2019-02-01

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Dale, H

10.1093/femsec/fiy234
FEMS Microbiology Ecology
Oxford University Press (OUP)

FEMS Microbiology Ecology, 95, 2019, fiy234

doi: 10.1093/femsec/fiy234
Advance Access Publication Date: 14 December 2018
Research Article

RESEARCH ARTICLE

Polychaete mucopolysaccharide alters sediment microbial diversity and stimulates ammonia-oxidising functional groups

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One sentence summary: Mucus secreted by invertebrates living in sediment can increase the abundance of nitrogen-processing microbial groups, but this invertebrate–microbe interaction is not currently considered in sediment nitrogen models.

Editor: Lee Kerkhof

ABSTRACT

Sediment nitrogen cycling is a network of microbially mediated biogeochemical processes that are vital in regulating ecosystem functioning. Mucopolysaccharides (mucus) are produced by many invertebrates and have the potential to be an important source of organic carbon and nitrogen to sediment microorganisms. At present, we have limited understanding of how mucopolysaccharide moderates total sediment microbial communities and specific microbial functional groups that drive nitrogen cycling processes. To start addressing this knowledge gap, sediment slurries were incubated with and without *Hediste diversicolor* mucus. Changes in dissolved inorganic nitrogen (ammonia, nitrite and nitrate) concentrations and bacterial and archaeal community diversity were assessed. Our results showed that mucopolysaccharide addition supported a more abundant and distinct microbial community. Moreover, mucus stimulated the growth of bacterial and archaeal ammonia oxidisers, with a concomitant increase in nitrite and nitrate. *Hediste diversicolor* mucopolysaccharide appears to enhance sediment nitrification rates by stimulating and fuelling nitrifying microbial groups. We propose that invertebrate mucopolysaccharide secretion should be considered as a distinct functional trait when assessing invertebrate contributions to sediment ecosystem function. By including this additional trait, we can improve our mechanistic understanding of invertebrate–microbe interactions in nitrogen transformation processes and provide opportunity to generate more accurate models of global nitrogen cycling.

Keywords: bioturbation; mucopolysaccharide; sediment; nitrogen cycling assemblages

Received: 10 July 2018; **Accepted:** 12 December 2018

INTRODUCTION

The nitrogen cycle is a globally vital network of biogeochemical processes that can often limit ecosystem productivity (Elser *et al.* 2007). Marine sediments have important roles in the nitrogen cycle because they provide up to 40% of the nitrogen required by shelf sea and coastal pelagic primary producers (Boynton and Kemp 1985). Sediment nitrogen transformations convert complex organic and inorganic nitrogen sources to more ecologically accessible forms (e.g. nitrate and ammonia), and are controlled by a series of redox-dependent biogeochemical processes that are performed by a range of microbial functional groups (Jetten 2008; Laverock *et al.* 2011). Generally, nitrification by ammonia- and nitrite-oxidising microbes occurs in the oxic sediment layer, with denitrification and other anaerobic pathways occurring in the deeper sub-oxic and anoxic zones (Vanderborght and Billen 1975).

Bioturbating invertebrates have significant impacts on sediment nitrogen cycling and sediment/seawater exchange (Laverock *et al.* 2011) because burrow ventilation and particle reworking activities redistribute organic matter, increase the sediment–water interface, and modify redox conditions (Kristensen *et al.* 2012). For example, ventilating behaviour by infaunal invertebrates can increase sediment oxygenation, which allows burrow walls to support greater abundances of aerobic nitrifying microbes and greater nitrification rates (Dollhopf *et al.* 2005; Satoh, Nakamura and Okabe 2007). Ventilation activity also increases the area of the oxic/anoxic interface in sediment, which promotes nitrification/denitrification (Sayama and Kurihara 1983; Rysgaard, Christensen and Nielsen 1995). Invertebrates with different burrowing activities (e.g. ventilation rates) can also have species-specific effects on nitrification rates and on the abundance ratio of ammonia-oxidising bacteria (AOB) and archaea (AOA) (Gilbertson, Solan and Prosser 2012). To gain a full mechanistic understanding of sediment nitrogen cycling and other sediment ecosystem functions, we must understand the specific links between all bioturbator activities and microbial processes (Graham *et al.* 2016).

Mucopolysaccharides (mucus) are complex polysaccharides containing amino groups that are secreted by invertebrates to aid locomotion and feeding, and to stabilise burrow structures (Wotton 2004). Mucus secretions are an important source of labile organic carbon and nitrogen to sediment microorganisms, and may be partly responsible for previously observed increases in microbial activity and nitrogen cycling associated with burrows (Aller and Yingst 1985; Aller and Aller 1986; Nielsen *et al.* 2004). Mucus lined burrows can support more abundant bacterial communities than the surrounding sediment (Papaspyrou *et al.* 2006), and there is potential for mucus to prime anaerobic nitrogen remineralisation (Hannides and Aller 2016) and to stimulate nitrification rates (Kristensen, Jensen and Andersen 1985). However, at present, the extents to which nitrogen cycling microbial communities or transformations are affected by invertebrate mucus secretions are unknown.

Here, we determined the impacts of mucus on the structure and function of sediment microbial communities and nitrogen cycling microbial groups, focusing specifically on aerobic nitrification processes. Sediment slurries were incubated with and without mucus obtained from the commonly occurring polychaete *Hediste diversicolor* which forms semi-permanent gallery burrows (Hale *et al.* 2014). We assessed the effect of mucus on inorganic nitrogen concentrations, total bacterial and archaeal abundance and diversity, and the abundance of AOB and AOA over time using linear-mixed effects models. We hypothesised that AOB and AOA populations would be stimulated by the presence of mucus and lead to increases in the production of nitrite (NO_2^-).

METHODS

Sediment (<3 cm depth) was collected in July 2016 from the Plym Estuary (UK) (50°22.281' N, 004°06.289' W) and sieved (500 μm) to remove macrofauna and detritus, before being settled in seawater over 7 days to ensure retention of the fine sediment fraction (<63 μm) (Sediment fraction used: 0–500 μm). Overlying seawater was removed, and the sediment homogenised before use. *Hediste diversicolor* were collected from the Orwell estuary (Online baits, Essex, UK), and acclimatised at ambient temperature for one week in a continuous flow seawater tank. To harvest mucopolysaccharide, 20 individuals were rinsed in UV-sterilised, filtered seawater and placed in a continuous flow seawater tank for 7 days. Rubber hose (internal diameter 10 mm) was laid on the bottom of the tank to act as a burrow mimic in the absence of sediment. Mucus was harvested from the outside and the inner 3 cm of the tubing using sterile forceps. Harvested mucus was washed in sterilised seawater and used to set up three sediment slurry treatments. The sediment-only treatment was prepared by mixing 2.7 g (wet weight) sediment with 5.4 ml artificial seawater (0.2 μm filtered, 35 psu) in 120 ml serum bottles (n = 3). The sediment–mucus treatment was prepared by mixing 0.3 g (wet weight) mucus with 2.7 g sediment and 5.4 ml artificial seawater (n = 3). The mucus-only treatment was prepared by mixing 0.3 g (wet weight) mucus with 5.4 ml of artificial seawater (n = 3). The bottles were plugged with cotton wool and incubated in the dark at 18°C (subtidal seawater temperature, July 2016) for 14 days with continual shaking to maintain the sediment suspension. Bottles were regularly weighed to assess evaporation with sterilised distilled water added to compensate for water loss (7.1% ± 1.1% of original water mass replaced with distilled water over the total incubation period).

Samples were taken at three points during the experimental period (T_0–T_2): Day 0 (30 minutes after setup), Day 7, and Day 14. Two 0.5 ml samples were taken from each treatment and centrifuged at 1677 × *g* for five minutes. The supernatant was removed for quantification of nitrogen compounds and the pellets were immediately stored at −20°C for carbohydrate quantification and DNA extraction. Carbohydrate concentration was measured using a phenol–sulfuric acid assay (Underwood, Paterson and Parkes 1995). Samples were weighed before 2 ml of distilled H_2O, 1 ml of 5% (v/v) aqueous phenol and 5 ml of concentrated H_2SO_4 were added. Absorbance was measured at 485 nm and calibrated using a glucose standard. Supernatant samples were filtered (0.2 μm) and diluted in low nutrient seawater (15 ml; North Atlantic Gyre 2015, 0.1 μm filtered, dark incubated) before quantification of NO_2^-, NO_3^- and NH_4^+ using standard autoanalyser protocols (Brewer and Riley 1965; Grasshoff 1976; Mantoura and Woodward 1983). Replicate samples of the initial starting sediment and *H. diversicolor* mucus (n = 4) were freeze-dried and analysed for %C and %N using a vario PYRO cube elemental analyser (Elementar Analysensysteme). DNA was extracted from 0.25 g sediment (wet weight) using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The DNA yield was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and stored at −20°C.

Q-PCR was used to determine the abundance of 16S rRNA genes, *amoA* genes and the bacterial *nirS* gene (see Supplementary Methods for full protocol and Table S2 (Supporting Information) for reaction efficiency data). Ten microlitre reactions

contained 5 μl 2× SensiFast SYBR No-ROXl master mix (Bioline, UK), 0.1 μl 10 pM forward and reverse primers, 1 μl template DNA and 3.8 μl molecular grade H_2O and were run in a Rotor-Gene 6000 (Corbett Life Science), with duplicate technical replicates for each sample. Results were converted from ng μl^{-1} to copy number.mgwwsediment^{-1}. Data is reported according to MIQE guidelines.16S rRNA gene sequencing was performed on the Illumina MiSeq platform using V6–V8 primer sets (Comeau, Douglas and Langille 2017), and sequences were analysed as previously published (Taylor and Cunliffe 2015; Taylor and Cunliffe 2017) (see Supplementary Methods for detail). Sequence data have been deposited in the European Nucleotide Archive (accession code PRJEB22034).

Data from the mucus-only controls were excluded from the analyses and used for comparative purposes only as high variability in the Q-PCR gene abundance data skewed the statistical trends (Figs S1–S9, Supporting Information). However, microbial community composition and nitrogen compound concentrations were similar to the sediment–mucus treatment or followed expected patterns. Changes in bacterial and archaeal community structure between treatments were calculated from weighted UniFrac distance matrices in R (version 3.2.2, R Development Core Team) using Permutational Multivariate Analysis of Variance (PERMANOVA, 999 permutations) and visualised using Multidimensional Scaling (MDS) techniques (vegan package, version 2.4–2; Oksanen *et al.* 2016). Relative abundances of taxa (>1% in at least one sample) and phylogenetic diversity (QIIME output) were also calculated. The identities of key taxa were confirmed using online BLAST searches. To assess more general changes in the presence and abundance of bacterial families and archaeal classes, ANOVA tables and Venn diagrams were produced in Calypso (Zakrzewski *et al.* 2017) from imported BIOM tables. This analysis was repeated with and without filtering to remove operational taxonomic units (OTUs) present at low levels in the community (<0.01% across all samples), and with and without the mucus only seawater control.

Linear mixed effect (LME) models were developed for each dependent variable (Table S1, Supporting Information), with mucus (2 levels: presence, absence) and time (3 levels: Day 0, 7, 14) treated as independent nominal variables. Relative abundance (%) was arcsine transformed. The use of LME models allowed bottle identity to be incorporated as a random effect (Zuur *et al.* 2009), and variance-covariate terms to be incorporated to account for any heteroscedasticity (Pinheiro and Bates 2000) (see Supplementary Methods). Spearman rank order correlations were run between each analysed gene, and the concentration of carbohydrates, NH_4^+, NO_2^- and NO_3^-. Statistical analyses were carried out using the nlme package (version 3.1–120, Pinheiro *et al.* 2013) in R (version, 3.2.2, R Development Core Team). R code is provided in the supplementary material.

RESULTS

Mucopolysaccharide degradation and microbial abundance

Elemental analysis showed that the *H. diversicolor* mucus (0.3 g) contained 0.007 g of C (17% dw) (583 μmol) and 0.001 g of N (2.5% dw) (71.4 μmol), with a C:N ratio of 7:1. The sediment (2.7 g) contained 0.028 g of C (2.4% dw) (2330 μmol) and 0.0026 g of N (0.22% dw) (182 μmol), so the introduction of mucus represents a 25% increase in C, a 38% increase in N, and altered the C:N ratio to 10:1. Sediment carbohydrate concentration was affected by the independent effects of mucus treatment and time (Fig. 1A; Table S1, Supporting Information; model 1). The addition of mucus raised the sediment carbohydrate concentration by 30% (from 5.30 ± 0.9 to 6.94 ± 1.0 glucose equivalents.mg wet weight sediment $^{-1}$; n = 9, day 0), and there was an overall decline in concentration over the incubation period.

Bacterial (Fig. 1B; Table S1, Supporting Information; model 2) and archaeal (Fig. 1C; Table S1, Supporting Information; model 3) abundances, determined by quantitative PCR (QPCR), were dependent on the interactive effect of mucus treatment and time. Bacterial abundance correlated positively with sediment carbohydrate concentration (Spearman Rank Correlation: r_s 0.7, *P* 0.045), and was 67% greater in the sediment–mucus treatment over the initial seven days of the incubation period. On Day 14, bacterial abundance had reduced to the same level observed in the sediment-only treatment. Similarly, archaeal abundance increased by 75% in the sediment–mucus treatment after seven days and then declined to similar levels as the sediment-only treatment. Unlike the bacterial community, there was no initial increase in archaea in the presence of mucus or significant correlation with carbohydrate concentration.

Microbial diversity

The diversity of bacterial (Fig. 2A; Table S1, Supporting Information; model 4) and archaeal (Fig. 2B; Table S1, Supporting Information; model 5) communities, determined by Illumina MiSeq 16S rRNA gene sequencing and Faith's index of phylogenetic diversity (Faith 1992), were also dependent on the interactive effect of mucus treatment and time. Bacterial diversity was initially reduced in the sediment–mucus treatment but returned to the levels observed in the sediment-only control within seven days. Conversely, archaeal diversity was lower in the presence of mucus than in the sediment-only control throughout the experimental period.

Mucus treatment and time also had an interactive effect on bacterial community structure, based on PERMANOVA of OTUs ($F_{1, 14}$ 40.04, *P* 0.001). Bacterial community structure was dependent on the presence of mucus throughout the incubation period (Fig. 2C), with an additional community composition shift over the initial seven days in both the presence and absence of mucus. Communities were dominated by the families *Rhodobacteraceae*, *Flavobacteriaceae*, unclassified *Bacteroidales* and unclassified *Chromatiales*, with the proportions of *Rhodobacteraceae* and *Flavobacteriaceae* in the bacterial community increasing by 100% in the presence of mucus (Fig. S3A, Supporting Information). PERMANOVA analysis of the archaeal community demonstrated no significant effect of mucus treatment or time (Fig. 2D). Overall, the archaeal community in the sediment-only controls was dominated by *Bathyarchaeota* and *Euryarchaeota*. In the sediment–mucus treatments, however, the community was dominated by Marine Group I, which continued to increase in abundance throughout the incubation period (Fig. S3B, Supporting Information).

Dissolved inorganic nitrogen (DIN)

Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were all dependent on the interactive effect of mucus treatment and time (Table S1, Supporting Information; models 6–8). Ammonium (NH_4^+) concentrations were similar in both treatments at the start of the incubation period, but in the final seven days there was an increase in the sediment-only treatment with no observable increase in the sediment–mucus treatment (Fig. 3A). Nitrite (NO_2^-) increased after seven days in the

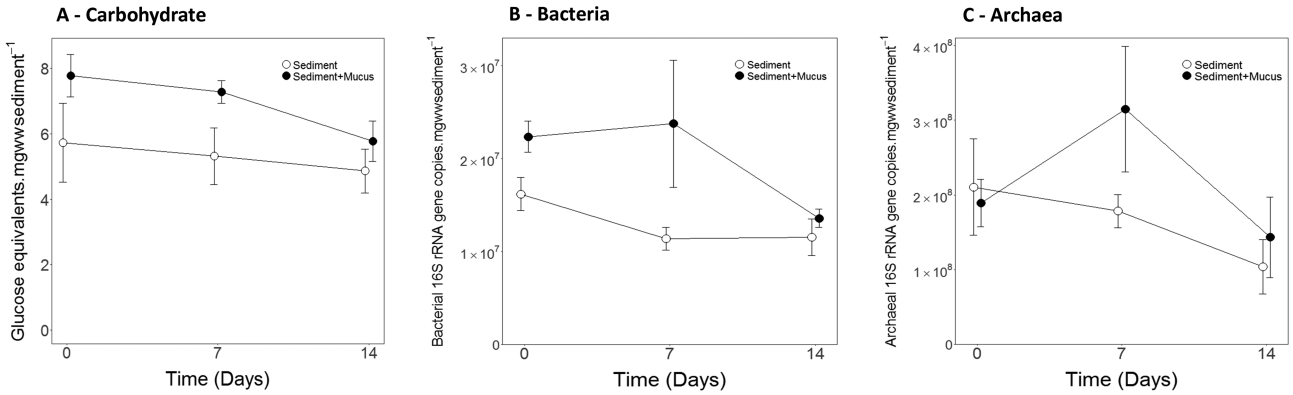

Figure 1. The effect of mucopolysaccharide on sediment carbohydrate concentration **(A)**, and bacterial **(B)**, and archaeal **(C)**, 16S rRNA gene copy numbers. Data shown are means ± SD (n = 3).

sediment–mucus treatment before returning to similar concentrations as the sediment-only treatment, where the concentration remained consistently low (Fig. 3B). Nitrate (NO_3^-) was also consistently low in the sediment-only treatment but increased steadily throughout the incubation period in the sediment–mucus treatment (Fig. 3C). After the initial seven days of the incubation, the sediment–mucus treatment contained 7.9 μmol of bioavailable nitrogen (total NH_4, NO_2, NO_3), which is 6.2 μmol more than the sediment-only treatment (1.7 μmol) and represents 8.7% of the total N available in the mucus.

Abundance of microbial nitrogen-cycling functional genes

AOB and AOA abundance, assessed by QPCR of *amoA* genes encoding ammonia monooxygenase, were affected by the interactive effect of mucus treatment and time (Fig. 4A and B; Table S1, Supporting Information; model 9–10). The abundance of both ammonia-oxidising groups initially increased in the sediment–mucus treatment before declining at the end of the incubation period, with AOA abundance reducing to the same levels observed in the sediment-only treatment. Additionally, the AOB:AOA abundance ratio increased from 1.0 to 6.2 in the presence of mucus (Fig. 4C; Table S1 (Supporting Information), model 11). The abundance of bacterial denitrifying groups, assessed by QPCR of *nirS* genes encoding nitrite reductase, was also dependent on the interactive effect of mucus treatment and time (Fig. 4D; Table S1 (Supporting Information), model 12), and again increased in the sediment–mucus treatment over the first seven days of the incubation period before declining to similar levels as the sediment-only treatment. Changes in carbohydrate concentration were positively correlated with both AOB and AOA abundance (r_s 0.54, *P* 0.022; r_s 0.57, *P* 0.013), and the AOB:AOA ratio (r_s 0.56, *P* 0.016). Abundance of the three-assessed functional genes and the AOB:AOA ratio also correlated positively with changes in both NO_2^- and NO_3^- concentrations ($P < 0.01$) (Fig. S7, Supporting Information).

Abundant nitrifying bacterial and archaeal taxa

The relative abundance of the nitrite-oxidising bacterial (NOB) family *Nitrospiraceae* was affected by the interactive effect of mucus treatment and time (Fig. S6A, Table S1, Supporting Information; model 13). The proportion of *Nitrospiraceae* in the community was initially similar in both treatments, but increased in the sediment–mucus treatment throughout the incubation period. This can mostly be attributed to the abundance of unidentified genera within this family, though identified *Nitrospira sp.* was present in low abundances (<0.05% relative abundance). The sequence data did not have sufficient resolution to assess the presence of comammox bacteria. Other known nitrifying families were either absent or were not abundant in any sample (<1%), but of these rarer families *Nitrosomonadaceae* (AOB) was 10 fold more abundant and *Nitrospinaceae* (NOB) was 2 fold more abundant in the sediment–mucus treatment than in the sediment-only treatment.

The proportion of the AOA Marine Group I was also dependent on the interactive effect of mucus treatment and time (Fig. S6B, Table S1, Supporting Information; model 14). The relative abundance of Marine Group I was greater in the sediment–mucus treatment than in the sediment-only treatment within 30 minutes of mucus addition, and continued to increase over the incubation period. The high abundance of this group was predominantly due to the dominance of a single OTU, which was homologous to *Nitrosopumilus sp.* PSO (Nitrosopumilaceae, Nitrosopumilales, Thaumarchaeota) (KX950759.1). At the genus level, this is supported by the immediate dominance of *Nitrosopumilus sp.* in the sediment–mucus treatment and the continued increase in relative abundance of this genus throughout the incubation period. Other known nitrifying archaeal taxa were not abundant in any sample (i.e. none > 1% relative abundance).

'Seeding' of microbial taxa from the *H. diversicolor* mucus microbiome

To determine whether mucus introduces novel nitrogen cycling microbial taxa to sediment, bacterial and archaeal communities were also assessed in an additional incubation containing only mucus and artificial seawater. Overall, mucus did not introduce novel nitrogen cycling bacterial taxa to the sediment. Immediately after the addition of mucus, 12 bacterial families were shared exclusively by the two mucus-amended treatments (Fig. S8A, Supporting Information), but these shared families are not currently known to be ammonia or nitrite oxidising. Known nitrogen cycling bacterial families were present in all three treatments (e.g. *Nitrosomonadaceae, Nitrospinaceae, Nitrospiraceae*), and the presence of mucus did increase the abundance of two of these nitrogen cycling bacterial families (ANOVA: *Nitrospiraceae*: $P = 0.0013$, *Nitrosomonadaceae*: $P = 0.0037$). However, only the less abundant *Nitrosomonadaceae* family (<0.6% of community in

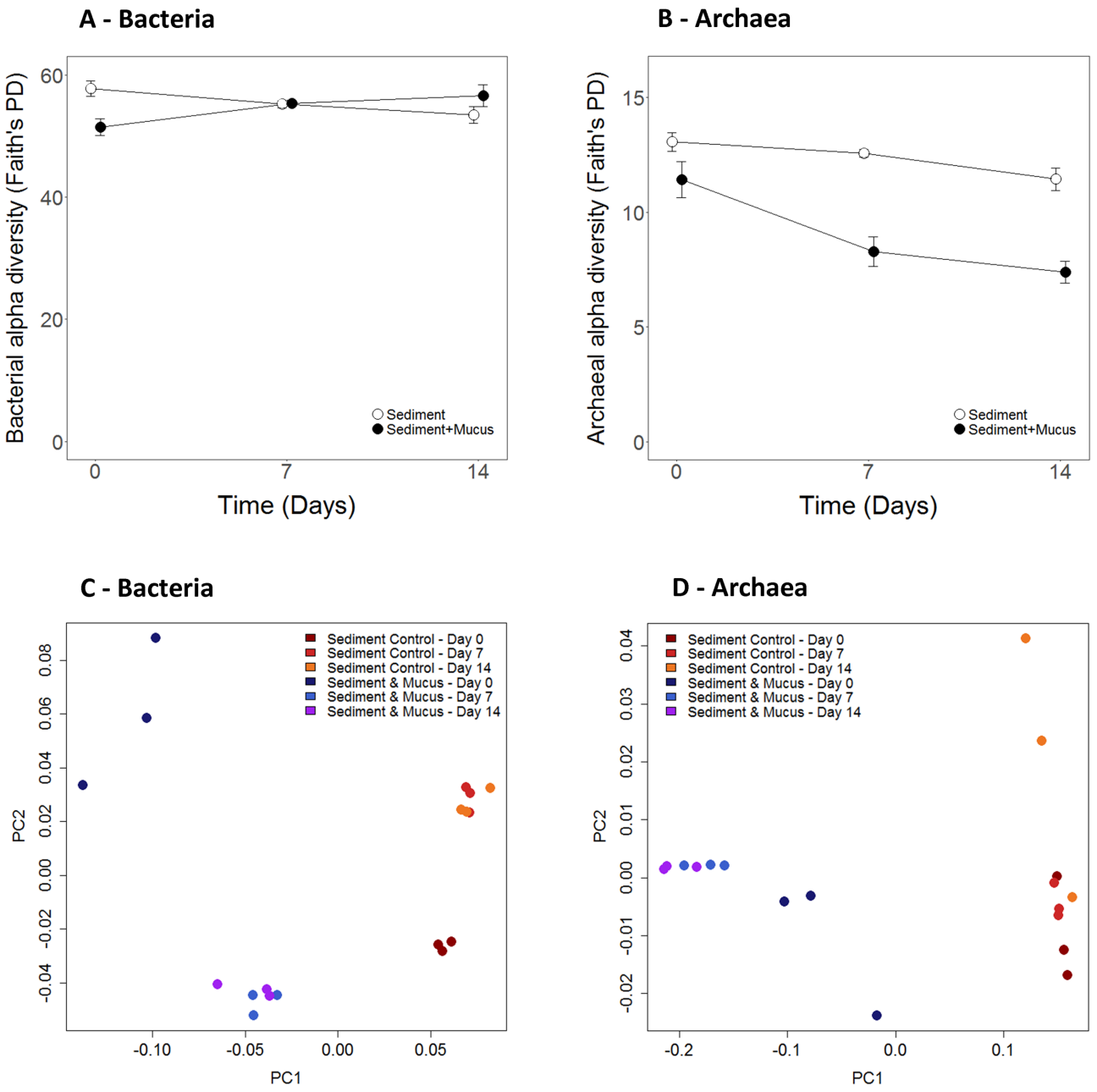

Figure 2. The effect of mucopolysaccharide on bacterial **(A)**, and archaeal **(B)**, alpha diversity. Data shown are means ± SD (n = 3). MDS (UNIFRAC) plots of bacterial **(C)**, and archaeal **(D)**, community structure.

any sample) was more abundant (seven-fold) in the sediment–mucus treatment than the sediment-only treatment immediately after mucus addition.

No archaeal classes were shared exclusively by the two mucus-amended treatments (Fig. S8B, Supporting Information). In the mucus-only treatment, the dominant class present was Marine Group I (Fig. S3B, Supporting Information). Immediately after the introduction of mucus, Marine Group I was significantly more abundant in both the mucus only control and the sediment–mucus treatment than in the sediment-only treatment ($P < 0.001$). All other classes present were either unaffected by mucus or declined in the presence of mucus. In terms of total OTU abundance, the taxonomic resolution of the bacterial community was much greater than the archaeal community on day 0 of the incubation, with either a limited number of OTUs, or no OTUs, shared between the mucus-only treatment and sediment–mucus treatment (Figs S8C and S8D, Supporting Information).

DISCUSSION

Previous studies have focused primarily on the oxygenating effects of invertebrate bioturbation on the abundance and activity of nitrogen-processing microbial groups (Laverock *et al.* 2011), and have not generally considered the potential effects of other behaviours such as mucus secretion. Here, we show that invertebrate mucopolysaccharide is most likely a source of nitrogen for microbial functional groups that drive nitrogen transformations. Our findings indicate that mucopolysaccharide serves as

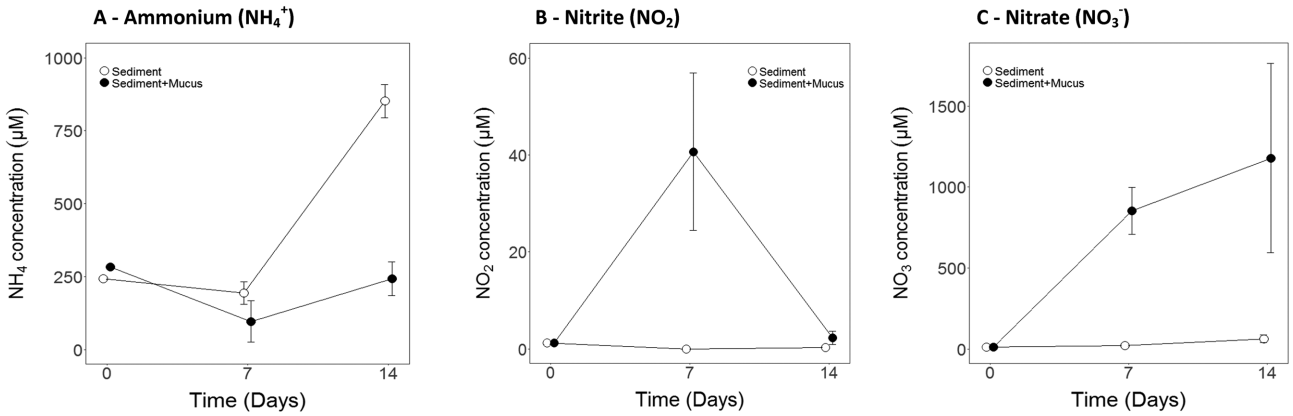

Figure 3. The effect of mucopolysaccharide on ammonium **(A)**, nitrite **(B)**, and nitrate concentrations **(C)**. Data shown are means ± SD (n = 3, except sediment Day 0 and sediment + mucus Day 7 n = 2).

an additional source of organic matter that stimulates bacterial growth and supports a distinct bacterial community, as well as stimulating ammonia-oxidising communities. AOB and AOA abundance increased in the presence of mucopolysaccharide which, with the concomitant increase in NO_2^- and NO_3, indicates increased rates of nitrification.

Invertebrate mucopolysaccharide is known to introduce reactive substances to sediment (Kristensen 2000), and has previously been suggested as a potential cause for increases in bacterial abundance within and around invertebrate structures (Aller and Yingst 1985; Dufour *et al.* 2008). Our study supports this by providing evidence that the addition of invertebrate-derived mucopolysaccharide leads to an increase in both carbohydrate concentration and bacterial abundance. Hence, observed increases in bacterial abundance around invertebrate burrow structures (Aller and Aller 1986; Papaspyrou *et al.* 2005) are likely, at least in part, to be attributable to the presence of invertebrate-supplied mucopolysaccharide. Mucus also changed the structure of the bacterial community, not by introducing unique taxa to the sediment but by changing the relative abundance of specific bacterial families already present in the sediment. Both *Flavobacteriaceae* and *Saprospiraceae* became more dominant in the presence of mucus and are known to degrade complex organic compounds, including polysaccharides and proteins (McBride 2014; McIlroy and Nielsen 2014).

By altering the structure of the general bacterial community in burrow walls, *H. diversicolor* mucus secretions may have a wide range of impacts on various ecosystem processes through the selective enhancement of other specific functionally important bacterial groups. Organic matter introduction by invertebrate mucopolysaccharides has been proposed to potentially alter sediment nitrogen cycling (Kristensen, Jensen and Andersen 1985; Bonaglia *et al.* 2014), but empirical evidence to demonstrate a direct effect is scarce. In this study, the lack of observed NH_4^+ production and the significant increase in NO_2^- that positively correlated with the increased AOB and AOA abundance strongly suggests that the presence of mucus increased nitrification rates, though *amoA* gene abundance cannot directly show nitrification activity (Bowen *et al.* 2014). In the sediment-only treatment, the increased concentration of NH_4^+ observed at the end of the incubation period indicates that net ammonification did occur within the sediments, but at a slow rate as after 14 days it had yet to stimulate observable nitrification activity. Nitrification would likely start to increase after this point, but clearly the introduction of mucus had significantly stimulated and accelerated nitrification activity. As further support for this increase in AOB and AOA abundance, the 16S rRNA sequencing data demonstrated a significant increase in the NH_4^+-oxidising bacterial family *Nitrosomonadaceae* and archaeal class Marine Group I with mucopolysaccharide.

The potentially higher rates of nitrification in the sediment–mucus treatment suggest that mucopolysaccharide increased the production of NH_4^+. As the mucopolysaccharide had higher nitrogen content, the degraded mucus amino moieties most likely served as a source of NH_4^+. Moreover, the mucus likely represented a more labile source of organic matter and/or the addition of mucus carbon simply stimulated the degradation rates of existing organic nitrogen in the sediment (Hannides and Aller 2016). Increased NH_4^+ subsequently stimulated the proliferation of ammonia oxidisers, which rapidly converted NH_4^+ to NO_2^-, and so NH_4^+ did not accumulate (Reyes *et al.* 2017). The increase in NO_3^- also indicates that nitrite oxidation was stimulated and the four-fold increase in the relative abundance of the family *Nitrospiraceae* may support this, although the known nitrite-oxidising genus *Nitrospira* was only present in low abundances (Daims 2014). The sequence data resolution means it is not possible to assess whether the detected *Nitrospira* were affiliated with comammox (Daims *et al.* 2015), though comammox bacteria have been identified in both lake and coastal sediment systems (Pjevac *et al.* 2017, Yu *et al.* 2018) and so could be affected by mucopolysaccharide addition. Meanwhile, the increase in *nirS* abundance on Day 7 suggests that at least some of the NO_2^- produced by AOB/AOA would have been denitrified instead of being used by nitrite oxidising bacteria, which may explain the relatively lower nitrite-oxidiser abundance relative to AOB/AOA. The concurrent decline in carbohydrate concentration, NO_2^- concentration, and AOB/AOA abundance suggests that the reactive substrates introduced by mucus had been exhausted by the end of the incubation period, and therefore there was no longer sufficient NH_4^+ being produced to support the increased abundance of NH_4^+ oxidisers (Foshtomi *et al.* 2015). High levels of benthic protist bacterivory (101–105 bacterial cells.ciliate^{-1} hr^{-1}) could explain the rapid decline in the abundance of nitrifying microbial groups (Starink *et al.* 1994, Tuorto and Taghon 2014), which would subsequently limit the production of any further NO_2^- (Bowen *et al.* 2014). Not all of the mucus-introduced N was necessarily processed by the nitrifying community to produce NO_2^-. Apart from the denitrifying communities mentioned above, other microbial processes, such as assimilation of N for

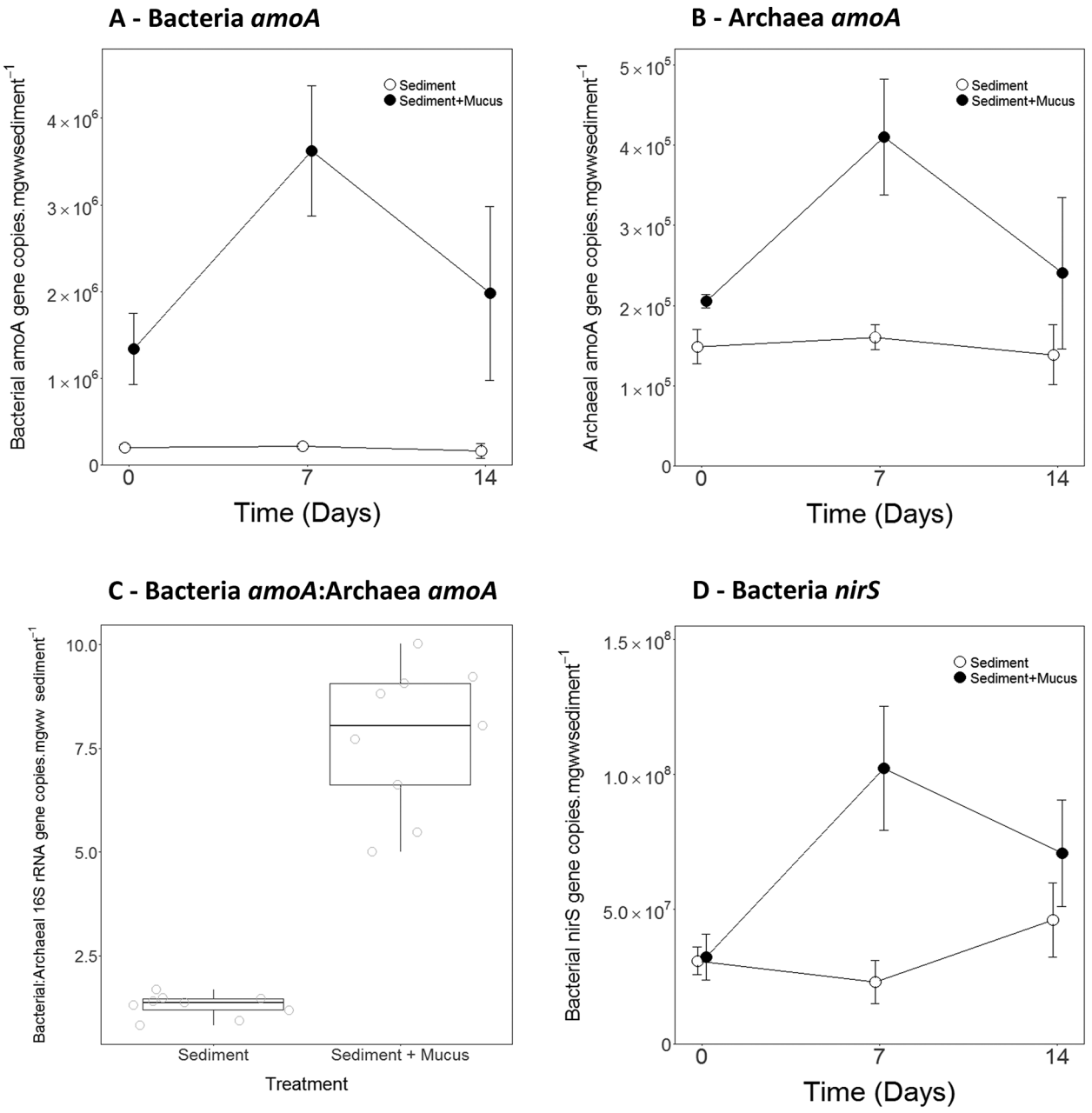

Figure 4. Variations in nitrogen cycling functional gene abundance in the presence of mucopolysaccharide. Bacterial *amoA* **(A)**, archaeal *amoA* **(B)**, bacterial:archaeal *amoA* copy ratio **(C)**, and bacterial *nirS* **(D)**. Data shown are means ± SD (n = 3).

growth and other anaerobic pathways in anoxic microniches (e.g. anammox, DNRA), might also have been co-occurring to influence the overall sediment nitrogen cycling. In natural burrow systems, however, the continuous production and turnover of mucus linings may well provide a continual source of organic carbon and nitrogen for microbial communities.

It is important to consider that mucopolysaccharide may act as an inoculum (evidenced here by the immediate increases in AOB and AOA abundance), as well as predominantly increasing the substrate available for nitrifying microbial communities. Although the presence of mucopolysaccharide did not lead to the introduction of novel microbial taxa to the sediment, it does appear to be a particularly important substrate for the bacterial family *Nitrosomonadaceae* and the archaeal class Marine Group I, with the majority of these being *Nitrosopumilus sp.* which tends to dominate in sediment AOA communities (Huang *et al.* 2016; Reyes *et al.* 2017). This suggests that invertebrate mucus secretions may affect nitrifying microbial communities by introducing reactive material to support the existing sediment community, and by acting as an inoculum that introduces mucus-associated bacterial and archaeal groups to the sediment. This inoculum effect may involve physical introduction of microbes to the sediment matrix, or it may simply represent a nitrifying microbial community that remains associated with introduced mucus. Either way, the presence of mucus within an invertebrate burrow system could increase total nitrification activity. Additionally, by examining AOB and AOA communities separately, this study was able to assess how invertebrate-derived mucopolysaccharide affects the relative contribution each group

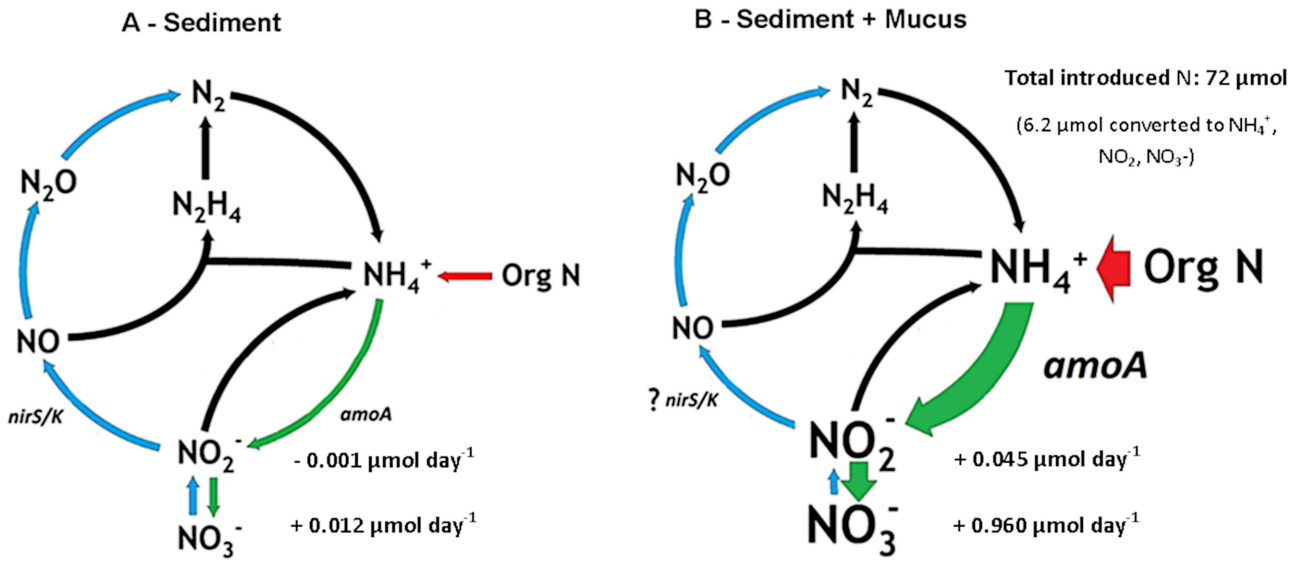

Figure 5. Summary of the proposed change in nitrogen cycling processes and microbial groups between sediments where invertebrate-derived mucopolysaccharide is **(A)**, absent or **(B)**, present, including rates of production for NO_2 and NO_3 between day 0 and day 7 of the incubation (Adapted from Jetten 2008).

makes to the overall AO community, which can vary depending on environmental conditions (Duff, Zhang and Smith 2017, Wang *et al.* 2017). Here, AOB dominated in all samples but the AOB:AOA ratio was substantially increased in the presence of *H. diversicolor*-derived mucopolysaccharide. This may be because AOA, in particular *Nitrosopumilus*, tend to dominate under NH_4^+-limited conditions (Martens-Habbena *et al.* 2009) and may have been outcompeted by AOB when exposed to the high levels of NH_4^+ production in the mucus-amended sediment.

It is important to note that this study primarily focused on aerobic processes in predominantly oxic incubations that would theoretically inhibit the majority of anaerobic microbial activities (Herbert 1999). Bacterial *nirS* abundance was therefore assessed to determine whether the presence of mucopolysaccharide in an oxic environment would affect the abundance of bacterial denitrifying groups. Overall, bacterial denitrifier abundance did increase in the presence of the mucopolysaccharide and this was concomitant with the increase in NO_2^- concentration. A relationship between bioavailable nitrogen concentrations and abundances of *nirS* genes has been observed *in situ* in oxic surficial sediment (Lee and Francis 2017), and so the increased activity of the ammonia oxidising community in the mucus-amended sediment may have stimulated nitrite reducers. However, the high concentration of NO_3^- in the mucus-amended incubation suggests that these anaerobic functional groups may not have been actively denitrifying in this generally oxic sediment environment; though oxygen content was not controlled in the incubation vials and so we cannot fully eliminate this possibility. Within invertebrate burrows, periodic irrigation creates oscillating oxic–anoxic environments (Volkenborn *et al.* 2012) in which mucopolysaccharides could potentially fuel coupled nitrification–denitrification (Gilbert *et al.* 2016). Increased coupling between nitrification and denitrification previously reported in bioturbated sediments (Howe, Rees and Widdicombe 2004; Dollhopf *et al.* 2005) may therefore be partially attributable to invertebrate mucopolysaccharide.

Fully extrapolating trends from sediment slurry incubations under laboratory conditions to natural sediment environments is not possible; however, it is important to examine the impacts of invertebrate mucus on sediment nitrogen cycling in the absence of the secreting organism and other environmental factors. The aim of this investigation was not to fully mimic natural sediment environments, but to develop a mechanistic understanding of invertebrate mucus impacts on sediment microbial communities. Through this we have been able to establish that *H. diversicolor*-derived mucopolysaccharide is able to alter the structure of the general bacterial community, and can enhance nitrification rates by fuelling, stimulating, or introducing nitrifying microbial groups (Fig. 5). There is a functional link between the presence of invertebrate mucopolysaccharide, nitrogen cycling microbial groups, and nitrogen biogeochemical transformations.

As natural population densities of *H. diversicolor* can reach 1150 individuals m^{-2} (Duport *et al.* 2006), the mucus production rate and nitrogen content observed in this study indicate that mucus excretions could exceed 15 g m^{-2} day^{-1} and contribute 0.34 g C m^{-2} day^{-1} and 0.05 g N m^{-2} day^{-1} to the surrounding sediment. In comparison, exopolymer production by epipelic diatoms in sediment contributes just 0.05 g C m^{-2} day^{-1} (Smith and Underwood 1998). Future work should examine how the trends observed in this study vary over a greater number of time points, assess the *H. diversicolor* associated microbiomes of natural populations, and determine how variations in mucus secretions between different invertebrate taxa (mucus nitrogen load, mucus lining thickness, mucus turnover, etc.) affect nitrification processes in sediments. This improved understanding will allow us to better develop nitrogen budget models and more accurately determine the effects of multiple stressors on sediment ecosystems.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

FUNDING

This work was supported by a Natural Environment Research Council (NERC) Southampton Partnership for Innovative Training of Future Investigators Researching the Environment Doctoral Training Partnership PhD studentship [SPITFIRE;

NE/L002531/1 to H.D.] and the Marine Biological Association of the UK (MBA) via a MBA Research Fellowship [to M.C.].

Conflicts of interest. None declared.

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