura filiformis* (Müller, 1776), is an active and well-studied bioturbator (Solan &
117 Kennedy 2002, Solan et al. 2004a, O'Reilly et al. 2006, Queirós et al. 2013, Queirós
118 et al. 2015). *Amphiura filiformis* is primarily a suspension feeder that remains buried
119 below the sediment surface and protrudes one or more arms into the water column.
120 It actively undulates its arms and pumps its disc for respiratory gas exchange,
121 burrow ventilation and irrigation, in addition to collection and expulsion of food and
122 waste (Vopel et al. 2003, Calder-Potts et al. 2015). *Amphiura filiformis* is also a
123 dominant species in many coastal and shelf areas of the NE Atlantic and its effects

124 on sediment properties may explain its structuring effect in infauna communities
125 (Queirós et al. 2006).
126 The effects of traditionally defined hypoxia on the biology of *A. filiformis* are relatively
127 well documented. Hypoxic exposure reduces *A. filiformis* disc diameter growth
128 (Hylland et al. 1996), reduces arm regeneration rates and delays spawning (Nilsson
129 & Sköld 1996, Nilsson 1999), reduces metabolic rates, reduces oocyte growth and
130 delays reproductive development (Calder-Potts et al. 2015). However, research that
131 examines the links between the biological, physiological and behavioural
132 consequences of more moderate reductions in DO and potential ecosystem effects
133 are limited.
134 In a 'random extinction' event simulation study focused on the North Sea, the
135 biogenic mixing depth (BMD), an indicator of bioturbation, was dependant on
136 whether *A. filiformis* was among the survivors (Solan et al. 2004a). Field data on
137 communities exposed to fishing pressure in the Irish Sea demonstrated that
138 community biomass and production dramatically decreased following the loss of the
139 dominant *A. filiformis,* a species which is highly vulnerable to physical damage
140 associated with trawling (Queirós et al. 2006). Therefore, in communities where
141 contributions to ecosystem function are dominated by one species, stress induced
142 loss or behavioural alterations of that dominant species can have consequences for
143 the entire community.
144 Consequently, we conducted a 14 day mesocosm experiment in which *Amphiura*
145 *filiformis* were exposed to 14 days moderate hypoxia in order to address the
146 following questions: 1) Does exposure to moderate hypoxia affect *A. filiformis*
147 behaviour, measured in terms of bioturbation activity? 2) Do any changes in *A.*
148 *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 2D imaging and particle tracing methods (Mahaut & Graf 1987, Gilbert et al. 2003, Solan et al. 2004b). Tracer data were then used to quantify two 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 set up and monitoring, and seawater O_2 manipulation methods. For full details relating to the experimental set up 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 the 25th 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° 07.961' W), using a 0.1 m^2 US-NL box-corer. Once retrieved the surface layers of sediment (top 10 - 15 cm) were placed into bags and

transported to the Plymouth Marine Laboratory (PML, Plymouth, UK) mesocosm facility where sediment was sieved (2 mm) in filtered sea water (10 μm diam. Hydrex filters). Fifty experimental glass aquaria (20 cm L x 5 cm W x 30 cm H) were filled with the sieved sediment to a depth of 19 cm (± 1 cm), leaving 11 cm of overlying water. Each aquarium was connected to a flow-through seawater system that delivered aerated, twice filtered (10 μm and 1 μm diam, Hydrex filters) sea water from a 450 L header tank, *via* a peristaltic pump (323E, Watson Marlow, Falmouth, UK) 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 sea water, 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^{th} -14^{th} 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 sea water. Only individuals with a disc diameter > 4 mm (based on the size at which adults reach sexual maturity (O'Connor et al. 1983)), plus five intact arms were placed into containers (vol. = 250

mL, 3 indiv. *per* container) containing freshly collected sea water 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 set up

Of the 50 sediment aquaria prepared, 42 were selected for use in the experiment. Aquaria were haphazardly assigned to one of two O_2 levels (normoxia = 8.09 ± 0.06 mg L^{-1} or hypoxia = 3.59 ± 0.04 mg L^{-1}) and one of 6 organism density levels (0, 5, 9, 13, 17, 21 indiv. *per* aquaria, equating to 0, 500, 900, 1300, 1700, and 2100 indiv. 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 six normoxic aquaria and six 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

Dissolved oxygen (DO) levels were reduced using a computerised control system (Walchem Webmaster Series, MA, USA), which regulated the addition of O_2 free

224 nitrogen gas to large header tanks (vol. 450 L) in order to purge the water of oxygen.
225 Modified water from these header tanks was then supplied to the experimental
226 aquaria *via* a peristaltic pump. The seawater within the header tanks and the
227 experimental aquaria were monitored daily for DO, temperature, salinity and pH
228 using a multiprobe (9828, Hanna Instruments, RI, USA). Within the normoxic
229 experimental aquaria the average DO of sea water was recorded as 8.09 ± 0.06
230 (mean ± 95 % CI), and the sea water in the hypoxic aquaria DO level was 3.59 ±
231 0.04 (mean ± 95 % CI). Experimental seawater conditions are documented in full in
232 Calder-Potts et al. (2015). Ample experimental evidence exists to challenge the
233 traditional hypoxic level of 2.0 mg O_2 L^{-1}, as insufficient to detect the onset of hypoxia
234 impacts for many organisms (Vaquer-Sunyer & Duarte 2008, Seibel & Childress
235 2013). Consequently, in this experiment a higher threshold of DO was used to
236 examine if any alterations in behaviour and functionality may occur.
237 Due to the large differences in brittlestar density within the aquaria, higher brittlestar
238 density treatments did have a slightly lower DO due to greater levels of organism
239 respiration. However, the differences were comparatively small and were unlikely to
240 have caused significant impacts to brittlestars. Within the normoxic aquaria, average
241 seawater DO within the lowest density treatment (5 indiv. *per* aquaria) was 8.22 ±
242 0.12 mg O_2 L^{-1}, whilst in the highest density treatment (21 indiv. *per* aquaria), DO
243 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
244 the hypoxic aquaria, average DO within the lowest density treatment (5 indiv. *per*
245 aquaria) was 3.78 ± 0.04 mg O_2 L^{-1}, whilst in the highest density treatment (21 indiv.
246 *per* aquaria), DO was 3.51 ± 0.08 mg O_2 L^{-1}. This is a difference in the means of 0.27
247 mg O_2 L^{-1}. Values are means ± 95 % CI.
248

Acquisition of bioturbation data

Image capture

Bioturbation data were acquired using a luminophore tracer technique (Mahaut & Graf 1987) and 2-D imaging under U.V. light to monitor the movement of luminophores over time. The luminophores (supplier, Partrac ltd., Glasgow, U.K.) used were chosen to match the sediment granulometry of the collection site (Cawsand, U.K.) and had a median grain size of 60 μm. The luminophore particles are naturally occurring quartz material coated with a fluorescent dye. Luminophores (0.2 g *per* cm^2 = 20 g *per* aquaria) were added to the experimental aquaria 5 d in advance of their allocated sampling day (T0, T6, T10 or T14) resulting in a staggered addition of luminophores across the experimental period. Luminophores were added to each aquarium by evenly pouring them into the overlying water. Settlement of luminophores took approximately 1 h, during which time water circulation to the aquaria was ceased.

Each aquarium was then photographed once every 24 h (± 1 h) for a total of 6 d ($n_{\text{images } per \text{ aquarium}}$ = 6). 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 was illuminated by a 8 W ultra violet (UV) light (see Schiffers et al. 2011, supporting material, figure 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). 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 (sized 3888 x 2592 pixels). After each photograph session, aquaria were returned to the experimental system and re-connected to their respective flow–through water treatment. The sixth and final photograph for each aquaria occurred on a sampling day (T0, T6, T10 or T14).

Image preparation and data extraction

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

299

300 *Quantifying bioturbation*

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

309

310 **Nutrient analysis**

311 Nutrient samples were taken from each aquarium on their designated sampling day
312 (T6, T10 or T14). Within each aquarium, water overlying the sediment and water
313 from the inflow pipe connected to the header tanks were sampled separately, both in
314 triplicate. Each individual sample (50 mL) taken was filtered through a 47 mm ø GF/F
315 filter and stored in an acid washed Nalgene bottle. In total, 150 mL (3 x 50 mL
316 samples) of water was collected for analysis from each aquarium and a further 150
317 mL of water was collected from each water inflow pipe. This created three paired
318 samples which were used to calculate nutrient fluxes within each aquarium. The
319 water samples collected from the overlying water within each aquarium were all
320 carefully taken at the same height above the sediment surface (1 ± 0.5 cm), but at 3
321 different points across the length of the aquarium (5, 10 and 15 cm). Samples were
322 stored and frozen at T = -20 °C until analysed using a segmented flow nutrient auto-
323 analyser (AAIII, SEAL Analytical, Fareham, UK). Standard methods were used to

determine ammonium, nitrate, nitrite, silicate and phosphate concentrations (Brewer & Riley 1965, Grasshoff 1976, Mantoura & Woodward 1983, Kirkwood 1989). Nutrient fluxes were calculated using Eq. (1) from Widdicombe and Needham (2007). Fluxes across the sediment-water interface provide an estimation of the net change of nutrient x within the experimental aquaria and give an indication of the alterations in biogeochemical cycling caused by a reduction in dissolved oxygen concentrations and also by changes in brittlestar activities and abundance.

$$F_x = \frac{(C_i - C_o) \times Q}{A} \qquad (1)$$

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 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. Ammonium flux data were the exception and could only be normalised using a 'sine' transformation. Each parameter was analysed using a general linear model analysis of variance (ANOVA), with 'water treatment'

348 (normoxic or hypoxic), 'brittlestar density' (0, 5, 9, 13, 17 and 21 indiv. *per* aquaria),
349 and 'experimental time' (0, 6, 10 and 14 d) as the factors. Prior to analyses of
350 nutrient flux data within the experimental aquaria, nutrient measurements originating
351 from the header tanks were tested for 'tank effects'. Header tank nutrient data could
352 not be normalised using any transformation and was analysed using the non-
353 parametric Mann-Whitney U rank sum test.
354 The treatments containing no *A. filiformis* (i.e. a brittlestar density of zero) were
355 excluded from analyses on maximum luminophore depths (MLD) and % of surface
356 sediment reworked (% SSR) because, as expected, luminophores were not
357 disturbed or bioturbated within these treatments. By excluding the zero density
358 treatment, MLD and % SSR relationships with brittlestar density are not artificially
359 strengthened or skewed due to the addition of a zero activity data point due to no
360 brittlestars being present. The zero brittlestar density treatments were included in the
361 nutrient flux analyses because they provide insight into background nutrient cycling
362 rates in the absence of *A. filiformis*.
363

364 **RESULTS**

365 **Bioturbation activity**

366 *Maximum luminophore depths (MLD)*
367 The average maximum luminophore depth (MLD) measured across all aquaria
368 (excluding the zero density treatment) was 7.99 ± 0.57 cm (mean ± 95% CI).
369 Analyses revealed no significant effects of the experimental parameters on MLD
370 (Table 1 a).
371

372 *Percentage of sediment surface reworked (% SSR)*

In both the normoxic and hypoxic water treatments, the percentage of sediment surface reworked (% SSR) was significantly greater as brittlestar density increased (Figure 1, Table 1 b). There was also a significant effect of 'experimental time' whereby on average, in both water treatments, less sediment surface was reworked the longer the brittlestars remained in the experimental system (Table 1 b). For example, the average % SSR across both water treatments and all density treatments at T0 was 41.67 %, which decreased to 30.56 % at T6, 28.02 % at T10 and 22.49 % at T14. 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 1 b). For example, the largest differences in % SSR between the normoxic and hypoxic aquaria occur in the highest brittlestar density treatment (21 indiv. *per* aquaria) at T6 and T14. At T6 within the normoxic aquaria % SSR = 54.80 %, whilst in the hypoxic aquaria % SSR = 29.68 %. At T14 within the normoxic aquaria % SSR = 64.16 %, whilst in the hypoxic aquaria % SSR = 26.10 %. There were no significant effects of 'water treatment' in isolation and no interaction effects between 'water treatment' and 'time' (Table 1 b).

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 sea water from the same source (Table 2). Consequently, header tank nitrate and phosphate data were examined in greater detail.

398

399 *Header tank nitrate*

400 The differences in nitrate concentrations between the two header tanks started at
401 T10 and increased with experimental time, with the largest differences occurring at
402 T14. Nitrate concentration within the normoxic header tank at T10 was 6.46 µM (±
403 0.080) and the corresponding hypoxic nitrate concentration was 5.91 µM (± 0.059), a
404 decrease of 8.5 % (Mann-Whitney U statistic = 1.00, t = 494.00, n = 18, p = < 0.001).
405 At T14 nitrate concentration within the normoxic header tank had increased to 7.14
406 µM (± 0.12), whilst nitrate concentrations in the hypoxic header was 4.71 µM (±
407 0.24), a decrease of 34.0 % compared to the normoxic tank (Mann-Whitney U
408 statistic = 0.00, t = 153.00, n = 18.00, p = < 0.001).

409

410 *Header tank phosphate*

411 Phosphate concentrations between the normoxic and hypoxic header tanks
412 remained closely matched until T14. At T14, phosphate concentration in the
413 normoxic header tank was 0.27 µM (± 0.015), whilst concentrations in the hypoxic
414 header tank were significantly lower at 0.17 µM (± 0.032), a decrease of 37.0 %
415 (Mann-Whitney U statistic = 35.00, t = 460.00, n = 18, p = <0.001).

416

417 *NO_x fluxes in experimental aquaria*

418 To investigate the effects of hypoxia, brittlestar density and time within the
419 experimental aquaria, combined nitrate and nitrite measurements (hereafter known
420 as NO_x) were examined. During the experiment NO_x influx (from the overlying water
421 into the sediment) predominantly occurred in both the normoxic and hypoxic aquaria
422 (Figure 2 a - c). Analyses revealed that no significant effects were detected between

423 'water treatments', 'brittlestar density' and 'experimental time', however there was a
424 significant interaction effect between 'water treatment' and 'experimental time' (Table
425 3 a). This is due to the slight increase in NO_x flux into the sediment within the
426 normoxic aquaria after 14 d experimental exposure (Figure 2 c).
427
428 *Ammonium fluxes in experimental aquaria*
429 In aquaria containing no brittlestars there were minimal amounts of ammonium flux
430 but efflux of ammonium consistently occurred in aquaria that contained brittlestars,
431 irrespective of the different O_2 regimes (Figure 2 d - f). Analyses revealed that 'water
432 treatment', 'brittlestar density', 'experimental time' and their interactions did not
433 significantly affect ammonium flux (Table 3 b). However, Figure 2 (f) indicates that
434 ammonium efflux at T14 has increased in the aquaria exposed to hypoxic seawater
435 that contain brittlestar densities of 13, 17 and 21 indiv. *per* aquaria. A subsequent
436 GLM ANOVA was conducted on T14 ammonium flux data from the high brittlestar
437 density treatments (13, 17 and 21 indiv. *per* aquaria). At T14, within the high
438 brittlestar density treatments, ammonium efflux was significantly greater within the
439 hypoxic aquaria compared to the normoxic aquaria (Figure 2 f, Table 3 c).
440
441 *Ratios of NH_4^+ : NO_x*
442 Concentrations (μM) of ammonium (NH_4^+) and NO_x in the experimental aquaria are
443 presented as ratios [NH_4^+ **:** NO_x] (Fig. 24 g – i). Comparing ratio concentrations of
444 NH_4^+ and NO_x better convey which sedimentary processes, such as nitrification,
445 ammonification and denitrification, are favoured within the experimental aquaria (Fig.
446 2 g - i). A higher ratio value indicates greater NH_4^+ concentrations, favouring
447 processes that produce NH_4^+ such as nitrate ammonification, or processes that have

448 decreased nitrate and nitrite such as denitrification. A low ratio value indicates
449 greater NO_x concentrations, and favours processes that produce nitrite and nitrate
450 and decrease NH_4^+, such as nitrification. 'Water treatment', 'brittlestar density' and
451 'experimental time' all had a significant effect on the NH_4^+ **:** NO_x ratios, with the
452 normoxic aquaria displaying lower NH_4^+ **:** NO_x ratios compared to the hypoxic
453 aquaria, with differences increasing over the experimental time period (Table 3 d). At
454 T6, normoxic NH_4^+ **:** NO_x ratios were an average of 6.07 % lower than the hypoxic
455 aquaria ratios. At T10, this difference increased to 32.38 % and at T14, the normoxic
456 NH_4^+ **:** NO_x ratios were an average of 51.35 % lower than the hypoxic aquaria. At T6
457 and T10, NH_4^+ **:** NO_x ratios increase steadily with brittlestar density (Figure 2 g, h). At
458 T14, the normoxic NH_4^+ **:** NO_x ratios peak at brittlestar density 9, and slightly
459 decrease and plateau at the higher brittlestar density treatments (Figure 2 i). Within
460 the hypoxic treatment at T14, NH_4^+ **:** NO_x ratios remain similar to normoxic levels but
461 only in the low density treatments (0 – 9 indiv. *per* aquaria). In the high density
462 treatments (13 – 21 indiv. *per* aquaria) NH_4^+ **:** NO_x ratios increase (Figure 2 i). The
463 interactions between 'water treatment' and 'brittlestar density' and 'water treatment'
464 and 'experimental time' had no significant effect on NH_4^+ **:** NO_x ratio data (Table 3 d).
465
466 *Phosphate flux*
467 Phosphate influx primarily occurred throughout the experimental period, but a certain
468 degree of variability was observed within the data, with some points indicating
469 phosphate efflux (Figure 3 a - c). There were no significant effects of any
470 experimental parameter on phosphate flux (Table 3 e).
471
472 *Silicate flux*

473 At T6, silicate efflux consistently occurred in brittlestar density treatments of nine
474 indiv. *per* aquaria or greater, irrespective of water treatment. After T6, variability in
475 the silicate flux results increased, resulting in some data points representing silicate
476 efflux and others representing silicate influx (Figure 3d - f). Analyses using all of the
477 silicate data revealed that 'brittlestar density' significantly affected silicate flux (Table
478 3 f). Figure 3 indicates that higher density treatments increased the efflux of silicate.
479 There was also an interaction effect between 'water treatment' and 'experimental
480 time', whereby, similarly to ammonium, silicate efflux within hypoxia at T14, in the
481 high brittlestar density treatments increased (Table 3 f). Further analyses focusing on
482 silicate flux at T14 within the high brittlestar treatments (13, 17, and 21 indiv. *per*
483 aquaria), revealed that silicate efflux was significantly greater in aquaria exposed to
484 hypoxia compared to the corresponding normoxic treatment (Figure 3 f, Table 3 g).
485

486 **DISCUSSION**

487 Exposure of *Amphiura filiformis* to moderate hypoxia for 14 days significantly
488 increased ammonium (NH_4^+) and silicate (SiO_4^{4-}) efflux and caused an increase in
489 NH_4^+: NO_x ratios when brittlestar densities were high (> 1300indiv. m^{-2}). Additionally,
490 there were idiosyncratic alterations in brittlestar activity (in terms of sediment surface
491 reworked) with significant interaction between the 'water treatments' and 'brittlestar
492 density'. There are several possible explanations for these results: the impact of
493 moderate hypoxia on individual *A. filiformis* was so small it only became detectable
494 at high densities; and/or there was an interaction between high-density aggregations
495 and low dissolved O_2 that exacerbated the effects of hypoxia. We were unable to
496 identify which scenario was most likely to have initiated the observed changes in
497 brittlestar activity and behaviour, but either way our results demonstrate a potential

498 impact on this species from a mild level of hypoxia when living in dense
499 aggregations.
500 In earlier work, based on the same experiment, Calder-Potts et al. (2015) found that
501 prolonged hypoxia (>14 d) resulted in reduced respiration rates and hindered female
502 oocyte growth and development, but brittlestar density had no effect on the
503 physiological parameters measured. They concluded that during hypoxia *A. filiformis*
504 may strategically allocate its energy into locomotory arm movements to increase
505 burrow irrigation rates and prevent the build-up of toxins. This conclusion supports
506 the results presented here, with brittlestars in the high density and longest incubation
507 treatments potentially increasing burrow irrigation rates, explaining in part, the rise in
508 NH_4^+ and SiO_2 efflux and alterations in sediment surface bioturbation patterns. This
509 also demonstrates that individuals of *A. filiformis* have considerable tolerance to
510 short-term hypoxia, probably due to their life-mode, natural habitat and potential
511 exposure to diel-cycling in changing DO conditions.
512
513 **Bioturbation of *Amphiura filiformis* under normoxic conditions**
514 Under normoxic conditions, there was a consistently positive relationship between
515 brittlestar density and the percentage of sediment surface reworked (% SSR). . At all
516 densities, brittlestars appeared to continue with routine burrow maintenance, with
517 visible excavated mounds, feeding arms protruding, and all individuals buried within
518 the sediment. Therefore it is reasonable to assume that each individual (which were
519 similar in size) may have equally contributed to surface sediment bioturbation
520 activities, producing the observed additive relationship with density. Previous
521 measurements of % SSR by *A. filiformis* from the same location, measured at natural
522 field densities, (214.28 indiv. *per* m^{-2}) over the same incubation time, ranged

523 between 1 – 27 % (Queirós et al. 2015). This is comparable to the % SSR
524 measurements for our lowest experimental densities of 500 – 1300 indiv. m^{-2}, with a
525 mean of 5 - 27 % SSR respectively. However, when calculated *per* individual, our
526 results showed lower sediment surface mixing than found by Quieros et al. (2015).
527 Several factors could explain these differences including confinement within aquaria,
528 deployment into mesocosm conditions, the use of sieved homogenised sediment,
529 and/or an increase in brittlestar densities compared to the field location. We
530 recognise that there is a considerable difference between brittlestars natural field
531 density (214.28 indiv. *per* m^{-2}, reported in Queirós et al. (2015)) and even the lowest
532 aquairium densities (500 indiv. m^{-2}) used in this experiment. However, the current
533 experiment was also designed to allow for robust examination of the biological
534 effects of hypoxia on *A. filiformis*, (results reported in Calder-Potts et al. 2015), 500
535 indiv. m^{-2} (equating to 5 indiv. *per* aquaria) was the lowest manipulated density
536 treatment we could use during this experiment. Although this raises questions about
537 the effects of increasing population density when brought into the laboratory and the
538 ecological relevance of this experiment to the specific *A. filiformis* population at
539 Cawsands, Plymouth, the range of brittlestar densities used here are comparable to
540 other *A. filiformis* populations found across Europe (e.g. O'Connor et al. 1983, Sköld
541 et al. 1994, Rosenberg et al. 1997, Gilbert et al. 2003, Solan et al. 2004b).
542 Additionally, in order to asertain how population density may affect brittlestar
543 functionality or resilience, the experiment needed to contain a range of brittlestar
544 densities above (or indeed below) the natural field densities. Thus the densities used
545 here allow for the controlled testing of hypothese within a carefully monitored
546 mesocosm environment, and provide valuable data that are widely applicable to *A.*
547 *filiformis* in general, rather than to the specific population used within this study.

548 In acknowledging this discrepancy in densities, we also need to consider the
549 potential differences in mesocosm experiments compared to natural environments.
550 For example, previous laboratory experiments have shown that once *A. filiformis*
551 buries itself, it can remain within the burrow cavity for weeks or even months if
552 conditions are favourable (Woodley 1975). Other experiments have shown that *A.*
553 *filiformis* can exhibit density-dependent migration, moving both within and on top of
554 the sediment to less populated areas, given the space to do so (Rosenberg et al.
555 1997). Observations of a natural population have shown that *A. filiformis* individuals
556 can distribute themselves in alternating patterns of disc chamber placements such
557 that they are one shallow, one deep; ranging from depths of 2.0 to 6.5 cm (O'Reilly et
558 al. 2006). Clearly it is difficult to pinpoint the exact effects of being confined within
559 aquaria, but it is likely that experimental procedures limit migratory movements within
560 sediments, which could affect optimal dispersal patterns. Despite this, we would still
561 expect bioturbation activities for burrow maintenance, irrigation and feeding to be
562 maintained. During this experiment, food availability was comparable to levels within
563 the local environment (see Calder-Potts et al. 2015), water velocity within aquaria
564 was low, as *per* the collection site (Uncles & Torres 2013), and conditions between
565 the normoxic and hypoxic aquaria (except DO levels) were comparable, all of which
566 strengthen our confidence in the results.
567
568 Experimental time significantly affected the % SSR, with decreased surface mixing,
569 in both water treatments, as the experiment progressed. This is likely due to the time
570 the brittlestars spent within aquaria prior to luminophore addition: Although T0
571 brittlestars were allowed a 6 h period before luminophores were added, they were
572 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 days 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 *Amphiura 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 T10the 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 sightings of individuals on the sediment surface were observed. Although the experiment was not monitored during night-time, 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 bioirrigation 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

598 stages of moderate hypoxic impacts and could underlay the differences in surface
599 sediment bioturbation patterns.
600 The luminophore imaging technique provided no evidence that moderate hypoxia
601 affected the maximum burrow depths of *A. filiformis*. This is somewhat surprising
602 given that previous studies (e.g. Sturdivant et al. 2012) have shown a relationship
603 between hypoxia and burrowing depth. However, if brittlestar (or disc chamber)
604 placement had moved closer to the sediment surface during the current hypoxic
605 exposure, it is possible that remnant burrows, which were formed prior to hypoxic
606 exposure, were still present and some tracer particles could have found their way
607 into these now unoccupied burrow structures. Although some brittlestars were
608 occasionally spotted on the sediment surface for brief periods of time, it is also
609 possible that the hypoxic treatment level used here was not severe enough to reduce
610 burrow depths.
611

612 **Cycling of NO_x and NH_4^+ during normoxia**

613 The majority of recycled N released from the sediments to the overlying water is in
614 the form of ammonium (NH_4^+), which is generally regenerated from the
615 decomposition and deamination of organic matter. It then passes from the sediments
616 to the overlying water *via* diffusion or advection (bio-irrigation), where it can be
617 assimilated by phytoplankton (Kemp et al. 1990). Before it escapes the sediments,
618 and when oxygen is present, a portion of this NH_4^+ is oxidised to NO_3^- (nitrate), a
619 process known as nitrification. NO_3^- can then be used by denitrifying bacteria (Kemp
620 et al. 1990).
621 In our experiment, NO_x influx and NH_4^+ efflux were persistent features and our data
622 agrees with previous studies using sediments collected from nearby sites within

623 Plymouth Sound. These previous studies have documented sediments acting as a
624 source of NH_4^+ and a sink for NO_x (Wood et al. 2009, Murray et al. 2013), and
625 suggested that rates of nitrification were insufficient to totally support levels of
626 denitrification with the sediment. Formal statistical analysis suggested that under
627 normoxic conditions, 'brittlestar density' had no significant effect on the sediment
628 uptake of NO_x or the release of NH_4^+. Other mesocosm studies using similar
629 sediment type and densities of *A. filiformis,* also found that brittlestar density had no
630 significant effects on NO_x or NH_4^+ fluxes under control conditions (Wood et al. 2009,
631 Murray et al. 2013). However, in the current study an increase in the ratio of NH_4 to
632 NO_x in the overlying water, indicated that there was a significant shift in the balance
633 between these nutrients at higher brittlestar densities. The balance between NH_4 and
634 NO_x is set by a number of interdependent biogeochemical processes occurring both
635 in the sediment and the overlying water. Small changes in these individual processes
636 may be not be statistically significant but when combined in an integrative measure,
637 such as the NH_4:NO_x ratio, significant impacts may become detectable. These
638 impacts may also build up over time making differences more apparent towards the
639 end of the exposure experiment, as was seen in the current study. If *A. filiformis*
640 either had no impact on any N-cycling processes or had an equal impact on all N-
641 cycling processes, you would expect the NH_4:NO_x ratio to remain constant. In the
642 current study this ratio increased with brittlestar density suggesting the presence and
643 activities of *A. filiformis* was favouring processes that produced NH_4^+ (e.g. excretion
644 of metabolic ammonium by the brittlestars or from microbes) and/or removed NO_x
645 (e.g. denitrification) over those processes that oxidised NH_4^+ and produced NO_x (e.g.
646 nitrification and, to some extent anammox). This does not mean that *A. filiformis*
647 activities only stimulated ammonium production and NOx oxidation, it is likely that it

also stimulated ammonium oxidation but to a lesser extent. It is reasonable to expect NH_4^+ production to increase with brittlestar density as excretion products rise and bacterial mineralisation of organic matter could intensify as burrow structures increase in numbers and surface area (Papaspyrou et al. 2005). Bacterial abundance and activity can be 10-fold higher in burrow walls compared to the surrounding environment, aiding 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 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 suggest that *A. filiformis* is 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 has reduced the sediment uptake of NOx, reduce the release of ammonium and therefore lower 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 way from processes that produce ammonium 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 maybe this is only the case above certain densities of bioturbators and in low density areas the main impact of bioturbation could be to increase ammonium 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

673 For the first 2 sampling points (T6, T10), there was no difference between the
674 normoxic and hypoxia treatments in terms of the effects of *A. filiformis* density on
675 NH_4^+ release, NO_x uptake or NH_4^+ : NO_x ratio. At the final sampling point (T14),
676 however, high brittlestar densities (≥1300 indiv. m^{-2}) produced a NH_4^+ efflux in
677 hypoxia treatments which was significantly greater than in normoxic conditions. With
678 little change occurring in the NOx uptake rates, this increase in ammonium release
679 also drove a large increase in the NH_4^+ : NO_x ratio. This result is supported by
680 Villnäs et al. (2012) who reported that an increase in the duration of hypoxic
681 exposure significantly increased the efflux of NH_4^+. Whilst it is difficult to separate out
682 how hypoxia, bioturbation, brittlestar excretion and bacterial remineralization are
683 independently affecting NH_4^+ fluxes, results from Villnäs et al. (2012) also highlighted
684 the importance of considering benthic abundance and biomass when studying N-
685 cycling in sediments. Calder-Potts et al. (2015) showed that hypoxic exposure
686 resulted in a decrease in oxygen uptake rates by brittlestars indicating that metabolic
687 activity had decreased. Therefore, it is possible that the observed increases in NH_4^+
688 within the high density treatments, were due to excretion processes linked to
689 increased brittlestar biomass and through bio-irrigation of burrow structures, which
690 enhanced the advection of NH_4^+ into the overlying water.
691 Additionally, as was generally seen under normoxic conditions, microbial processes
692 responsible for NH_4^+ removal (i.e. nitrification) again appear to be unable to keep
693 pace with processes of anaerobic NH_4^+ generation, especially at high brittlestar
694 density treatments, and thereby cannot maintain the balance that was observed
695 under normoxic conditions. However, our data would also suggest that under
696 hypoxic conditions there was little evidence for enhanced stimulation of nitirifcation in
697 the densest aggregations of brittlestars, contrary to the situation observed in the

698 normoxic treatments. Although at the very highest brittlestar density there is some
699 evidence that this nitrification stimulation is beginning to occur. Consequently,
700 moderate hypoxia may have indirectly changed sedimentary microbial processes
701 and nutrient cycling by altering the behaviour of bioturbating organisms.
702
703 **Cycling of phosphate and silicate during normoxia**
704 In oxygenated conditions, and oxidised areas, such as burrow walls, PO_4^{3-}
705 (phosphate) sorption onto insoluble iron-manganese compounds can readily occur,
706 resulting in PO_4^{3-} influx into the sediment. The capacity of this process is determined
707 by the supply of Fe III in the sediment, with macrofaunal activities increasing the
708 amount of oxidised surface area available for PO_4^{3-} accumulation (Karlson et al.
709 2007). During our experiment, PO_4^{3-} primarily fluxed into the sediment, with
710 experimental parameters having no effect. Previous laboratory experiments using *A.*
711 *filiformis* and sediment from Plymouth Sound have reported contradictory results.
712 Wood et al (2008) found brittlestar density significantly increased sediment uptake of
713 PO_4^{3-}, whilst Murray et al (2013) found no significant effects on PO_4^{3-} flux when *A.*
714 *filiformis* was present compared to aquaria with no macrofauna. We suggest that,
715 given the high degree of variability within the PO_4^{3-} flux results, statistically significant
716 outcomes were unlikely.
717
718 SiO_4^{4-} (silicate) fluxes are thought to be a balance between oxic precipitations into
719 the sediment and excretion of SiO_4^{4-} rich waste from infauna and diatom
720 decomposition. Infaunal bioturbation activities contribute to nutrient fluxes through
721 promotion of an oxidised environment within the sediment adjacent to burrows within
722 which, compound oxidation may occur. In this experiment, the majority of aquaria

723 exhibited SiO_4^{4-} efflux, representing SiO_4^{4-} regeneration into the water column, but at
724 T10 some measurements indicated influx of SiO_4^{4-}, possibly explained by microalgal
725 uptake or adsorption processes at the sediment-water interface (Bartoli et al. 2009).
726 We found that brittlestar density significantly increased silicate efflux, possibly due to
727 increased mobilisation of silicate from porewaters (Bartoli et al. 2009). Previous
728 mesocosm experiments failed to detect a significant effect of *A. filiformis* on SiO_4^{4-}
729 flux (Wood et al. 2009), although this could be due to discrepancies in the amount of
730 organic matter and the degradation of benthic diatoms within sediments (Villnäs et
731 al. 2012) between the different studies.
732
733 **Cycling of phosphate and silicate during hypoxia**
734 In hypoxic conditions iron-bound PO_4^{3-} is generally released into the pore-water as
735 Fe(III) and is reduced to Fe(II), causing efflux of PO_4^{3-} (Belias et al. 2007). However,
736 in our experiment, PO_4^{3-} generally fluxed into the sediment, with 'water treatment'
737 having no effect. During our experiment, oxygen was limited (i.e. hypoxic) but not
738 unavailable (i.e. anoxic). It may be reasonable to assume that with some oxygen still
739 present in the hypoxic treatment, PO_4^{3-} adsorption onto ferric iron still occurred.
740 However, Villnäs et al. (2012) did not observe an increase in PO_4^{3-} efflux from
741 sediments exposed to hypoxia, and concluded that it was likely to be due to the low
742 content of PO_4^{3-} in the sediment. This may be true for our experiment, and could
743 mask any potential effects of hypoxia and brittlestars. Unfortunately, no analyses of
744 dissolved and particulate PO_4^{3-} in our sediments were carried out.
745
746 Similarly, to the case with ammonium, 14 d exposure to hypoxia, in high brittlestar
747 densities (1300 – 2100 indiv. m^{-2}), resulted in increased SiO_4^{4-} efflux compared to

748 the normoxic aquaria. In support of our results, previous studies have also
749 documented a rise in SiO_4^{4-} efflux during prolonged hypoxia (Villnäs et al 2012). It is
750 likely that a combination of bioturbation and bio-irrigation activities, degradation of
751 benthic diatoms, and release of SiO_4^{4-} from surfaces of hydrated oxides of iron due to
752 reduced oxic precipitation into the sediments, contributed to the SiO_4^{4-} efflux results
753 observed here (Villnäs et al. 2012).
754
755 **Experimental limitations**
756 *Changes in header tank nutrient concentrations*
757 Reductions of NO_3^- (nitrate) and PO_4^{3-} (phosphate) concentrations occurred within
758 the hypoxic header tank after 10 d (NO_3^-) and 14 d (PO_4^{3-}) experimental exposure,
759 despite both header tanks receiving filtered seawater from the same source.
760 Unfortunately, at the time of experimentation, samples to test for microbial growth
761 within the header tanks and aquaria were not taken. Despite these alterations,
762 nutrient flux measurements can be interpreted with confidence as they are calculated
763 using differences between the corresponding header tank and aquaria, thus, whilst it
764 may not be ideal to have differences in absolute values between the normoxic and
765 hypoxic header tanks, it is not critical to the comparisons of nutrient fluxes. For
766 example, the reduction in PO_4^{3-} concentrations within the hypoxic header tank at T14
767 did not cause any significant effects to the aquaria flux results, and the high levels of
768 variability in PO_4^{3-} flux data occurred within both the normoxic and hypoxic
769 treatments. Additionally, NO_3^- levels within the hypoxic header tank, the hypoxic NO_x
770 flux and NH_4^+ **:** NO_x ratio values can be evaluated with confidence for several
771 reasons: (1) There is no difference in NO_x fluxes between the hypoxic and normoxic
772 experimental aquaria at T10 (Figure 2 b), 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 (Figure 2 c) 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 reduction 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, this set up could be considered as pseudo-replication, 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; all equipment used was well "seasoned" and have 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 (MPB).

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

798 function. We have demonstrated how population density plays an important role in
799 determining the impacts of hypoxia: Dense patches of *A. filiformis* may exhibit larger
800 changes in behaviour and shifts in ecosystem function, compared to sparse patches,
801 as competition for oxygen and resources heighten, and O_2 diffusion into the
802 sediment reduces. The duration of a hypoxic event will also be important in
803 determining the individual and community effects, as different species have varying
804 thresholds and sensitivities to decreased O_2 concentrations. In the current study, and
805 previous work (Calder-Potts et al. 2015), *A. filiformis* exhibited an initial tolerance to
806 hypoxia, with significant effects only occurring after 14 days exposure. The results
807 from Calder-Potts et al. (2015) were consistent with the view that *A. filiformis* is an
808 'oxyconformer', reducing its metabolic rate with declining pO_2. However, when
809 oxygen is still available 'oxyconformers' can be behavioural 'oxyregulators',
810 attempting to maintain constant levels of oxygen in their burrows or body fluids
811 through compensatory adjustments in ventilatory efforts, such as burrow irrigation
812 (Pörtner 2010). This concept supports our conclusions that after prolonged hypoxic
813 conditions *A. filiformis* may have increased burrow irrigation rates in an attempt to
814 maintain oxygen levels within the burrow, and to avoid the build-up of toxins. This
815 subtle change in brittlestar behaviour under hypoxic conditions altered sediment
816 surface bioturbation patterns, and increased the efflux of NH_4^+, possibly reducing
817 nitrification rates. In areas where persistent hypoxia and reduced O_2 diffusion into
818 the sediments occur, inhibition of nitrification, and the subsequent decrease in
819 denitrification, could result in a build-up of nitrogen. This build up would further the
820 unpredictable eutrophication phenomena (Huesemann et al. 2002), which would
821 inhibit an area's ability to recover and rehabilitate, and cause a loss of biodiversity
822 and functionality.

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1037 Table 1. General linear model ANOVA for maximum luminophore depths (MLD) (a);
1038 percentage of sediment surface reworked (% SSR) (b); Degrees of freedom (DF);
1039 adjusted sum of squares (Adj SS); adjusted mean squares (Adj MS); F-value (F);
1040 and probability value (*p*). (Bold *p*-values indicate significance 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			

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1043 Table 2. Mann-Whitney U rank sum test on header tank nutrient concentrations (μM).

1044 N = 54; t-value (t); Mann-Whitney U statistic (MWU); probability value (*p*). (Bold *p*-

1045 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**

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Table 3. General linear model ANOVA for NO_x flux (a); ammonium (NH_4^+) flux (complete data set) (b); ammonium flux at T14 within the high brittlestar density treatments (13, 17, and 21 indiv. *per* aquaria) (c); NH_4^+ **:** NO_x ratios (d); phosphate (PO_4^{3-}) flux (e); silicate (SiO_4^{4-}) flux (complete data set) (f); and silicate flux at T14 within the high brittlestar density treatments (13, 17, and 21 indiv. *per* aquaria) (g). Degrees of freedom (DF); adjusted sum of squares (SS); adjusted mean squares (MS); F-value (F); probability value (*p*). (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) Ammonium flux					
Water treatment	1	0.12	0.12	0.23	0.640
Density	5	3.16	0.63	1.14	0.372
Time	2	0.15	0.07	0.13	0.875
Water treatment*Density	5	2.53	0.51	0.92	0.490
Water treatment*Time	2	0.82	0.41	0.74	0.489
Error	19	10.49	0.55		
Total	34	17.16			
(c) Ammonium flux (T14, high density treatments)					
Water treatment	1	58240.00	58240.00	9.18	**0.009**
Density	2	14844.00	7422.00	1.17	0.339
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) Phosphate flux					
Water treatment	1	16.75	16.75	0.73	0.402
Density	5	74.66	14.93	0.65	0.663
Time	2	39.76	19.88	0.87	0.434
Water treatment*Density	5	50.79	10.16	0.44	0.812
Water treatment*Time	2	0.13	0.06	0.00	0.997
Error	20	457.30	22.87		
Total	35	639.40			
(f) Silicate flux					
Water treatment	1	427.60	427.60	0.61	0.445
Density	5	17675.20	3535.00	5.02	**0.004**
Time	2	3106.10	1553.00	2.21	0.136
Water treatment*Density	5	1288.40	257.70	0.37	0.866
Water treatment*Time	2	10017.10	5008.60	7.11	**0.005**
Error	20	14082.80	704.10		
Total	35	46597.10			
(g) Silicate flux (T14, high density treatments)					
Water treatment	1	41393.90	413 93.90	17.30	**0.001**
Density (high)	2	146.80	73.40	0.03	0.970
Error	14	33507.40	2393.40		
Total	17	75048.10			

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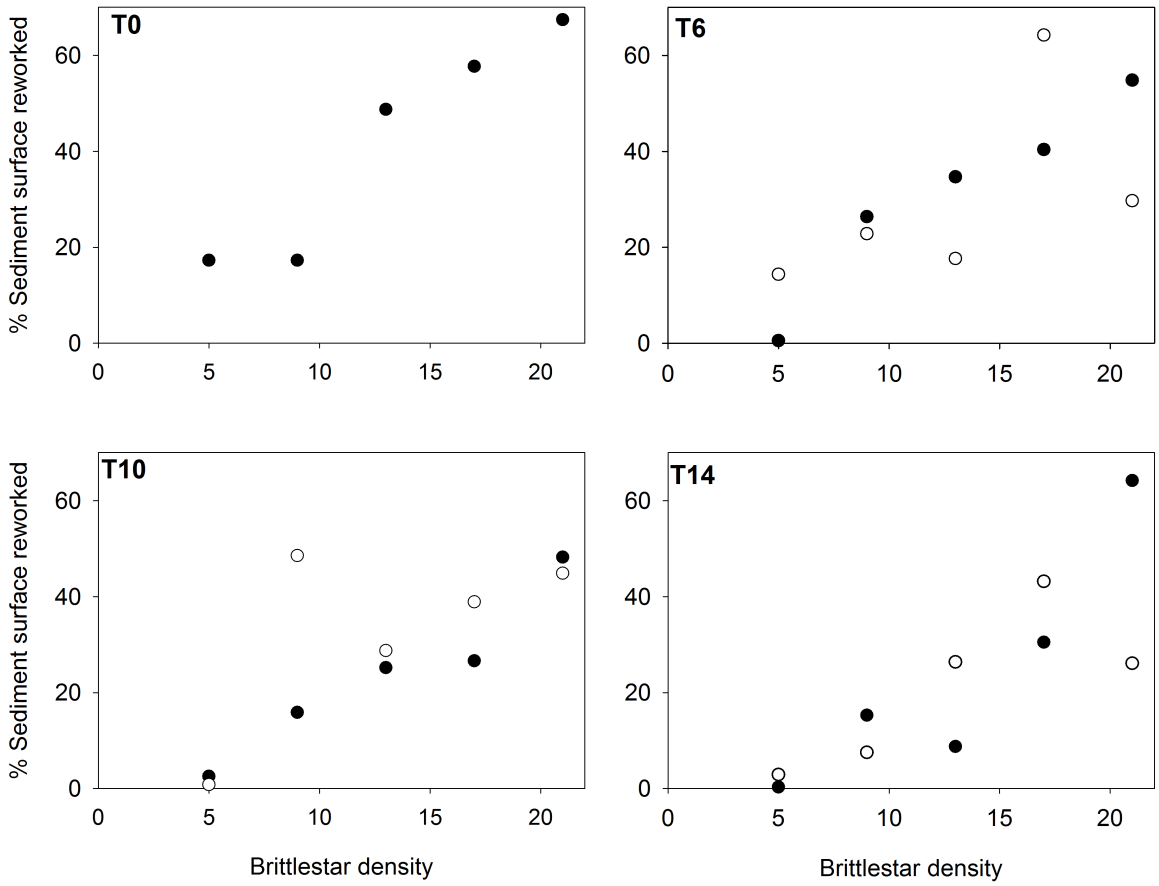

Figure 1. Percentage of sediment surface reworked (% SSR) (top 1 cm only) against brittlestar density at time points T0, T6, T10 and T14. Points represent individual aquaria, ● = normoxia, ○ = hypoxia.

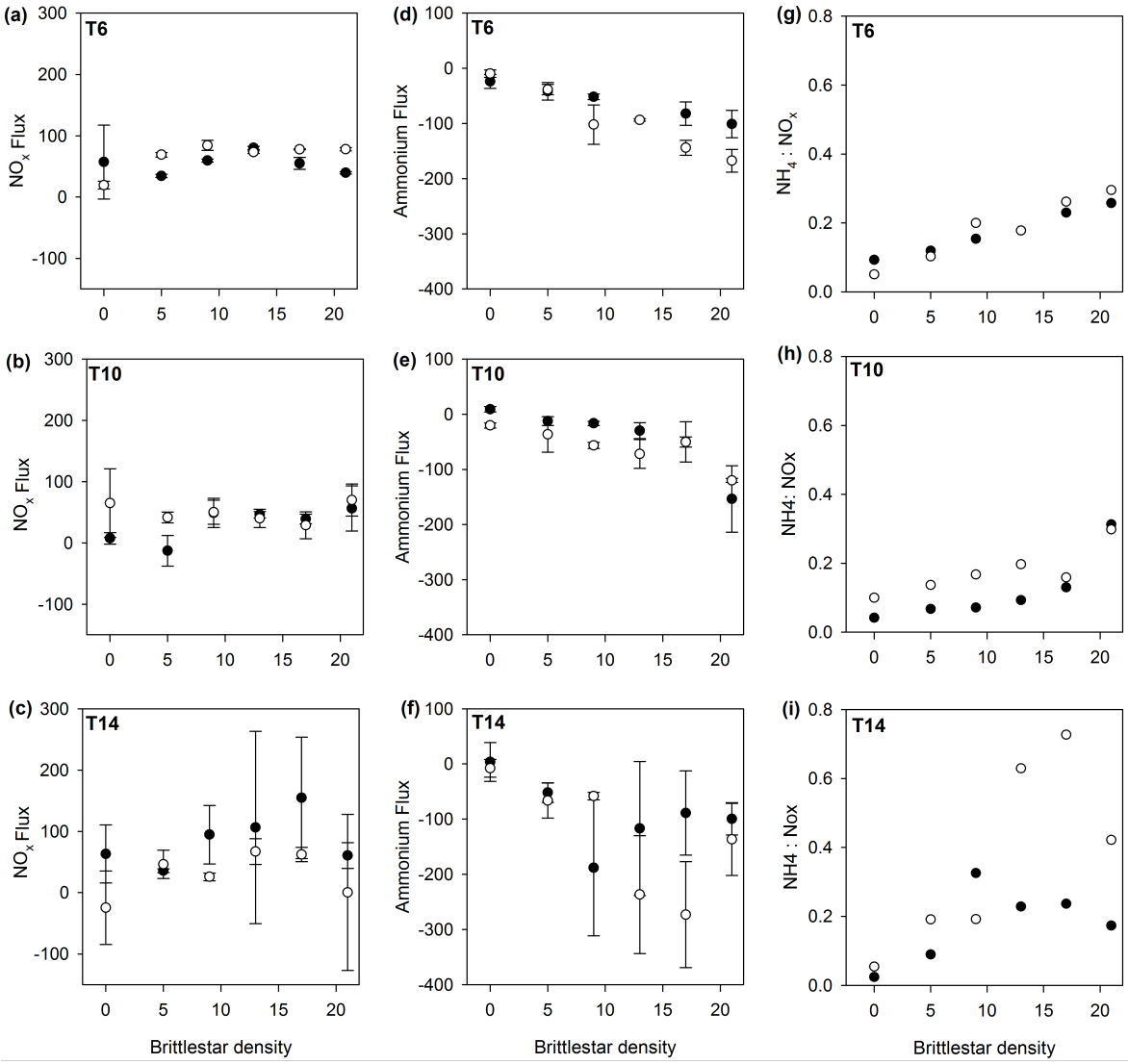

Figure 4: NO_x flux (µmol m^{-2} h^{-1}) (a - c), ammonium (NH_4^+) flux (µmol m^{-2} h^{-1}) (d – f) and NH_4^+ **:** NO_x ratios (g – i) in experimental aquaria at time points T6, T10 and T14. Data for NO_x and ammonium fluxes are means ± 95 % confidence intervals, NH_4^+ **:** NO_x ratio data calculated from mean concentrations. For NO_x and ammonium flux, positive results represent nutrient influx, whilst negative results represent nutrient efflux, ● = normoxia, ○ = hypoxia.

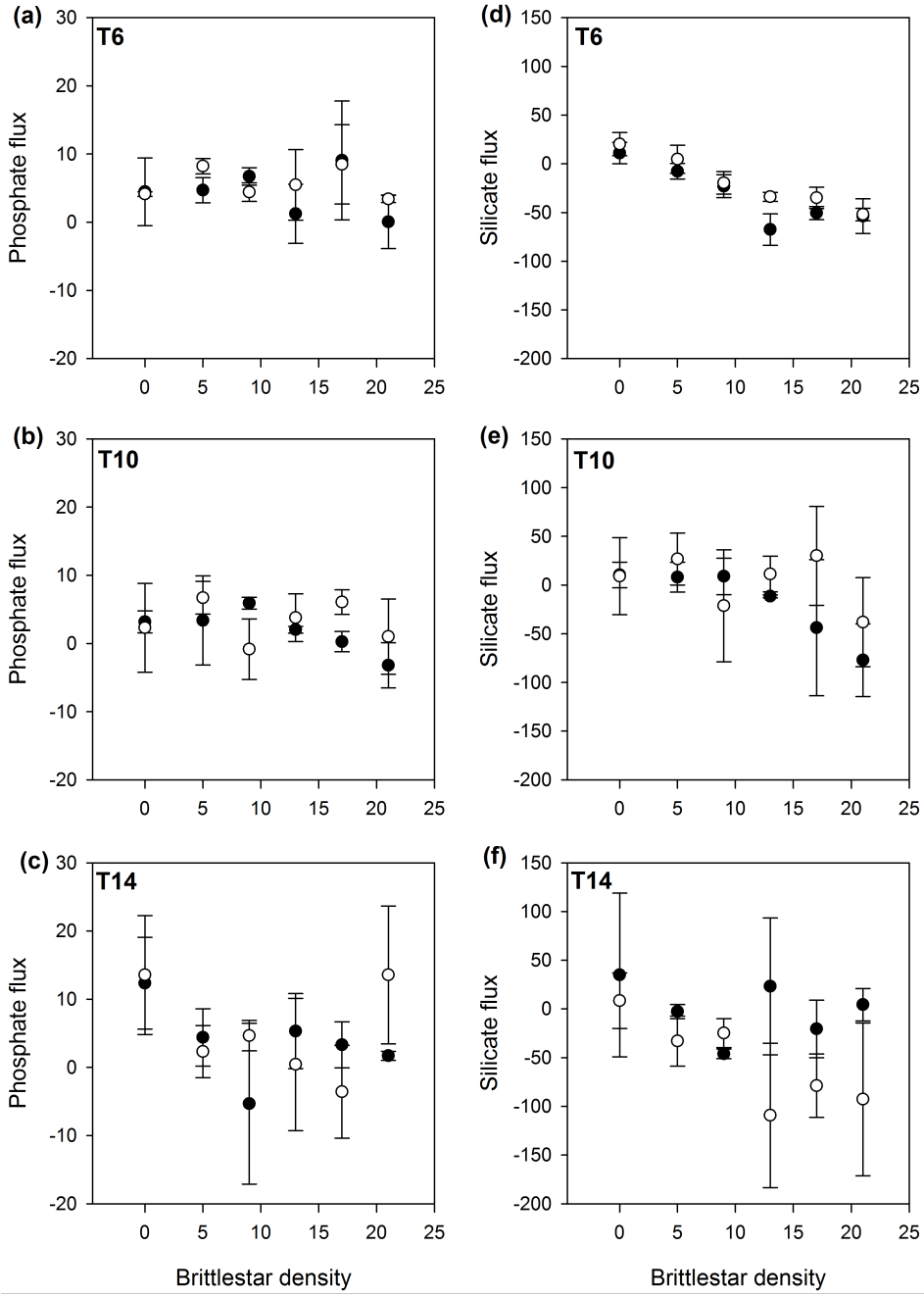

Figure 5: Phosphate (PO_4^{3-}) flux (µmol m^{-2} h^{-1}) (a – c) and Silicate (SiO_4^{4-}) flux (µmol m^{-2} h^{-1}) (d – f) in experimental aquaria at time points T6, T10 and T14. Data are means ± 95 % confidence intervals. Positive results represent nutrient influx, whilst negative results represent nutrient efflux, ● = normoxia, ○ = hypoxia.