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Ocean and Earth Science

The Impacts of Invertebrate Activities on Sediment Microbial Community and Functional Ecology

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Abstract

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By Harriet Jane Dale

Sediment nitrogen cycling is a network of microbially-mediated biogeochemical processes that can regulate ecosystem functioning. As invertebrate activity is known to affect sediment microbial communities, producing accurate models of benthic nitrogen cycling requires an understanding of the interactions between invertebrates, microbial communities and biogeochemical processes. Few invertebrate activity assessments have included detailed microbial analysis and there is a general bias towards a narrow range of invertebrate activities that relate to particle reworking and burrow ventilation. The aim of this study was to use contemporary molecular techniques to expand our understanding of invertebrate-microbe interactions in relation to three invertebrate activities. First, despite previous research on burrow ventilation, burrow morphology effects on microbial communities remain poorly characterised. This study identified clear differences in the abundance and activity of ammonia-oxidising microbial groups between open and closed burrow morphologies, but only at mid-mixing sediment depths. Vertical depth variation should therefore be considered in future invertebrate trait assessments. Second, this study confirmed previously unverified claims that nitrogen-rich invertebrate mucopolysaccharide secretions stimulate nitrification and denitrification processes. These effects can also be altered by mucus concentration and redox oscillations, and could therefore differ between invertebrate taxa with varying mucus lining thicknesses or ventilation periodicities. Finally, using high-throughput sequencing, this study examined two additional understudied invertebrate traits: internal gut transit and external surface transport. These findings demonstrated that the gut tracts of the marine worm *Hediste diversicolor* contain a unique transitory microbial community that could support a distinct assemblage of ammonia-oxidising archaea, and identified a unique external microbial community. Both of these traits could play a role in mediating microbial distribution and transport within sediment environments. Overall, this study identified several understudied invertebrate activities and characterised their effects on sediment microbial communities and specific nitrogen cycling functional groups. The burrow morphology research also highlighted the importance of re-examining invertebrate activities with contemporary molecular techniques to gain further insight into invertebrate-microbe interaction mechanisms. Incorporating these additional traits and more robust microbial analyses into sediment ecosystem models could help to build the ecological complexity needed to better predict future changes to sediment nitrogen budgets and other ecologically critical biogeochemical processes.

List of Contents

List of Figures and Tables

1. Introduction

2. The role of burrow morphology in moderating ventilation effects on nitrifying microbial groups

3. Invertebrate mucopolysaccharide effects on sediment microbial community composition and nitrogen cycling processes

4. Mucopolysaccharide secretion effects on nitrogen cycling communities under oscillating redox conditions

5. Exploring the effect of marine polychaete gut passage on sediment microbial assemblage structure

6. General Discussion

List of Appendices

Appendix I: Chapter 2 Supplementary Information

Appendix II: Chapter 3 Supplementary Information

Appendix III: Chapter 4 Supplementary Information

Appendix IV: Chapter 5 Supplementary Information

List of Accompanying Materials

Full dataset available in University of Southampton online repository (PURE)

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UNIVERSITY OF Southampton

Research Thesis: Declaration of Authorship

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Dale H, Taylor JD, Solan M, Lam P, Cunliffe M (2018) Polychaete mucopolysaccharide alters sediment microbial diversity and stimulates ammonia-oxidising functional groups. *FEMS Microbiology Ecology* **95**: fiy234 (Appendix II).

Dale H, Solan M, Lam P, Cunliffe M (2019) Sediment microbial assemblage structure is modified by marine polychaete gut passage. *FEMS Microbiology Ecology* **95**: fiz047 (Appendix IV).

Signature:		Date:	21.03.2019

Declaration of authorship continued: outline of work conducted jointly with or by others presented in this thesis (information pertaining to point 6 on previous page).

Chapter 2: The role of burrow morphology in moderating ventilation effects on nitrifying microbial functional groups

The majority of the experimental work was carried out by H. Dale with the following exception:
[1] The photographs presented in Figure 2.4 were taken by A. Harvey from the Marine Biological Association.

This work is currently in review at *Marine Ecology Progress Series*.

Chapter 3: Invertebrate mucopolysaccharide effects on sediment microbial community composition and nitrogen cycling processes

The majority of the experimental work was carried out by H. Dale with the following exceptions:
[1] Dr M. Cunliffe conducted the carbohydrate assay described in Chapter 3.3.3.
[2] NH_4^+, NO_2^-, and NO_3^- were measured by Dr M. Woodward from Plymouth Marine Laboratory as described in Chapter 3.3.3.
[3] B. Hambach from the National Oceanography Centre aided in the elemental analysis described in Chapter 3.3.3. H. Dale prepared the samples for analysis.
[4] Dr J. Taylor from the University of Salford completed the pre-processing analysis of the high-throughput sequencing data as described in Chapter 3.3.4.

This work was published in full in Dale *et al.* 2019 (Appendix II).

Chapter 4: Mucopolysaccharide secretion effects on nitrogen cycling communities under oscillating redox conditions

The majority of the experimental work was carried out by H. Dale with the following exception:
[1] NH_4^+, NO_2^-, and NO_3^- were measured by Dr M. Woodward from Plymouth Marine Laboratory as described in Chapter 4.3.2.

Chapter 5: Exploring the effect of marine polychaete gut passage on sediment microbial assemblage structure

The majority of the experimental work was carried out by H. Dale with the following exceptions:

[1] The photographs presented in Figure 5.1 were taken by D. Laundon from the Marine Biological Association.
[2] Dr S. Simpson from the Marine Biological Association carried out half of the dissections described in Chapter 5.3.1.

This work was published in full in Dale *et al.* 2019 (Appendix IV).

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List of Abbreviations

AIC	Akaike Information Criteria
Anammox	Anaerobic Ammonium Oxidation
ANOVA	Analysis of Variance
AOA	Ammonia-Oxidising Archaea
AOB	Ammonia-Oxidising Bacteria
bp	Base Pairs
cDNA	Complementary Deoxyribose Nucleic Acid
CFU	Colony Forming Units
Comammox	Complete Ammonia Oxidiser
Ct	Cycle Threshold
CT	Computed Tomography
DGGE	Denaturing Gradient Gel Electrophoresis
DIN	Dissolved Inorganic Nitrogen
DNA	Deoxyribose Nucleic Acid
DNRA	Dissimilatory Reduction of Nitrate to Ammonium
DNTP	Deoxyribonucleic Triphosphate
dw	Dry Weight
EPS	Extracellular Polymeric Substances
HTS	High-throughput sequencing
ind	Individuals
$L_{surface}$	Surficial sediment depth
L_{mid}	Mid-mixing sediment depth
L_{max}	Maximum sediment mixing depth
LME	Linear Mixed Effect
MDS	Multidimensional Scaling
MIQE	Minimum Information for Publication of Q-PCR Experiments
ML	Maximum Likelihood
MPN	Most Probable Number
mRNA	Messenger Ribose Nucleic Acid
nanoSIMS	Nanometre-scale Secondary Ion Mass Spectrometry
nanoSIP	Nanometre-scale Stable Isotope Probing
nMDS	Nonmetric Multidimensional Scaling
NOB	Nitrite-Oxidising Bacteria
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PET	Positron Emission Tomography
PLFA	Phospholipid Fatty Acid
PSU	Practical Salinity Unit
PVC	Polyvinyl Chloride
Q-PCR	Quantitative Polymerase Chain Reaction
Q-Q	Quantile-Quantile
REML	Restricted Maximum Likelihood
RFLP	Restriction Fragment Length Polymorphism
RISA	Ribosomal Intergenic Spacer Analysis
rRNA	Ribosomal Ribonucleic Acid
RT-QPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SRB	Sulfate Reducing Bacteria
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UV	Ultraviolet
v/v	Volume/Volume
w/w	Weight/Weight
ww	Wet Weight

1. Introduction

1.1. Sediment nitrogen cycling

Primary productivity in marine ecosystems is often limited by the supply of available nitrogen compounds (Bristow *et al.*, 2017), as most organisms are unable to directly fix nitrogen (N_2) and instead rely on external sources to undertake many biological functions (Bertics *et al.*, 2010; Canfield *et al.*, 2010). In shallow marine sediments, the transformation of these essential nitrogen compounds through interlinked oxidation and reduction biogeochemical reactions is therefore a key ecosystem function (Galloway *et al.*, 2004; Hulth *et al.*, 2005). These transformations also support productivity in the overlying water column by altering sediment fluxes of dissolved inorganic nitrogen (DIN) (Herbert, 1999; Elser *et al.*, 2007; Ekeroth *et al.*, 2016), which can provide up to 80% of phytoplankton nitrogen requirements (Boynton & Kemp, 1985; Dale & Prego, 2002; Laverock *et al.*, 2011). Consequently, a clear understanding of sediment nitrogen cycling processes can help us to understand and predict current and future changes to ecosystem productivity.

Nitrogen transformations are regulated by specialised bacterial and archaeal functional groups (Herbert, 1999; Jetten, 2008; Canfield *et al.*, 2010). In sediments, an array of these groups degrade organic nitrogen-based macromolecules to ammonium (NH_4^+) (ammonification; Figure 1.1 [1]) (Herbert, 1999), though this production is often masked by subsequent nitrification (Figure 1.1 [3 & 4]), which oxidises NH_4^+ to nitrite (NO_2^-) and nitrate (NO_3^-) (Reyes *et al.*, 2017). Nitrification is predominantly mediated by ammonia-oxidising bacteria and archaea, which possess genes encoding ammonia monooxygenase (*amoA*), and nitrite-oxidising bacteria, which possess genes encoding nitrite oxidoreductase (*nxr*) (Herbert, 1999; Francis *et al.*, 2005; Konneke *et al.*, 2005; Treusch *et al.*, 2005; Jetten, 2008; Reyes *et al.*, 2017). Recent research has also identified comammox bacteria from the genus *Nitrospira* in lake and coastal sediment systems (Pjevac *et al.*, 2017; Yu *et al.*, 2018), that are able to actively undertake both oxidation processes (Daims *et al.*, 2015).

Both ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) can be more abundant (Abell *et al.*, 2010; Bowen *et al.*, 2014; Li *et al.*, 2015; Smith *et al.*, 2015; Jiang *et al.*, 2017; Wang *et al.*, 2017), or more active (Bowen *et al.*, 2014; Duff *et al.*, 2017), in ammonia-oxidising communities. This variation in dominance and activity appears to be related to varying niches (Jiang *et al.*, 2017), with AOA tending to be better adapted to low NH_4^+ and low O_2 conditions (Martens-Habbena *et al.*, 2009; Qin *et al.*, 2017; Wang *et al.*, 2017), though the isolation of NH_4^+ tolerant AOA groups reduces support for this niche differentiation (Lehtovirta-Morley *et al.*, 2016). Both ammonification

and nitrification processes recycle reactive nitrogen to benthic and pelagic systems, with nitrification also playing a role in nitrogen removal as the only known link between organic nitrogen inputs and the anaerobic pathways that remove reactive nitrogen from sediment (Damashek & Francis, 2017).

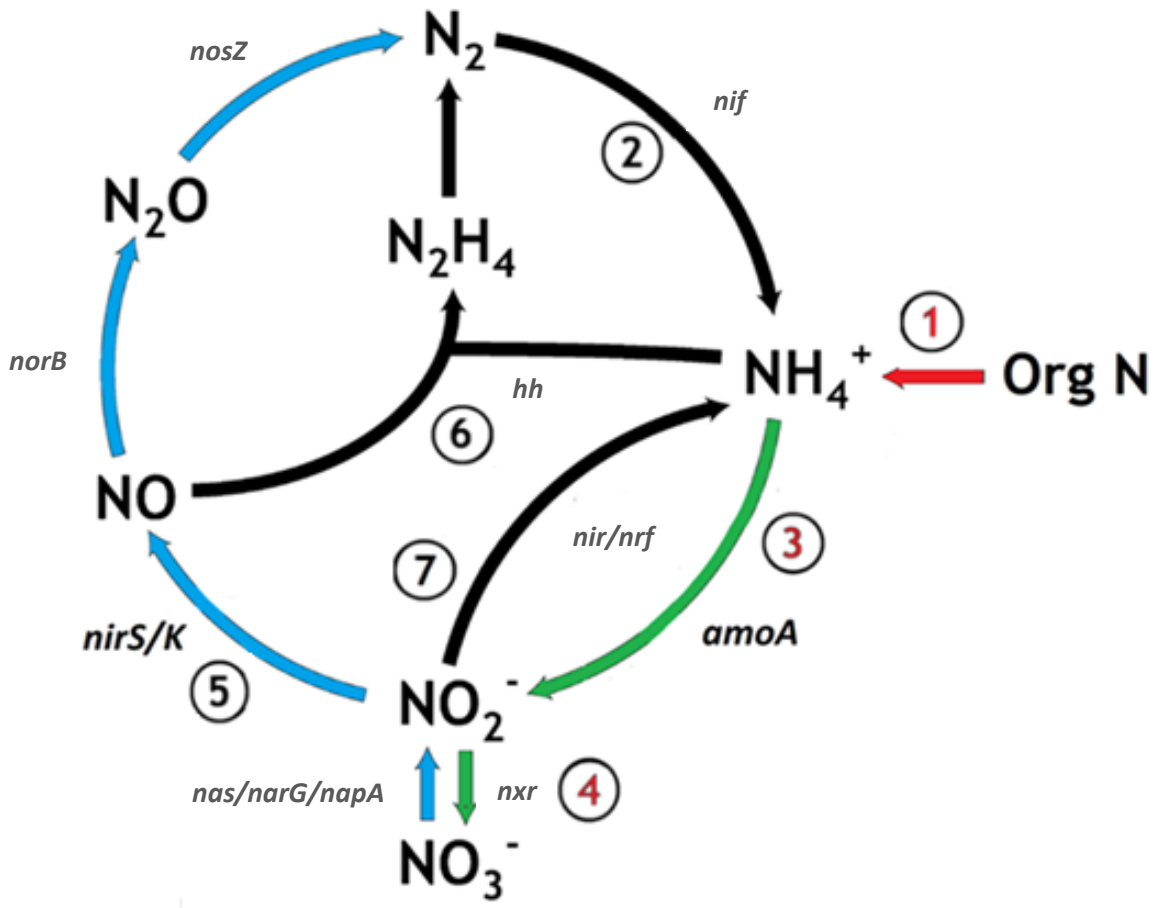

Figure 1.1 Known reactions of the microbial nitrogen cycle, with representative genes that encode key enzymes.

1. Ammonification **2.** Nitrogen fixation **3.** Ammonia oxidation **4**. Nitrite oxidation **5.** Nitrite reduction **6.** Anammox **7.** DNRA. Green arrows represent nitrification processes, blue arrows represent denitrification processes. Red numbers indicate processes that can occur aerobically. Representative genes most frequently examined in sediment systems are highlighted in bold (Adapted from Jetten, 2008).

Anaerobic pathways that transform reactive nitrogen to less reactive forms can help to alleviate the negative impacts of excess nitrogen inputs, and are therefore significant transformation processes in coastal and estuarine sediments (Hulth *et al.*,

2005; McTigue *et al.*, 2016). Denitrification (Figure 1.1 [5]) reduces NO_3^- to N_2 and is the most well studied of these pathways, responsible for removing 10-80% of estuarine reactive nitrogen (Galloway *et al.*, 2004). Although fungal denitrification is now recognised as an important component (Wankel *et al.*, 2017), the majority of research has focused on bacterial denitrifiers, particularly nitrite-reducing bacteria that possess the gene encoding nitrite reductase (*nirS* or *nirK*) (Jetten, 2008; Bowen *et al.*, 2014). These bacteria tend to be facultative anaerobes that only use NO_3^- as a terminal electron acceptor when O_2 is not available. This means that denitrification tends to occur below the oxidised zone of sediment or in anoxic microniches at the sediment surface (Herbert, 1999; Howe *et al.*, 2004). As denitrifying bacteria can use NO_2^- and NO_3^- produced by nitrifying microbial groups, nitrification and denitrification processes can be coupled (Jenkins & Kemp, 1984). These aerobic and anaerobic processes occur in different sediment redox zones however, so the extent of this coupling depends on the extent of the oxic-anoxic boundary (Sørensen, 1978). Other anaerobic pathways, such as anammox (anaerobic ammonium oxidation) (Figure 1.1 [6]) and DNRA (dissimilatory reduction of nitrate to ammonium) (Figure 1.1 [7]), can also have significant effects on reactive nitrogen concentrations. Bacteria capable of anammox transform NH_4^+ to N_2 using NO_2^- as an electron acceptor (Strous *et al.*, 1999; Thamdrup & Dalsgaard, 2002). This process can form half of N_2 production in shelf sea sediments (Burgin & Hamilton, 2007) and is therefore able to remove significant amounts of reactive nitrogen. Conversely, microorganisms undergoing DNRA recycle reactive nitrogen by using NO_3^- as an electron acceptor and converting it back to the more biologically available NH_4^+ (Burgin & Hamilton, 2007).

Overall, nitrogen cycling is complex because it is formed of multiple transformations and mediated by a range of functionally important microbial groups. It has already been well established that biogeochemical processes and fluxes, such as nitrogen cycling, are significantly affected by the diversity, composition, and activity of microbial communities (Reed & Martiny, 2013; Bowen *et al.*, 2014; Graham *et al.*, 2016). A detailed understanding of the role of nitrogen cycling microbial groups, and how they may be impacted by potential abiotic and biotic changes to sediment environments, is therefore required to understand and predict current and future changes to nitrogen cycling processes and their impacts on ecosystem productivity and nitrogen pollution.

1.2. Bioturbation

Bioturbation is defined as any faunal activity that alters sediment properties. These activities are generally split into particle reworking (burrowing, locomotion, and feeding) and irrigation (ventilation of biogenic structures) (Kristensen *et al.*, 2012), and act to increase sediment column heterogeneity by redistributing resources, such as organic matter, and by introducing O_2 and solutes deeper into the sediment column (Aller & Yingst, 1985; Krantzberg, 1985). Burrow structures formed by many taxa further enhance these processes by increasing the area of sediment in direct contact with overlying water (Widdicombe & Needham, 2007).

Invertebrate burrows are unique microenvironments with steep chemical gradients, high concentrations of organic matter and distinct redox conditions, that subsequently alter the structure and activity of sediment microbial communities (Kristensen, 2000; Nielsen *et al.*, 2004; Webb & Eyre, 2004; Volkenborn *et al.*, 2010). Increased O_2 penetration through bioturbation activity allows burrow walls to support greater abundances of aerobic microbes (Fenchel, 1996; Satoh *et al.*, 2007), and therefore support greater rates of aerobic processes such as organic matter decomposition (Kristensen & Blackburn, 1987; Papaspyrou *et al.*, 2007) or oil degradation (Chung & Gary, 1999; Taylor & Cunliffe, 2015). Invertebrate burrows can also enhance exchange and coupling between aerobic and anaerobic processes. Burrow walls extend the area of the oxic-anoxic interface (Kristensen, 2000), while intermittent irrigation causes burrow environments to oscillate between oxic and anoxic conditions which allows aerobic and anaerobic microbially-mediated processes to occur sequentially (Kristensen *et al.*, 1991; Volkenborn *et al.*, 2012; Delefosse *et al.*, 2015). As bioturbating taxa are highly abundant and globally distributed, these impacts on sediment properties and microbial communities are significant and widespread (Laverock *et al.*, 2011).

Nitrogen transformations in sediment are mediated by interactions between invertebrate activity and microbes (Magri *et al.*, 2017). By increasing the volume of oxidised sediment in which aerobic processes can occur and supplying organic nitrogen, bioturbation and excretion by invertebrates can increase the proliferation of nitrifying microbial communities around burrow structures, and subsequently increase rates of nitrification (Sayama & Kurihara, 1983; Welsh, 2003; Nielsen *et al.*, 2004; Satoh *et al.*, 2007; Black & Just, 2018) (Figure 1.2). Burrow walls are therefore sites of high NH_4^+ consumption and NO_2^-/NO_3^- production (Satoh *et al.*, 2007), which can then affect nitrogen fluxes between the sediment and the water column (Ieno *et al.*, 2006; Godbold *et al.*, 2011; Ekeroth *et al.*, 2016). Additionally, the increased production of NO_2^- and NO_3^- has

the potential to stimulate anaerobic removal pathways, such as denitrification and anammox, as the close proximity of oxic and anoxic sediment in burrows allows exchange between these aerobic and anaerobic processes (Kristensen *et al.*, 1991; Howe *et al.*, 2004; Dollhopf *et al.*, 2005). Hence, rates of denitrification are often greater in bioturbated sediment (Gilbert *et al.*, 1998). Oscillating oxic-anoxic conditions in burrows also support this coupling by allowing these aerobic and anaerobic processes to occur in succession (Gilbert *et al.*, 2016), which selects for facultative denitrifying bacterial groups (Wittorf *et al.*, 2016). Burrow walls therefore support distinct and diverse denitrifying communities (Stauffert *et al.*, 2014, Foshtomi *et al.*, 2018).

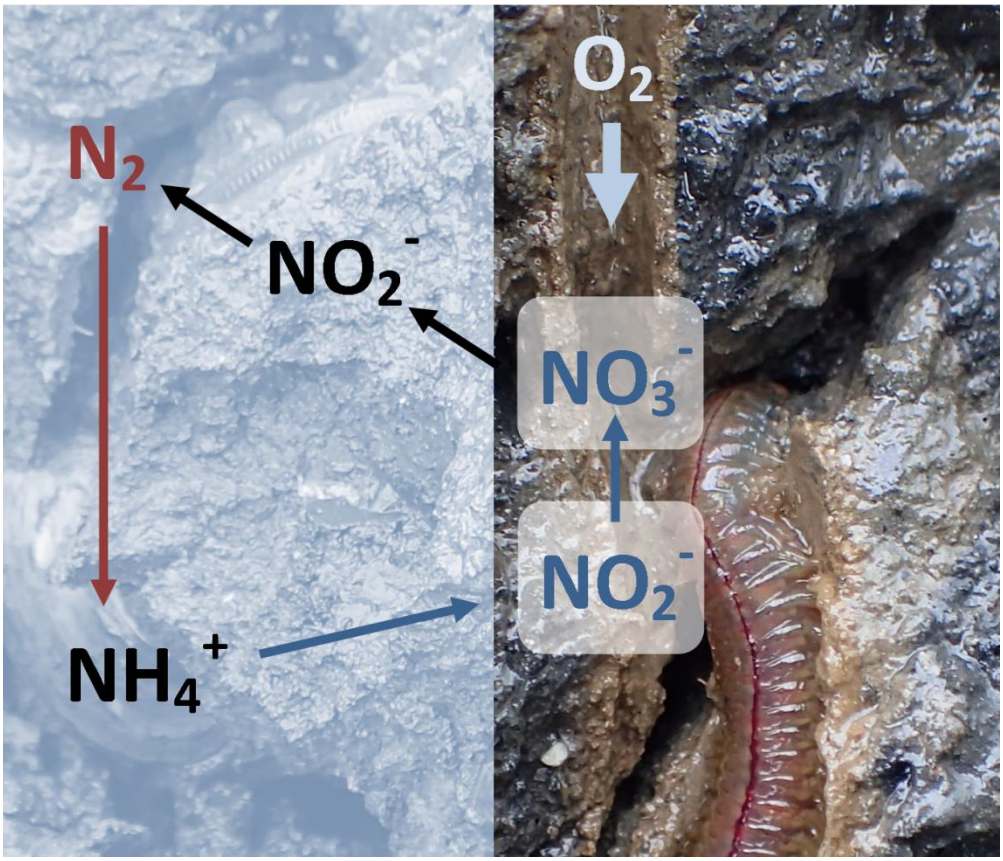

Figure 1.2 Conceptualised nitrogen cycling processes around *Hediste diversicolor* burrow.
Nitrification (blue), denitrification (black), and nitrogen fixation (red) (Adapted from Bertics *et al.*, 2010) (Photograph - Davis Laundon, MBA).

1.3. Factors limiting microbial assessment in bioturbation studies

There are currently a limited number of studies that accurately assess the microbial impacts of bioturbation, both in general and in terms of nitrogen cycling. This is due to a range of factors including a lack of microbial assessment in many bioturbation studies, the use of dated techniques, limited analysis of microbial functioning and limited spatiotemporal resolution. For future research to continue closing knowledge gaps related to invertebrate-microbe interactions, we need to understand the limitations of previous bioturbation studies and how these can be improved upon with contemporary molecular techniques.

No microbial assessment - One reason for the lack of microbial data in bioturbation studies is that impacts on biogeochemical processes are often attributed to changes in microbial activity with no assessment or analysis of the microbial community (Kristensen, 1985; Christensen *et al.*, 2000; Michaud *et al.*, 2006; Bonaglia *et al.*, 2014). Previous research showing the stimulation of organic carbon loss in the presence of the polychaete *Alitta (Nereis) virens* suggested that this loss was due to increased microbial activity in organically enriched burrows (Kristensen & Blackburn, 1987), but this was not confirmed by any direct measure of microbial abundance, community structure or activity. Although these assumptions are based on previous research or sound scientific reasoning, there is no way to verify these theories if microbial assessment is excluded.

Use of non-gene microbial markers - Other sediment ecology studies that have assessed the effect of bioturbation on microbial communities have used techniques that provide limited information. For example, epifluorescence microscopy techniques have been successfully used to demonstrate the positive effect bioturbation can have on bacterial abundance (Aller & Yingst, 1985; Aller & Aller, 1986; Branch & Pringle, 1987) (Figure 1.3), however, this methodology can provide no additional information on the presence, abundance, or activity of functionally important microbial groups, such as those involved in nitrogen cycling. This has been improved by culture based techniques, such as the most probable number method (MPN) or assessments of colony forming units (CFU) (Goñi-Urriza *et al.*, 1999) (Figure 1.3), which have been used to determine the abundance of specific microbial groups of interest in burrow walls, such as the sulfate-reducing bacteria (Hansen *et al.*, 1996). Although this allows a more targeted approach, these techniques are biased by the large proportion of unculturable bacterial taxa (>90%) (Teske *et al.*, 1996; Katayama *et al.*, 2003). Microbial marker techniques, such as phospholipid fatty acid (PLFA) analysis (Figure 1.3), have therefore been used in sediment ecology studies as they can provide information on changes in biomass and

diversity across a much greater range of the microbial community (Steward *et al.*, 1996; Bird *et al.*, 2000; Marinelli*, et al.*, 2002). Specific phospholipid markers that are known to be associated with a particular taxonomic or functional group can also be used to target microbes of interest (Dobbs & Guckert, 1988). This technique can then be combined with other methods, like stable isotope labelling, to assess how bioturbation affects the activity of groups like chemoautotrophic bacteria, or to determine the dominance of aerobic and anaerobic taxa (Vasquez-Cardenas *et al.*, 2016). Yet, the use of these markers is time consuming and still lacks taxonomic and functional resolution because it relies on all members of the group possessing specific known PLFA markers (Matsui *et al.*, 2004).

Use of gene-based techniques - By utilising techniques such as quantitative PCR (Q-PCR) that target known genes specific to phylogenetic groups (e.g. bacterial 16S rRNA), we can make more accurate evaluations of microbial abundance (Chen *et al.*, 2017). Meanwhile, precise community profiling assessment of diversity and structure (DGGE, RISA, RFLP) can determine if bacterial communities differ between burrow walls, surficial sediment, and anoxic sediment (Papaspyrou *et al.*, 2005; Bertics & Ziebis, 2009; Laverock *et al.*, 2010), and assess the role of bioturbators in modifying oil remediation communities (Cuny *et al.*, 2007; Stauffert *et al.*, 2013) (Figure 1.3). Genetic methodologies can also be used to target specific microbial functional groups by targeting known functional genes (O'Mullan & Ward, 2005). By using Q-PCR (Figure 1.3) to target the bacterial and archaeal *amoA* gene and bacterial *nirS* gene, studies have demonstrated that burrowing invertebrates alter the abundance and structure of ammonia-oxidising communities (Satoh *et al.*, 2007; Gilbertson *et al.*, 2012) and identified spatiotemporal variations in both nitrifying and denitrifying bacterial groups (Smith *et al.*, 2015). These data still need to be interpreted with caution as the presence of ammonia-oxidising genes does not necessarily indicate the presence of active ammonia oxidation. This activity can be better captured by using mRNA reverse transcription and Q-PCR in combination (RT-QPCR) to assess transcript abundance. Overall, this provides a better assessment of ammonia-oxidising and nitrite-reducing bacterial and archaeal activity (Bowen *et al.*, 2014) and therefore a better understanding of how bioturbation impacts microbial respiration. To further explore any functional implications of these invertebrate-microbe interactions, molecular microbial analyses can also be used in combination with assessments of relevant sediment biogeochemical processes. This can include measurements of nitrogen compound concentrations in the porewater or overlying water column to examine potential effects on productivity or sediment fluxes (Mermillod-Blondin *et al.*, 2005; Papaspyrou *et al.*, 2010), or the use of inhibition (e.g. acetylene) and stable isotope techniques to

investigate changes in nitrogen cycling process rates (Nielsen *et al.*, 2004; Laverock *et al.*, 2013; Gilbert *et al.*, 2016).

Some bioturbation studies are now also beginning to use high-throughput sequencing (Figure 1.3) methods to rapidly acquire greater community composition data in more detail and with improved accuracy (Sun *et al.*, 2013; Ma *et al.*, 2015). Through this, research has examined how redox changes influence the composition of the active bacterial community (Frindte *et al.*, 2016), how bioturbation and oil pollution alter bacterial community structures (Taylor & Cunliffe, 2015), and how biogeochemical gradients impact nitrogen cycling communities (Reyes *et al.*, 2017; He *et al.*, 2018). To date, however, few studies have used these sequencing techniques to establish the impact of sediment invertebrate activity, particularly with regard to nitrogen cycling (Foshtomi *et al.*, 2018). Additionally, the large data sets obtained from such a thorough community-wide analysis mean it is important to remain hypothesis-driven, and to focus on specific bioturbation impacts rather than simply looking at descriptive community changes (Prosser *et al.*, 2007; Prosser, 2015). Overall, these genetic techniques, especially when used in combination, can improve our understanding of bioturbation impacts on microbial communities as a whole, but also provide the ability to target functionally important groups more accurately and therefore draw more functionally significant ecological conclusions.

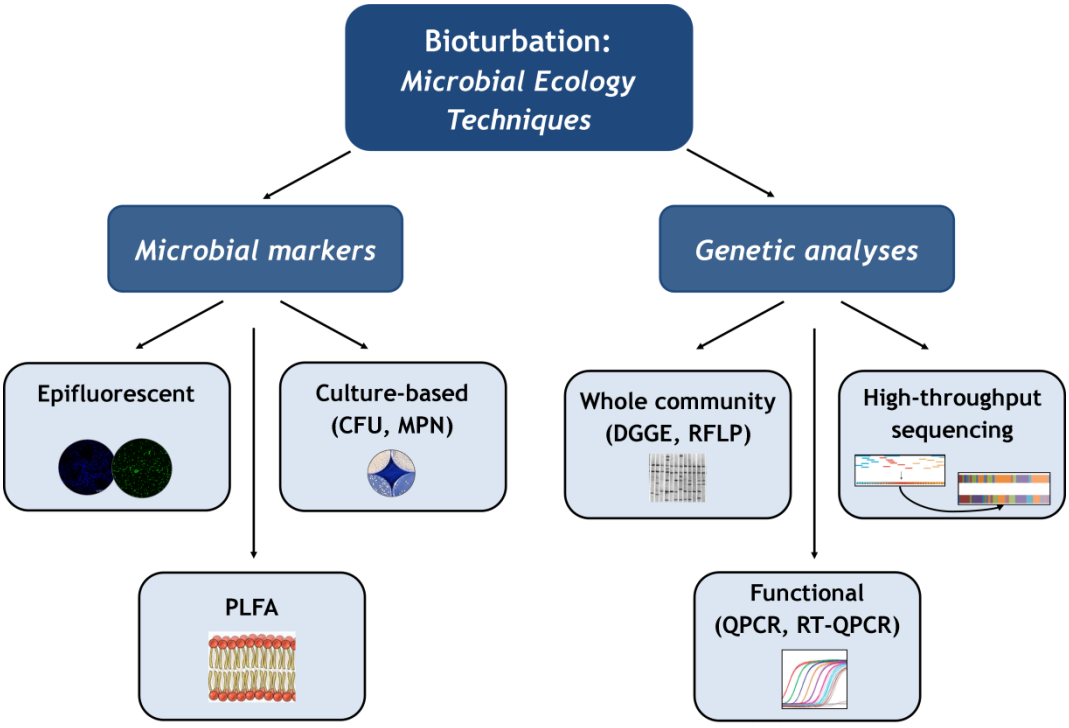

Figure 1.3 Microbial ecology techniques previously used in bioturbation studies, divided into marker and genetic based methodologies.

Limited spatial analysis - Another factor that is currently limiting understanding in bioturbation studies, particularly those using genetic analyses, is a lack of spatial assessment. Nitrogen cycling microbial abundance and activity are known to vary with depth through the sediment column (Papaspyrou *et al.*, 2014). Bioturbation impacts on nitrogen cycling microbial communities may also only be observable at depth, where the introduction of O_2 to the bulk anoxic sediment has a greater impact than in the oxidised surficial sediment (Satoh *et al.*, 2007). Yet many studies are assessing nitrogen cycling genes in just the initial 1-2 cm of surficial sediment (Gilbertson *et al.*, 2012; Stauffert *et al.*, 2014; Zhao *et al.*, 2017) because, outside of invertebrate burrows, this is the average maximum penetration depth of O_2 in sediment (Foshtomi *et al.*, 2015). Other studies have only sampled from subsurface sediments (Shen *et al.*, 2017), which still limits spatial comparison. Studies that observe changes in nitrogen fluxes with no impact on surficial nitrogen cycling communities (Sciberras *et al.*, 2017) should therefore be regarded with caution, as there may be unobserved impacts on microbial communities at depth that may be influencing nitrogen transformations. Assumptions made about the effects throughout the sediment column based on data retrieved from the effects in the surficial sediment could lead to misinformation about the role of bioturbation in biogeochemical regulation. By treating sediments and sediment microbial communities as three-dimensional structures, and by assessing these communities using multiple, accurate molecular techniques, the role of bioturbation-nitrogen cycling interactions in sediment biogeochemical cycling may be better resolved and understood.

1.4. Variations in invertebrate bioturbation traits

Our knowledge of the ecologically complex interactions between invertebrate activity and microbial functional groups is also limited by substantial interspecies variations in feeding and bioturbation activities (Welsh, 2003). Differences in traits, such as burrow structure and feeding mode (Kristensen, 2001; Kristensen *et al.*, 2012), mean that invertebrates have taxon-specific effects on particle reworking, O_2 introduction and solute fluxes (Zorn *et al.*, 2006; Quintana *et al.*, 2007; Solan *et al.*, 2008). In diverse sediment communities, these impacts can then also be mediated by varying interaction effects between species (Emmerson *et al.*, 2001), with the most dominant or effective bioturbator often masking the impacts of other taxa (Mermillod-Blondin *et al.*, 2005; Norling *et al.*, 2007). Overall, this means that some invertebrate species play a greater role in mediating sediment ecosystem processes (Wrede *et al.*, 2017) and so the loss or gain of certain key species or traits, or a change in the dominant species, may have significant effects on nitrogen cycling processes (Kauppi *et al.*, 2018).

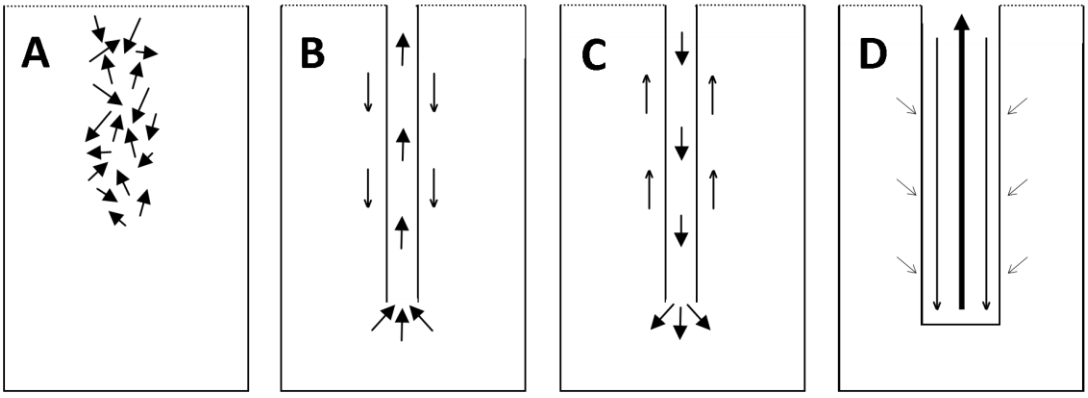

Figure 1.4 Typical invertebrate functional groups based on particle reworking. Biodiffusers (A), upward-conveyors (B), downward-conveyors (C), regenerators (D) (Adapted from François-Carcaillet & Poggiale, 1997).

Sediment communities are likely to alter rapidly under current rates of anthropogenic change (Waters *et al.*, 2016), and accurate predictions of any potential impacts require a mechanistic understanding of how varying bioturbation traits regulate biogeochemical cycling. To simplify this, taxa can be separated into functional groups based on bioturbation or burrowing mode (François-Carcaillet & Poggiale, 1997; Figure 1.4). All members of each group are then considered to be functionally equivalent, and therefore have similar traits and effects on sediment environments (Hooper *et al.*, 2005). Functional groupings that are based on a single set of traits or a single ecosystem

function, however, will not necessarily be functionally equivalent when different traits or functions are considered (Hooper *et al.*, 2005). Three closely related species that feed in a similar way can have varying impacts on solute fluxes because of differences in burrow morphology and depth (Renz & Forster, 2013; Renz & Forster, 2014). To truly understand the role that bioturbation can play in biogeochemical cycling, each invertebrate trait needs to be assessed individually to determine how variations within this trait affect both the process of interest and the related microbial functional groups.

The impacts of some bioturbation traits on bacterial communities have already been examined. Bacterial abundance and activity are generally higher in deeper or more strongly irrigated burrows (Aller & Yingst, 1985; Mermillod-Blondin *et al.*, 2004), and distinct bacterial communities are observed between irrigated and non-irrigated structures (Bertics & Ziebis, 2009). This may be because irrigation and increased surface area both act to increase O_2 exchange, and therefore encourage the proliferation and activity of aerobic bacteria. Potential impacts on nitrogen cycling, however, have been less widely assessed. Although many studies have demonstrated that taxa with different bioturbating behaviours can have different impacts on sediment nitrogen fluxes (Mermillod-Blondin *et al.*, 2004; Ieno *et al.*, 2006; Braeckman *et al.*, 2010; Godbold *et al.*, 2011), the complex nature of the nitrogen cycle means that flux assessments do not necessarily provide an accurate measure of which nitrogen transformations are occurring and to what extent. Studies using gene-based microbial assessments have advanced this by demonstrating that functionally different invertebrate species can also have different effects on the abundance and structure of ammonia-oxidising (Gilbertson *et al.*, 2012; Zhao *et al.*, 2017) and nitrite-reducing (Sciberras *et al.*, 2017) microbial communities. Yet, by only assessing surficial microbial communities, these studies may again be missing potential variations occurring at depth. Additionally, taxa used in trait assessment studies are often selected because they represent extreme variations in burrowing or irrigating traits, and so it is likely that more than one bioturbation trait will differ between the selected species. This makes it difficult to determine which variations are contributing to observed nitrogen cycling impacts because no one trait variation was isolated. Species comparison studies also tend to focus on variations in the ‘classic’ bioturbation behaviours of ventilation and burrowing, which may overlook the potentially significant role of other invertebrate activities that can vary between taxa. Improving the mechanistic understanding of invertebrate-microbe interactions therefore requires more detailed and isolated assessments of ‘classic’ bioturbation impacts on microbial communities, as well as studies that delve into the potential impacts of less studied behaviours.

1.5. Understudied invertebrate activities related to microbial ecology

1.5.1. Burrow ventilation

Burrow ventilation is a well characterised invertebrate activity that plays a significant role in the introduction of O_2 and solutes to sediment and displays clear inter-species variation (Quintana *et al.*, 2007; Braeckman *et al.*, 2010; Norkko *et al.*, 2012). Ventilation rate and periodicity, and their effect on sediment O_2 penetration, have therefore been examined in a range of invertebrate taxa (Kristensen *et al.*, 1991; Kristensen, 2001; Volkenborn *et al.*, 2010; Volkenborn *et al.*, 2012). Analyses of ventilation traits, however, rarely include detailed microbial assessments that could better establish the wider effects of these variations on biogeochemical cycling (Foshtomi *et al.*, 2018). Additionally, the extent to which these ventilation effects are mediated by burrow morphology has received little attention.

Invertebrate burrows can broadly be grouped into open ("U-shaped") and closed ("J-shaped) morphologies with water exiting the burrow through an opening or through percolation from depth respectively (Figure 1.5), which influences the type of exchange that can occur between burrows and the surrounding sediment (Meysman *et al.*, 2006). These variations in burrow morphology can have subsequent effects on both biogeochemical processes and general bacterial activity (Renz & Forster, 2013; Renz & Forster, 2014; Vasquez-Cardenas *et al.*, 2016), but, again, few studies have undertaken detailed microbial assessments using molecular techniques. Furthermore, ventilation trait analyses are often conducted using live invertebrates which means the specific effect of the trait of interest cannot be accurately isolated for use in benthic modelling. For example, although invertebrates with different burrow morphologies have been shown to alter the aerobic and anaerobic activities of chemoautotrophic microbes (Vasquez-Cardenas *et al.*, 2016), additional irrigation traits may vary between the selected taxa and so it is not possible to confirm that the observed effects are attributable to burrow structure alone. Current bioirrigation indices that aim to predict community composition effects on sediment ecosystem functioning either do not consider burrow morphology because of this interdependency with other traits (Renz *et al.*, 2018), or have decided that one morphology has a greater effect on biogeochemical cycling despite a lack of microbial analysis (Wrede *et al.*, 2018). Irrigation traits, along with other classic bioturbation traits, therefore need to be assessed in isolation to determine the actual impact, and potential

importance, of a trait on a process of interest and whether it should be included in future indices to improve biogeochemical cycling predictions.

Previous studies have experimentally isolated bioirrigation traits by irrigating artificial burrow structures, which allow better control over irrigation conditions (e.g. rate, periodicity, residence time) than the use of live invertebrates (Hansen *et al.*, 1996; Matsui *et al.*, 2004; Na *et al.*, 2008). Artificial structures have been used to examine changes in O_2 uptake (Marinelli & Boudreau, 1996), sulfate reduction (Nielsen *et al.*, 2003), and have demonstrated, through PLFA analysis, that changes to irrigation periodicity only have minor impacts on bacterial community composition but increased burrow residence time allows a successional community to develop (Marinelli *et al.*, 2002). Artificial burrows have not yet been used in conjunction with genetic microbial analysis or to assess nitrogen cycling processes.

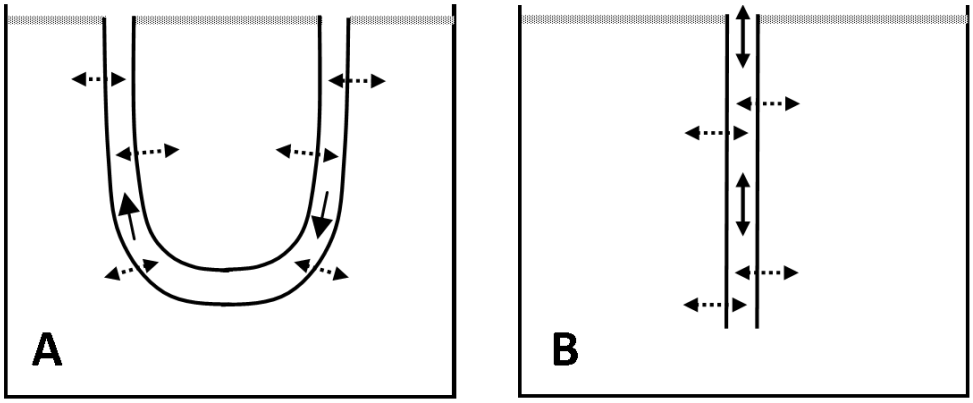

Figure 1.5 Typical burrow morphologies in impermeable sediment.
Open burrow structure with two or more openings at the surface (A), and closed burrow structure with a single opening at the surface (B) (Adapted from Kristensen *et al.*, 2012).

These artificial systems do not fully mimic natural irrigation activity because many use continuous irrigation which removes the complexity of invertebrate burrow ventilation (Kristensen *et al.*, 1991). Additionally, few systems account for invertebrate O_2 consumption (Chennu *et al.*, 2015), which would partially diminish O_2 exchange with sediment (Na *et al.*, 2008). O_2 exchange will also be altered by the use of point irrigation systems, which introduce water directly to depth in the sediment rather than through a permeable structure that allows exchange along the full burrow length (Matsui *et al.*, 2004; Na *et al.*, 2008). Similarly, isolating irrigation from other invertebrate activities ignores the reality that bioturbation is an interacting system and that these interactions may affect the importance of individual traits (Solan *et al.*, 2008). The goal with artificial experimental

systems is not to mimic nature but to improve overall functional understanding by allowing identification and characterisation of traits that play a significant role in mediating nitrogen cycling.

1.5.2. Invertebrate mucus secretions

Many sediment invertebrates secrete mucus (Kristensen, 2000) to aid with locomotion, burrowing, feeding, and to stabilise sediment structures (Nehring *et al.*, 1990; Wotton, 2004; Papaspyrou *et al.*, 2005). Invertebrate burrows are often lined with mucus (Aller & Yingst, 1985; Nielsen *et al.*, 2003; Webb & Eyre, 2004), which can alter sediment properties and processes by affecting solute and O_2 exchange between the burrow lumen and the sediment. The extent of this impact can vary between invertebrate taxa because of differences in mucus thickness (Hannides *et al.*, 2005; Zorn *et al.*, 2006). As mucus secretions are a complex of glycoproteins and glycosaminoglycans (Wotton, 2004), they also have the potential to be a source of reactive material to the sediment, with proteins and carbohydrates forming around 6-38% and 2-50% of mucus dry weight respectively (Talmont & Fournet, 1990; Santos *et al.*, 2009; Stabili *et al.*, 2014). Invertebrate mucus may therefore introduce significant quantities of organic carbon and nitrogen to sediment systems, which may be partially responsible for observed increases in bacterial abundance around burrow structures (Aller & Yingst, 1985; Kristensen, 2000). It has also been previously assumed that the organic nitrogen introduced by mucus will be broken down to NH_4^+ and therefore stimulate nitrification processes (McTigue *et al.*, 2016), as has been observed with diatom extracellular polymeric substances (EPS) in sediment (Bohórquez *et al.*, 2017). Yet, very few studies have examined if and how the presence of invertebrate mucus in sediment affects concentrations of reactive materials, composition of microbial communities, or rates of biogeochemical processes.

Variations in mucus lining thickness, and in bacterial abundance and activity, have been observed between *Hediste* (*Nereis*) *diversicolor* and *Alitta* (*Nereis*) *virens* burrows, which suggests that mucus may play a role in mediating microbial communities (Papaspyrou *et al.*, 2006). These differences in mucus secretion were not, however, isolated from other potential trait differences between these species (e.g. feeding mode, ventilation rate), which makes it difficult to assess how much of this impact is attributable to mucus variations. Other studies have therefore used seawater incubations to isolate mucus effects, which showed that mucus increases the abundance of heterotrophic bacteria capable of degrading proteins and saccharides (Herndl & Peduzzi, 1989; Peduzzi & Herndl, 1991; Alain *et al.*, 2002). This is in line with the idea that the introduction of organic compounds with mucus could encourage degradation and bacterial activity.

Sediment communities, however, will likely respond differently to seawater communities and only one study to date has isolated mucus in sediment or assessed the role of mucus in nitrogen cycling (Hannides & Aller, 2016). Through sediment incubations, this study demonstrated that mucus secretions can increase nitrogen remineralisation in sediment environments (Hannides & Aller, 2016). Yet, a lack of microbial assessment means it is difficult to conclude how this increase in NH_4^+ input impacted microbial functional groups involved in subsequent nitrogen transformations. To fully assess the role that mucus can play in sediment nitrogen cycling, we therefore need to be isolating the effects of mucus addition, incubating in realistic sediment conditions, and assessing impacts on both total microbial communities and specific nitrogen cycling microbial groups.

From diatom EPS studies, it is also clear that the impacts of mucopolysaccharide secretions on degradation rates and microbial communities within the sediment can alter with variations in O_2 regime (McKew *et al.*, 2013). Intermittent ventilation of invertebrate burrows means that burrow walls, including mucus linings, are exposed to regular oscillations between oxic and anoxic conditions (Furukawa, 2001; Pischedda *et al.*, 2012; Volkenborn *et al.*, 2012), and the extent of these oscillations will vary between invertebrate taxa due to differences in ventilation periodicity (Kristensen *et al.*, 1991; Kristensen, 2001; Volkenborn *et al.*, 2010; Delefosse *et al.*, 2015). The unique biogeochemical conditions created by these oscillations can lead to more rapid and complete organic matter degradation (Aller, 1994; Sun *et al.*, 2002), and an increase in facultative denitrifying bacteria to support sustained denitrification activity (Gilbert *et al.*, 2016; Wittorf *et al.*, 2016). As these O_2 variations in invertebrate burrows could mediate both mucopolysaccharide degradation and its subsequent effects on nitrogen cycling microbial communities and processes, the potential role of mucus secretions must be assessed under oxic, anoxic, and oscillating conditions to accurately determine its potential as an invertebrate functional trait. Overall, if there is a strong functional link between mucus secretions, nitrogen cycling microbial groups, and nitrogen cycling processes, then variations in mucus linings should be included as key traits in future bioturbation assessments to better understand why invertebrate taxa have variable impacts on microbial communities and nitrogen cycling.

1.5.3. Invertebrate gut transit

All metazoans have microbial communities living in and on internal and external surfaces which collectively form the individual's microbiome (Biron *et al.*, 2014). Both internal and external invertebrate surfaces can be important transporters of microbes within an

environment, with benthic crabs and shrimp able to transport gut bacteria up to a distance of 25 km (Troussellier *et al.*, 2017). The gut is one of the main interaction sites between invertebrates and microbial communities (Biron *et al.*, 2014), where communities are often formed of anaerobes (Wüst *et al.*, 2011; Sommer & Backhed, 2013) that can potentially provide the host with missing catabolic capabilities, such as the ability to degrade complex polysaccharides (Yoon *et al.*, 2015). Generally, invertebrate species are thought to have a core gut community that is found in all individuals (Lau *et al.*, 2002; Dishaw *et al.*, 2014), however, studies are now starting to demonstrate that host-microbiome interactions may be context-dependent (Biron *et al.*, 2014), and can therefore vary between geographically distinct populations (Wong *et al.*, 2013).

Significant interactions between invertebrates and sediment microbial communities may occur during sediment passage through the guts of deposit feeders. The digestive tracts of deposit feeders form a physicochemical gradient where redox conditions, organic matter concentrations, and enzyme activity all differ along the length of the individual (Plante & Jumars, 1992; Plante & Mayer, 1994; Mayer *et al.*, 1997). This means that different microbial communities are found at different points along the gut (Furlong *et al.*, 2002; Wilde & Plante, 2002; Li *et al.*, 2009), primarily because of the digestion and regrowth of microbes. Midgut digestion can be responsible for the loss of up to 97% of ingested bacteria (Plante *et al.*, 1989). As different bacterial taxa can have different sensitivities to enzymatic lysis, this digestion can affect microbial community composition (Plante & Shriver, 1998) and could vary between invertebrate species (Plante & Mayer; 1994, Mayer *et al.*, 1997). Microbial regrowth in the hindgut can cause a 20-100% increase in abundance compared to midgut communities, due to the high concentrations of dissolved organic matter, gut mixing, and a lack of competitors and predators (Plante *et al.*, 1989; Hymel & Plante, 2000; Andresen & Kristensen, 2002). This regrowth also has the potential to selectively alter microbial community composition as conditions may have different impacts on different microbial taxa (King, 2018). Greater bacterial abundances, and distinct bacterial communities, in deposit feeder faecal casts relative to the surrounding sediment indicate that this abundant and uniquely structured transitory community may be introduced from the gut to the sediment environment, where it could affect microbially mediated biogeochemical processes (Andresen & Kristensen, 2002; King, 2018). These selective and variable effects of both digestion and regrowth mean that gut transit may be a key invertebrate functional trait that could explain some of the variation in biogeochemical processes and bacterial communities between taxa, between disparate populations (Wohlgemuth *et al.*, 2017), and between individual burrows (Grossmann & Reichardt, 1991; Bird *et al.*, 2000).

Gut transit may also have direct impacts on nitrogen cycling microbial groups, as the ingestion of denitrifying taxa means that digestive tracts of aquatic deposit feeders can be sites of significant denitrification activity and nitrous oxide (N_2O) release (Stief *et al.*, 2009; Heisterkamp *et al.*, 2010). In terrestrial earthworms, ingested denitrifying microbes (Horn *et al.*, 2006a) undergo rapid proliferation under favourable anoxic gut conditions (Karsten & Drake, 1997), with the potential to be transferred back into the soil substrate as microbial isolates from earthworm faecal casts are more likely to reduce NO_2^- than isolates from the original soil (Furlong *et al.*, 2002). Deposit feeders may therefore be significant sources of denitrifiers or denitrification products to sediment environments. Detailed microbial assessments are required to determine the extent of this impact in marine environments because much of this work has focused on terrestrial systems. Overall, the passage of sediment through deposit feeder guts has the potential to impact sediment microbial communities, either through the introduction of the microbiome or through the selective effect of varying physicochemical gut conditions. The role of gut passage therefore needs to be assessed as an invertebrate trait to determine if these impacts are functionally important and whether they can explain some of the observed variation in microbial communities and biogeochemical processes in invertebrate communities.

1.6. Current study

The significant role that sediment nitrogen cycling plays in coastal and shelf sea ecosystem productivity means that it is important to understand how microbially-mediated nitrogen transformations are regulated in sediments. One ecological factor that has been relatively well assessed is the activity of sediment invertebrates, with particle reworking and ventilation activity altering the distribution of organic matter, O_2 and other electron acceptors in sediments, which create niches that support greater abundances and activity of nitrogen cycling microbial functional groups. Variations in bioturbation activity between different invertebrate taxa can have varying impacts on sediment properties, the general sediment microbial community and nitrogen cycling processes (Figure 1.6 A-B).

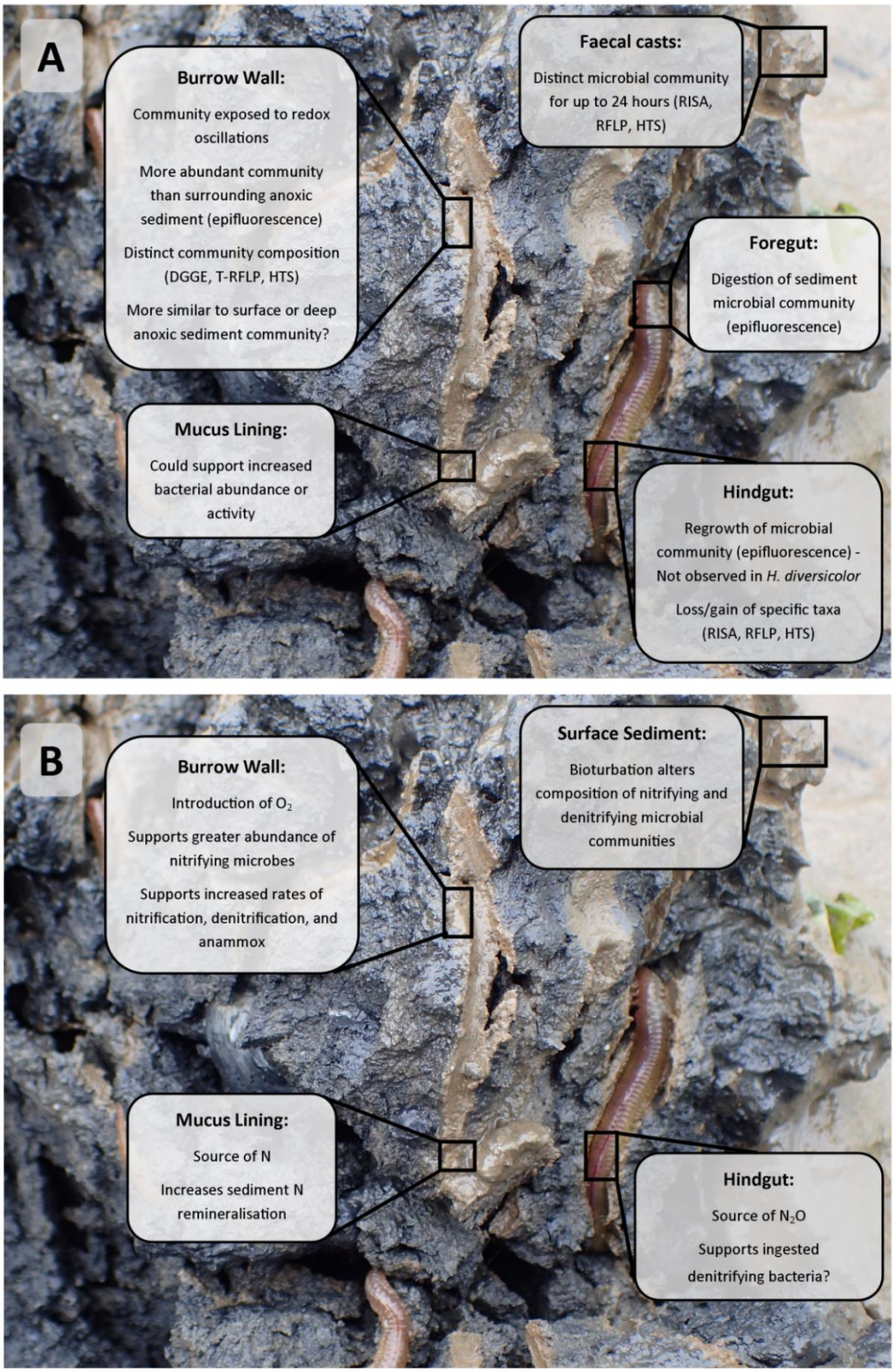

Figure 1.6 Current understanding of how sediment-dwelling invertebrate activities regulate whole sediment microbial communities (A) and nitrogen cycling microbial communities (B).

Text in parentheses represents microbial ecology technique used to observe stated trend **(**Photograph - Davis Laundon, MBA**)**.

As this review highlights, however, we only have a limited understanding of which of these variable traits are playing important roles in the regulation of sediment nitrogen transformations because few trait assessment studies have used contemporary molecular microbial ecology techniques. Additionally, few invertebrate traits have been isolated to confirm the effects that are attributable to specific traits. Traits that have been assessed have largely been drawn from 'classic' bioturbation activities with little consideration for the impacts of other activities, such as defecation or secretion. Identifying invertebrate traits that play a significant role in nitrogen cycling regulation will allow better predictions about how nitrogen cycling will be impacted by the loss, replacement or abundance change of invertebrate taxa in sediment communities, by improving the spatial scale and degree of ecological complexity in benthic biogeochemical models (Lessin *et al.*, 2018; Snelgrove *et al.*, 2018). More studies are therefore required to fill these knowledge gaps in our understanding of how invertebrate-microbe interactions can mediate biogeochemical processes. This overarching aim provides the basis for the investigations conducted within this thesis, which are outlined below.

Chapter 2: The role of burrow morphology in moderating ventilation effects on nitrifying microbial functional groups

Artificial burrows were used to examine how open and closed burrows moderate the effect of invertebrate ventilatory behaviour on nitrogen cycling microbial communities. The use of artificial burrows allowed the effect of burrow morphology to be isolated from other variations in ventilation behaviour (e.g. ventilation rate, ventilation periodicity), and nitrogen cycling communities were examined using Q-PCR and RT-QPCR in combination to assess changes in both the abundance and transcription of key nitrifying genes (bacterial *amoA* and archaeal *amoA*).

Chapter 3: Invertebrate mucopolysaccharide secretion effects on sediment microbial community composition and nitrogen cycling processes

Hediste diversicolor mucopolysaccharide secretions were isolated in sediment slurry incubations under continuously oxic conditions to investigate how relatively high concentrations of mucus affected sediment bacterial and archaeal communities. High-throughput sequencing was used in conjunction with Q-PCR to examine changes in whole community composition and diversity, and the abundance of key nitrogen cycling microbial functional groups. Changes in nitrogen compound concentrations were also assessed to confirm whether these microbial variations had an observable impact on biogeochemical processes.

Chapter 4: Mucopolysaccharide secretion effects on nitrogen cycling communities under oscillating redox conditions

To explore how the unique oscillating O_2 environment of invertebrate burrows may alter the effects of mucopolysaccharide secretions, sediment slurry incubations with and without mucopolysaccharide were exposed to continuously oxic, continuously anoxic, and oscillating oxic-anoxic conditions. In this study, however, significantly lower amounts of mucus were added to the sediment slurries compared to Chapter 3, to represent a low mucus environment. As nitrogen cycling processes were the focus of this investigation, Q-PCR and RT-QPCR were used to examine changes in the abundance and expression of nitrogen cycling functional genes. The extent to which these microbial community variations altered nitrogen cycling processes was examined through changing nitrogen compound concentrations.

Chapter 5: Exploring the effect of marine polychaete gut passage on sediment microbial assemblage structure

High-throughput sequencing of bacterial and archaeal communities was used to investigate how the diversity and composition of microbial communities varied during passage through the gut of the deposit-feeding polychaete *Hediste diversicolor*. The extent to which this may influence sediment communities was assessed by comparing these transitory gut communities to those observed in the surrounding burrow wall, surface, and unbioturbated sediment. Variations in nitrogen cycling functional gene abundance were also assessed using Q-PCR, to explore whether gut passage had specific effects on functionally important microbial groups.

2. The role of burrow morphology in moderating ventilation effects on nitrifying microbial groups

2.1. Abstract

Burrow ventilation by infaunal invertebrates is known to affect sediment nitrogen cycling by altering the activity, distribution and abundance of nitrifying microbial communities, however, the extent to which variations in burrow morphology can modify this relationship has received little attention. In this study, we use artificial burrows to isolate the effects of an open versus closed burrow arrangement on the abundance and activity of ammonia-oxidising bacteria (AOB) and archaea (AOA) at different depths across the sediment profile. Our findings demonstrate that burrow morphology had little effect on the nitrifying microbial community at the sediment-water interface or at depth. At intermediate mixing depths, however, AOB and AOA abundance were greatest around closed burrow mimics and activity levels were highest around the open burrow mimics. These data, coupled with visual observations of the sediment, indicate that variations in sediment-water exchange between these two burrow morphologies result in varying O_2 distributions that subsequently affect nitrogen cycling communities. This will be relevant for current attempts to produce functionally relevant bioirrigation indices, in which burrow morphology effects on biogeochemical processes have been assumed.

2.2. Introduction

The reworking of sediment particles and modification of porewater by invertebrates can alter microbial nitrogen transformations in marine sediments (Laverock *et al.*, 2011). Infaunal burrows support abundant nitrifying communities and increased nitrification and denitrification rates (Kristensen *et al.*, 1991; Satoh *et al.*, 2007), in part because active burrow ventilation by invertebrates increases the supply of O_2 that stimulates microbial activity (Aller *et al.*, 1983; Mermillod-Blondin *et al.*, 2005; Na *et al.*, 2008). Whilst burrow walls can sustain abundant nitrifying communities throughout the sediment column, O_2 fluxes and nitrogen cycling microbial groups around burrows are not homogeneously distributed with depth (Pischedda *et al.*, 2012; Papaspyrou *et al.*, 2014) and variations in microbial community structure between ventilated and non-ventilated sediment may only be observable at depths of several centimetres (Satoh *et al.*, 2007). Assessments of invertebrate-microbial interactions are, however, often limited to the upper 1-2 cm of sediment (Stauffert *et al.*, 2014; Sciberras *et al.*, 2017), as this corresponds to the generally accepted maximum penetration depth of oxygen (Foshtomi *et al.*, 2015). This lack of consideration of vertical variations in microbial community composition is unlikely to lead to complete assessments of invertebrate-microbe interactions and their mediation of biogeochemical processes, not least because invertebrate fauna redistribute organic matter to deeper sediment layers (Van Duyl *et al.*, 1992).

As variations in the rate, magnitude, and temporal pattern of ventilatory activity can modify biogeochemical processes, considerable effort has been made to characterise the ventilatory behaviour of sediment invertebrate communities (Solan *et al.*, 2019). Whilst burrow morphology (Hale *et al.*, 2014) and ventilatory dynamics can be complex (Forster & Graf, 1995; Jovanovic *et al.*, 2014) and vary with environmental context (Ouellette *et al.*, 2004), the effects that the burrow system will have on microbial activity and biogeochemical processes will largely be a function of the structure and configuration of the burrow (Capowiez *et al.*, 2014) and type of sediment-water exchange generated (Heron & Ridd, 2008; Renz & Forster, 2014; Vasquez-Cardenas *et al.*, 2016).

Open ('U-shaped') burrows are characterised by two or more openings at the sediment-water interface and are typically found in impermeable sediments where diffusive transfer drives exchange between the burrow lumen and the surrounding sediment, while closed ('J-shaped') burrows are characterised by a single opening and are typically found in permeable sediments that allow advective transport of spent burrow water through the sediment profile (Meysman *et al.*, 2006) (Figure 2.1). In closed burrows observed in less permeable sediment, return flow of porewater back to the surface tends

to be more localised and less evenly-distributed (Volkenborn *et al.*, 2010; Volkenborn *et al.*, 2012).

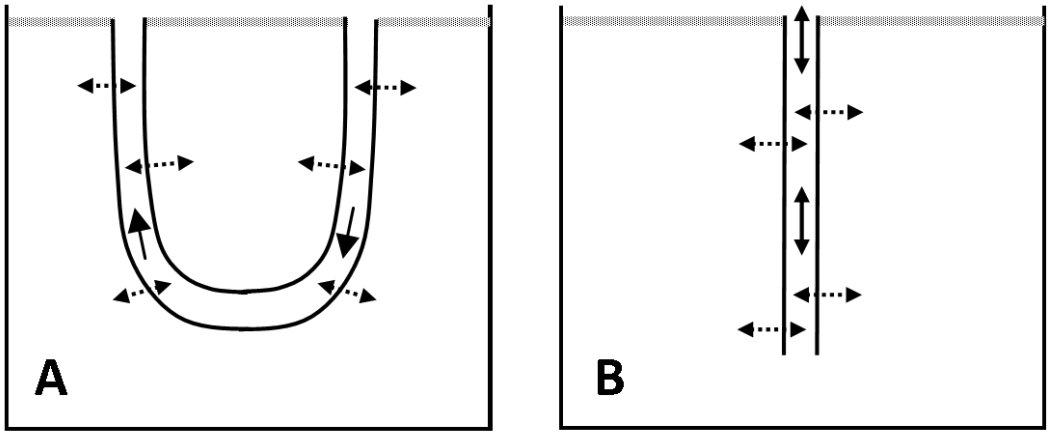

Figure 2.1 Typical burrow morphologies in impermeable sediment.
Open burrow structure with two or more openings at the surface (A), and closed burrow structure with a single opening at the surface (Adapted from Kristensen *et al.*, 2012).

Determining the impact of burrow morphology on microbial communities and processes is not straightforward because it is necessary to isolate the effect of burrow morphology from the confounding effects of other attributes of the burrow system and/or from intra-specific variations in ventilatory behaviour (Wohlgemuth *et al.*, 2017). Recent work has shown the utility of using replicate artificial burrows of a known morphology in which the confounding effects of other factors, such as invertebrate secretions, can be removed, whilst also allowing greater control of irrigation rates and burrow residence times (Hansen *et al.*, 1996; Marinelli *et al.*, 2002; Na *et al.*, 2008).

In this study, we used this artificial burrow approach to isolate the effects of burrow morphology (open vs. closed) from the effects of ventilation rate, and determine how these variables affect the abundance and activity of ammonia-oxidising bacteria (AOB) and archaea (AOA) at representative depths within the bioturbated region of the sediment profile. We hypothesised that open and closed burrows would stimulate nitrification activity across the full extent of the sediment profile, but that variations in sediment O_2 and solute distributions between the two morphologies would affect the vertical distribution of nitrifying microbial communities.

2.3. Method

2.3.1. Artificial burrow design

Initial artificial burrow designs consisted of a U-shaped length of tubing that ran from the top of each side of the mesocosm to the base of the sediment column, with perforations and oxygen permeable dialysis membrane along the sediment enclosed section (Figure 2.2). This was based on previous artificial irrigation studies (Marinelli, 1994; Nielsen *et al.*, 2003). A peristaltic pump transported seawater through a sealed connection into the burrow inflow, with a second sealed connection at the outflow, to create a closed and pressurised flow system in which the burrow outflow returned to the inflow reservoir. Small-scale trials of this design showed no sediment colour change around the artificial burrow to indicate sediment oxygenation (Lyle, 1983; Statham *et al.*, 2017). Subsequently, inflow and outflow reservoirs were separated to ensure the artificial burrows received well-oxygenated seawater without excess metabolite accumulation.

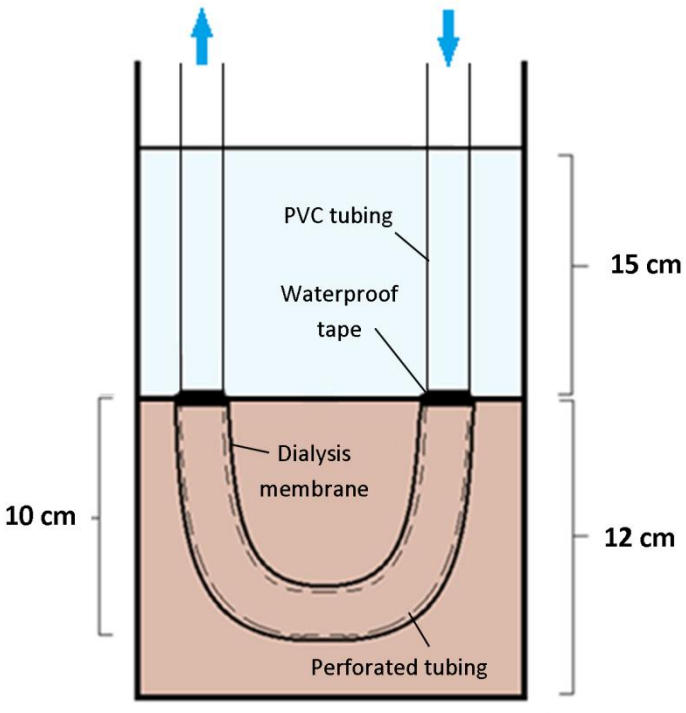

Figure 2.2 Schematic of the initial closed system U-shaped artificial burrow design. Arrows indicate water inflow and outflow.

In further trials, however, accurate ventilation rates were difficult to maintain, with some burrows exhibiting increasingly inconsistent flow rates or no flow at all. This appeared to limit sediment-water exchange within the system, which meant there was still no observable change in sediment oxygenation and no change in nutrient concentrations

in the overlying water column. To better control ventilation rates, the design was altered to an open system in which the outflow emptied directly into either the water column (U-shaped burrow design) or the sediment column (J-shaped burrow design) (Figure 2.3). These new designs were tested in seawater with a coloured dye to confirm that the dialysis membranes filled with inflowing seawater. In further sediment trials, irrigation rates were maintained at a consistent 1.86 ± 0.03 ml min^{-1} and 1.73 ± 0.05 ml min^{-1} for the U-shaped and J-shaped artificial burrows respectively, and so this open system was carried forward for the burrow morphology study.

2.3.2. Sediment collection and artificial burrows

Surficial sediment (<3 cm depth) was collected in June 2016 from the Plym Estuary (UK) (50˚22.281' N, 004˚06.289' W) and sieved (500 µm) in seawater to remove macrofauna and detritus, left to settle for 90 hours to retain the fine sediment fraction, then drained and homogenised.

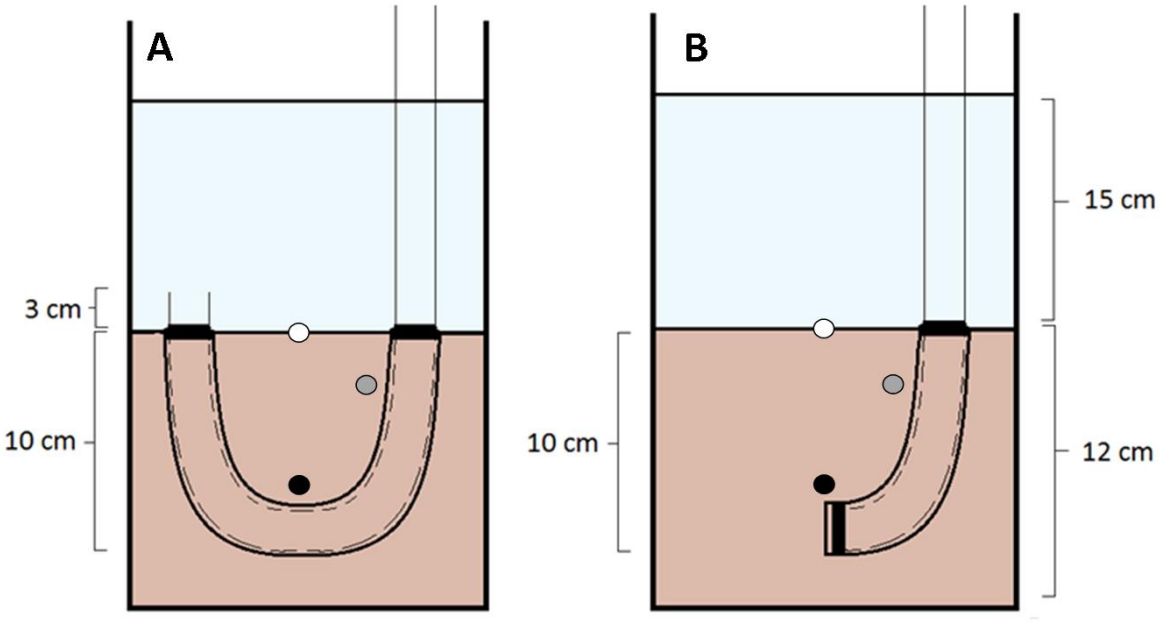

Figure 2.3 Schematic of open (A) and closed (B) artificial burrow design. Diagram indicates the three sediment sampling sites: sediment-water interface ($L_{surface}$, white), subsurface (L_{mid}, grey), and deep (L_{max}, black).

Replicate open (U-shaped, outflow above sediment surface, Figure 2.3 A) and closed (J-shaped, outflow 10 cm below sediment surface, Figure 2.3 B) artificial burrows were constructed using PVC tubing (6 mm diameter) with 5 mm diameter perforations, offset from one another at 15 mm intervals, to allow lateral exchange. Cellulose dialysis

membrane (50 mm circumference) formed a sheath over the PVC tubing and was attached 1 cm beyond the final perforation at the anterior and posterior end (Marinelli, 1994; Nielsen *et al.*, 2003). Artificial burrows were positioned in individual aquaria (internal diameter: 10 cm; height: 33 cm) and sediment homogenate was added to a depth of 12 cm and overlain with 15 cm of seawater (1 µm filtered; UV sterilised, ~34 psu; Figure 2.3). Three replicates of each artificial burrow were arranged randomly in a dark, temperature controlled room (12 ± 1 ˚C) and acclimatised for 96 hours before the seawater was exchanged.

2.3.3. Burrow ventilation

Artificial burrows were continuously flushed with 1 µm filtered, ultraviolet (UV)-sterilised seawater at 2 ml min^{-1} (Nielsen *et al.*, 2003) using a peristaltic pump (Ismatec, Germany) for 14 days (Marinelli *et al.*, 2002). Aquaria were maintained in the dark and were subject to a partial (80%) seawater change every 5 days. After 14 days, sediment samples were taken from the sediment-water interface (~0.2 cm depth, hereafter $L_{surface}$), the subsurface sediment adjacent to the artificial burrow entrance (~3 cm depth, hereafter L_{mid}), and the sediment adjacent to the upper side of the deepest section of the artificial burrow (~8 cm depth, hereafter L_{max}) using a sterile syringe shaft (Figure 2.3). All sediment samples were stored at -80 ˚C.

Table 2.1 Standard curve reaction efficiencies.
Summary of efficiencies, slope values and technical replicate variation for each reaction (bacterial and archaeal *amoA* DNA and cDNA abundance).

Gene	Efficiency	R^2	Slope	Intercept	Ct variation
Bacterial *amoA* DNA	0.69	0.929	-4.408	3.012	0.41
Archaeal *amoA* DNA	0.78	0.987	-3.974	4.504	0.57
Bacterial *amoA* cDNA	1.04	0.836	-3.222	3.697	0.41
Archaeal *amoA* cDNA	0.66	0.974	-4.549	6.435	0.69

2.3.4. Microbial analyses

DNA and RNA were extracted from 0.3 g of sediment (wet weight) using the RNeasy PowerSoil Total RNA Kit and the RNeasy PowerSoil DNA Elution Kit (Qiagen, Germany). The DNA and RNA yield was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80˚C. To assess the activity of the ammonia-oxidising community, bacterial and archaeal *amoA* RNA transcripts were reverse transcribed to cDNA using the Omniscript Reverse Transcription Kit (Qiagen, Germany), with the Domain-specific primers amoA-2R and ArchamoA-2R (Rotthauwe *et al.*, 1997; Francis *et al.*, 2005). The abundance and activity of ammonia-oxidisers was calculated using quantitative PCR (Q-PCR) to determine the abundance of bacterial and archaeal *amoA* DNA and cDNA respectively (Table 2.1). Bacterial *amoA* gene and transcript abundances were analysed using the primers amoA-1F and amoA-2R (amplicon length 491 bp; Rotthauwe *et al.*, 1997) with an annealing temperature of 60 ˚C. Archaeal *amoA* gene and transcript abundances were analysed using the primers ArchamoA-1F and ArchamoA-2R (amplicon length 635 bp; Francis *et al.*, 2005) with an annealing temperature of 53 ˚C. Ten µL reactions contained 5 µL 2x SensiFast SYBR No-ROX 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 Rotor-Gene 6000 (Corbett Life Science, Australia), with duplicate technical replicates for each sample. Both reactions were carried out with an initial denaturation step of 94 ˚C for 10 minutes, followed by 42 cycles of 94 ˚C for 30 seconds, annealing temperature for 30 seconds, and 72 ˚C for 60 seconds. Normalised gene expression was calculated as the ratio of cDNA to DNA, and all results were converted from ng μl^{-1} to copy number.mgwwsediment^{-1}. Data are reported according to MIQE guidelines (Bustin *et al.*, 2009).

2.3.5. Statistical analysis

Initially, linear mixed effect (LME) models were developed for each dependent variable (bacterial *amoA* abundance and transcript abundance, i.e. cDNA abundance, archaeal *amoA* DNA abundance and gene expression, bacterial:archaeal *amoA* DNA abundance (AOB:AOA)), with burrow type (open, closed) and sediment depth ($L_{surface}$, L_{mid}, L_{max}) treated as independent nominal variables. As the structure of the data differed between sediment depth treatment levels, we established independent LME models for $L_{surface}$, L_{mid}, and L_{max} sediments for each dependent variable with burrow type treated as an independent nominal variable (Table 2.2). As we used all Q-PCR replicates (n = 6), we

included replicate as a random effect. Where there was evidence of heteroscedasticity, variance-covariate terms were applied to allow residual spread to vary with individual explanatory variables (Pinheiro & Bates, 2000). We found no evidence of a violation of homogeneity of variance for any of the bacterial *amoA* DNA abundance, archaeal *amoA* DNA abundance, or AOB:AOA models, but diagnostic plots indicated residual spread varied with burrow type for normalised bacterial and archaeal *amoA* expression at all three sediment depths (Table 2.2). This was confirmed by comparing the initial LME model to an equivalent model with specific variance structures using Akaike Information Criteria (AIC) and visualisation of model residuals obtained by restricted maximum likelihood (REML) estimation. The optimal fixed structure was then determined by applying manual backward selection using the likelihood ratio test and maximum likelihood (ML) estimation to sequentially remove the highest order non-significant terms. The minimal adequate models (Table 2.2) were validated by assessing normality (Q-Q plots) and visual inspection of residuals versus fitted values (Zuur *et al.*, 2009; Zuur & Ieno, 2016). All analyses were carried out using the nlme package v. 3.1-120 (Pinheiro *et al.*, 2013) in R (version, 3.2.2, R Core Team, 2015).

2.4. Results

2.4.1. Observations of sediment

Sediments that contained open burrows developed a continuous and defined oxidised layer (brown coloration, ~ 0.2 cm layer) at the sediment-water interface (Figure 2.4 A-C). Below this depth, sediment did not appear to be oxidised (black coloration: Lyle 1983; Statham *et al.*, 2017). In contrast, sediments that contained closed burrows did not develop a defined oxidised layer at the sediment-water interface, but regions of oxidised sediment were visible around the artificial burrows that varied in intensity and in vertical and horizontal spread (Figure 2.4 D-F).

2.4.2. Abundance of ammonia-oxidisers

Bacterial and archaeal *amoA* gene abundance was not dependent on burrow type in the $L_{surface}$ or L_{max} sediments (Table 2.2, Models 1, 3, 4, 6), however in the L_{mid} sediment higher abundances were observed around the closed burrows (AOB, 8,450 ± 1,530 copies.mgwwsediment^{-1}; AOA, 11,400 ± 6,200 copies.mgwwsediment^{-1}) than around the open burrows (AOB, 2,240 ± 1,750 copies.mgwwsediment^{-1}; AOA, 2,340 ± 2,240 copies.mgwwsediment^{-1}) (Table 2.2, Models 2, 5; Figure 2.5 A-B). The AOB:AOA abundance ratio was not affected by burrow type at any sediment depth (Table 2.2, Models 13, 14, 15).

Although we were unable to statistically compare AOB and AOA abundances between sediment depths, there is some indication that abundances increased between the L_{mid} and L_{max} sediment surrounding the open burrow (L_{mid}: AOB, 2,240 ± 1,750 copies.mgwwsediment^{-1}; AOA, 2,340 ± 2,240 copies.mgwwsediment^{-1} and L_{max}: AOB, 13,700 ± 6,710 copies.mgwwsediment^{-1}; AOA, 10,400 ± 3,440 copies.mgwwsediment^{-1}; Figure 2.6 A-B).

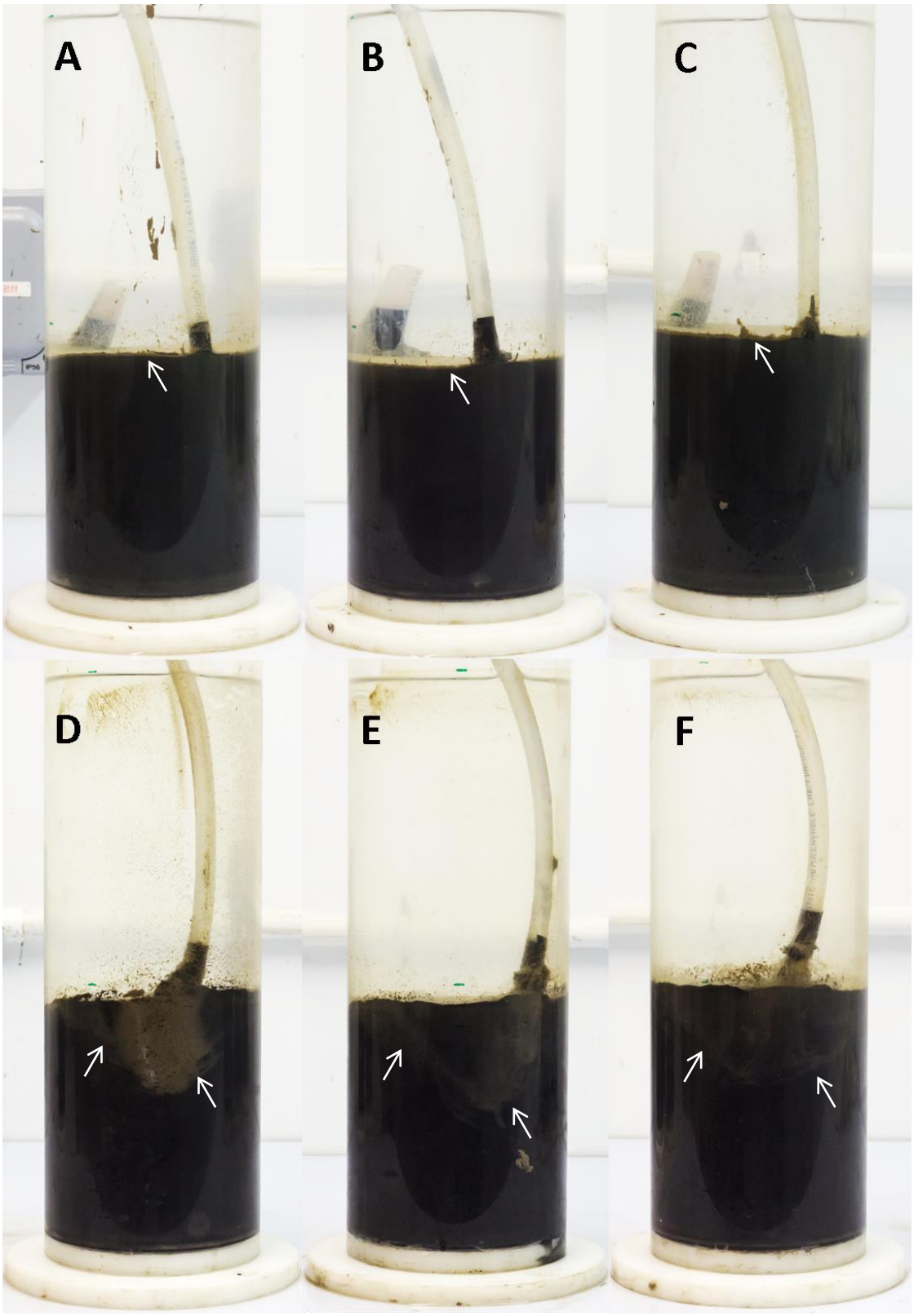

Figure 2.4 Open (A-C) and closed (D-F) artificial burrow treatments.

Cores shown at the end of the two week irrigation period, displaying distinct distributions of oxidised sediment (brown coloration, position indicated by arrows) (Core: external diameter = 11 cm, internal diameter = 10 cm) (Photographs - Alix Harvey, MBA)

Table 2.2 Summary of linear mixed effects models.

Models treat bacterial and archaeal *amoA* DNA abundance, bacterial and archaeal *amoA* normalised gene expression, and AOB:AOA as dependent variables and burrow type as the fixed explanatory variable. Significant results highlighted in bold. Initial linear model: Response variable ~ burrow type. (Appendix I, Statistical Information).

Model	Sediment depth	Response variable	Burrow type			Variance-Covariate
			L-ratio	d.f	p	
1	$L_{surface}$	Bacterial *amoA* abundance	0.00	1	0.993	-
2	**L_{mid}**	**Bacterial *amoA* abundance**	**12.01**	**1**	**< 0.001**	**-**
3	L_{max}	Bacterial *amoA* abundance	2.59	1	0.107	-
4	$L_{surface}$	Archaeal *amoA* abundance	0.01	1	0.908	-
5	**L_{mid}**	**Archaeal *amoA* abundance**	**4.73**	**1**	**0.030**	**-**
6	L_{max}	Archaeal *amoA* abundance	1.27	1	0.260	-
7	$L_{surface}$	Bacterial *amoA* expression	1.71	1	0.191	Burrow type
8	**L_{mid}**	**Bacterial *amoA* expression**	**2.89**	**1**	**0.090**	**Burrow type**
9	L_{max}	Bacterial *amoA* expression	0.30	1	0.581	Burrow type
10	$L_{surface}$	Archaeal *amoA* expression	1.32	1	0.251	Burrow type
11	**L_{mid}**	**Archaeal *amoA* expression**	**11.04**	**1**	**< 0.001**	**Burrow type**
12	L_{max}	Archaeal *amoA* expression	2.09	1	0.148	Burrow type
13	$L_{surface}$	AOB:AOA	0.58	1	0.446	-
14	L_{mid}	AOB:AOA	1.17	1	0.280	-
15	L_{max}	AOB:AOA	0.68	1	0.410	-

2.4.3. Activity of ammonia-oxidisers

We found no effect of burrow type on the normalised gene expression of bacterial and archaeal *amoA* in the $L_{surface}$ or L_{max} sediments (Table 2.2, Models 7, 9, 10, 12), however in the L_{mid} sediment we found that gene expression was higher around the open burrows (AOB, 27.34 ± 34.21 RNA/DNA; AOA, 35.42 ± 51.05 RNA/DNA) than in the closed burrows (AOB, 2.71 ± 2.58 RNA/DNA; AOA, 1.75 ± 2.74 RNA/DNA) (Table 2.2, Models 8, 11; Figure 2.5 C-D).

Although not statistically compared, our data indicated a decrease in bacterial *amoA* gene expression between the L_{mid} and L_{max} sediment surrounding the open burrow (L_{mid}: 27.3 ± 34.2 copies.mgwwsediment^{-}1 and L_{max}: 2.61 ± 2.68 copies.mgwwsediment^{-1}; Figure 2.6 C-D). This trend was not observed with archaeal *amoA* gene expression.

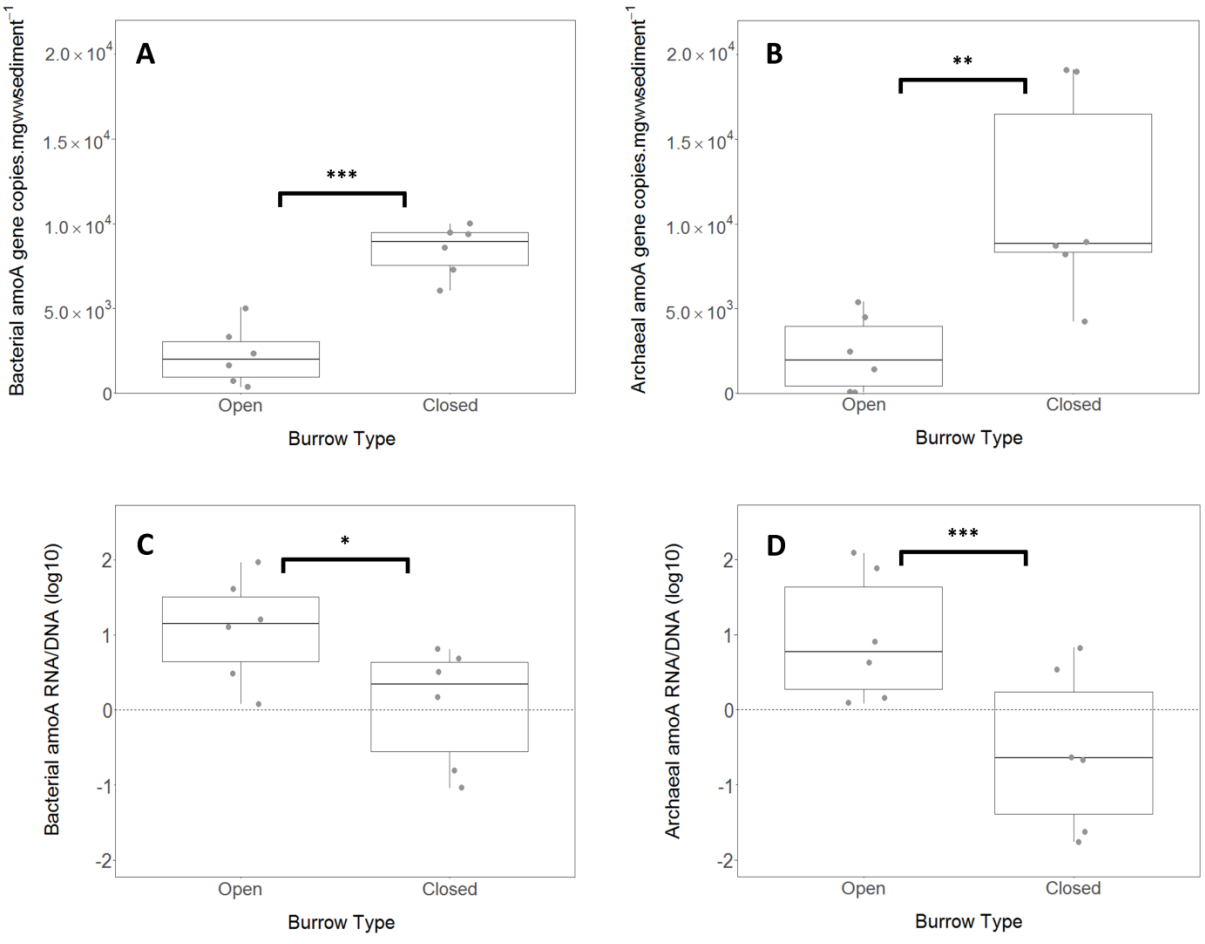

Figure 2.5 Variations in bacterial and archaeal *amoA* gene abundance (A-B) and normalised bacterial and archaeal *amoA* gene expression (C-D) with burrow type in the subsurface sediment (L_{mid} only).

Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread. (n = 6) (*** p < 0.001, ** p < 0.05, * p < 0.1). Gene expression data are log transformed (log_{10}) for ecological clarity.

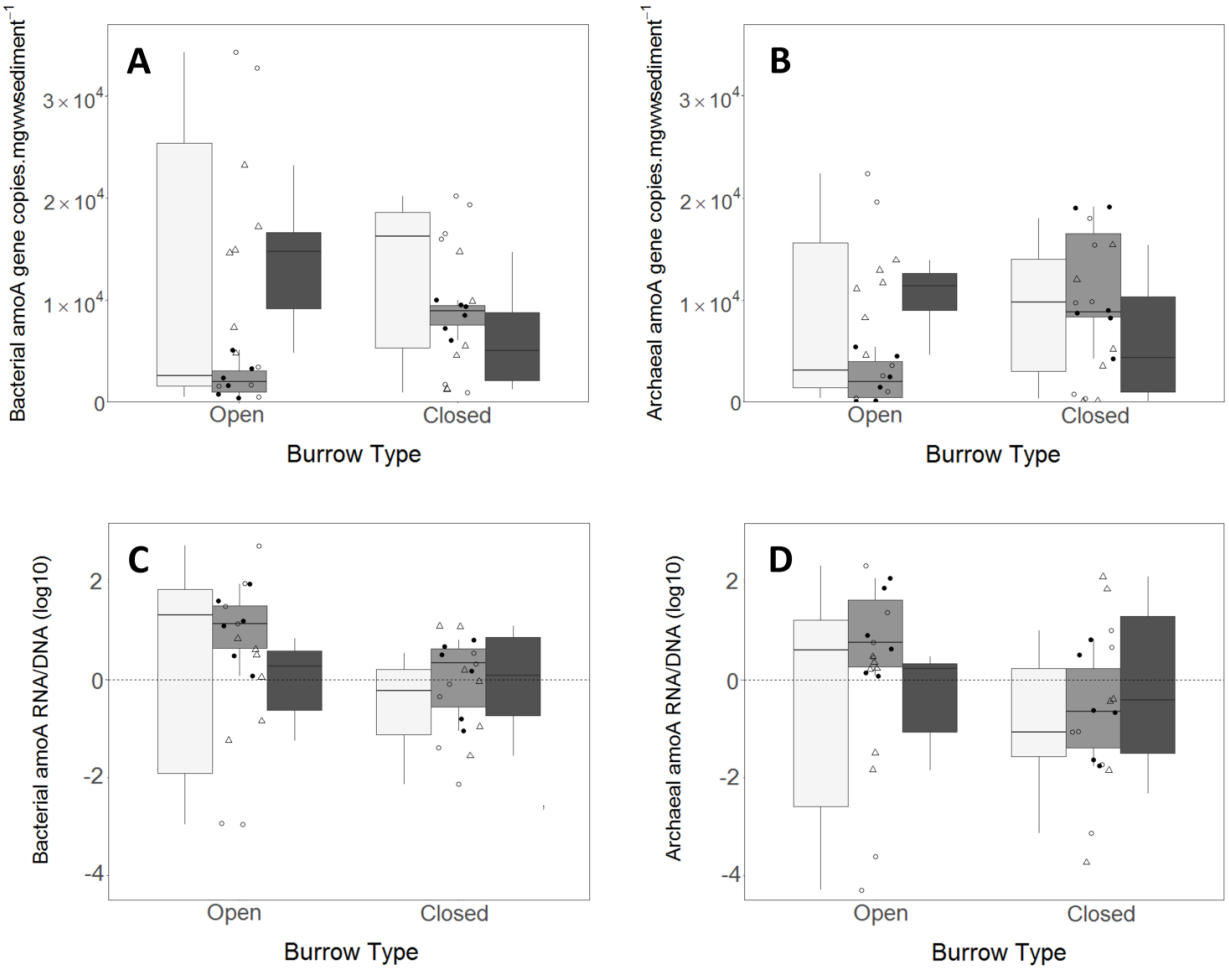

Figure 2.6 Bacterial and archaeal *amoA* gene abundance (A-B) and normalised bacterial and archaeal *amoA* gene expression (C-D) with burrow type at all sediment depths ($L_{surface}$, L_{mid}, and L_{max}).

Burrow type only significantly affected these variables at mid-mixing depths (L_{mid}). ($L_{surface}$: light grey, open circles; L_{mid}: medium grey, closed circles; L_{max}: dark grey, open triangles) (n = 6). Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread. Complete dataset is presented here for visual comparison of trends. Gene expression data are log transformed (log_{10}) for ecological clarity.

2.5. Discussion

Whilst it is recognised that the ventilatory activities of invertebrate fauna are important in moderating sediment microbial community structure and biogeochemical processes (Laverock *et al.*, 2011), efforts to separate the relative influence of different ventilation components have been less prominent (Marinelli *et al.*, 2002). Here, using an artificial burrow system in which ventilation activity and burrow architecture can be independently controlled and distinguished, we demonstrate that burrow morphology can lead to distinct changes in the abundance and activity of nitrifying microbial functional groups. These effects were most notable at mid-mixing depths, where there were observable differences in oxygen distribution between open and closed burrows. This indicates that the influence of invertebrate ventilatory behaviour does not solely relate to the rate and periodicity of burrow ventilation (Volkenborn *et al.*, 2012), but also to variations in porewater exchange due to specific burrow morphologies.

Increased ammonia-oxidiser abundance in the subsurface of closed burrows must be related to variations in organic matter and/or O_2 distribution (Herbert, 1999; Li *et al.*, 2018). As the process of sediment homogenisation in this study would have resulted in an even distribution of organic matter and carbon, we conclude that closed burrows may support greater diffusion of O_2 and other terminal electron acceptors to subsurface sediment because of the associated increase in water residence time relative to an open burrow system (Santos *et al.*, 2012). Indeed, in an open burrow system, the relatively unrestricted water flow is likely to minimise residence time in the subsurface sediment and lead to an accumulation of inhalant ventilated water at the maximum burrow depth. This greater potential for O_2 diffusion to occur in the deepest sections of the burrow (Nielsen *et al.*, 2004) could explain the increased proliferation of AOB and AOA with increasing depth. We acknowledge, however, the possibility that this is an artefact of using burrow mimics that do not replicate the complex periodicity of burrow ventilation (Kristensen *et al.*, 1991) as O_2 penetration tends to decline along the length of open ended burrows in natural systems (Pischedda *et al.*, 2012). Incorporating these periodicities into future artificial burrow morphology studies could further understanding by examining temporal variations in nitrifying microbial activity.

In contrast, the architecture of closed burrows means that expelled ventilation water can only exit the burrow via percolation through the burrow wall or at the terminus of the burrow (Delefosse *et al.*, 2015). The reliance on diffusive exchange increases residence time of oxygenated water through the length of the burrow, which increases the likelihood of a gradient of O_2 diffusion into the surrounding sediment. Our observations of

a relatively constant abundance of ammonia-oxidisers with depth around the closed burrows would support this view, and is generally consistent with observations of oxygen distributions in impermeable and semi-permeable sediment made elsewhere (Volkenborn *et al.*, 2012; Jovanovic *et al.*, 2014). Visual observation of the sediment cores, however, does suggest some degree of percolation, possibly through sediment fractures or meiofaunal burrows, as localised plumes of oxidised sediment in the upper 5 cm of the sediment column and a disrupted sediment-water interface were observed (Volkenborn *et al.*, 2010). This could also explain the greater proliferation of the nitrifying community at the mid-mixing depths around closed burrows. Nevertheless, irrespective of the mechanism, closed burrows seem to be able to support greater AOB and AOA abundances in the subsurface sediment by increasing sediment O_2 penetration.

The differential effects of burrow type on microbial activity are consistent with findings elsewhere (Vasquez-Cardenas *et al.*, 2016), and are likely to relate to organic matter availability (Van Duyl *et al.*, 1992). Burrow walls with high abundances of ammonia-oxidisers have high levels of ammonium (NH_4^+) consumption (Satoh *et al.*, 2007). Therefore, sites of increased ammonia-oxidiser abundance may become NH_4^+-depleted during bouts of irrigation which would lead to a subsequent decline in ammonia-oxidising activity (Stein & Arp, 1998). Activity would then appear to be higher in the less-oxygenated areas around the burrows, contrary to expectation (Satoh *et al.*, 2007). Whilst, these trends may not reflect the full range of impacts that burrow type can have on ammonia-oxidising activity, as activity was only assessed at the end of the ventilation period, these results indicate that burrow morphology moderates ventilation effects on nitrogen cycling communities. Future research should therefore examine how burrow morphology effects on nitrification activity vary temporally as ventilation occurs.

Variations in ammonia-oxidising microbial groups between O_2-rich microniches created by open and closed burrows could alter sediment fluxes of dissolved inorganic nitrogen (DIN). The potentially greater production of nitrite (NO_2^-) and nitrate (NO_3^-) in the subsurface sediment surrounding closed burrows, for example, may increase the release of DIN into the overlying water, with implications for ecosystem productivity (Hulth *et al.*, 2005). Burrow morphology may also alter the degree of coupled nitrification-denitrification (Sayama & Kurihara, 1983; Na *et al.*, 2008), as increased production of NO_2^- and NO_3^- at depth could facilitate exchange between aerobic and anaerobic processes and therefore increase denitrification activity. More detailed microbial assessments (Marinelli *et al.*, 2002) and flux analyses will be needed to better understand how and when different attributes of invertebrate ventilatory behaviour alter sediment nitrogen cycling and the depth distribution of nitrogen cycling microbes.

Previous studies have pooled burrow wall samples from the entire sediment column (Pischedda *et al.*, 2011) or have focused only on the surficial sediment because this is the perceived limit of O_2 penetration (Foshtomi *et al.*, 2015). Here, we have shown that visible changes in O_2 penetration at the sediment surface had little impact on the ammonia-oxidising community, whilst variations in nitrogen cycling microbial groups between the burrow types occurred at mid-mixing depths. Analysing changes in nitrogen cycling microbial groups with depth reveals distinct and context-specific spatial distributions (Satoh *et al.*, 2007; Papaspyrou *et al.*, 2014), while assessing only a single depth ignores the three-dimensional nature of sediment environments (Gilbertson *et al.*, 2012; Stauffert *et al.*, 2014).

In changing sediment ecosystems, strong species-specific effects on sediment microbial communities and biogeochemical processes mean that biodiversity loss, or changes to dominant invertebrate taxa, have the potential to significantly affect organic matter cycling (Mermillod-Blondin *et al.*, 2005; Solan *et al.*, 2008; Gilbertson *et al.*, 2012). Accurately predicting the effects of bioturbator community change requires a clear understanding of which irrigation traits significantly affect microbially-mediated processes across the sediment profile, to improve the degree of small-scale heterogeneity and ecological complexity in benthic system models (Lessin *et al.*, 2018; Snelgrove *et al.*, 2018). Current trait-based descriptions of invertebrate ventilation (Renz *et al.*, 2018) are useful but do not consider how the morphological complexity of the burrow mediates the effects of infaunal behaviour on nitrogen cycling communities across various sediment depths and environmental contexts.

The role of burrow morphology requires further assessment to fully understand the extent to which this trait regulates irrigation effects, but the findings outlined here clearly indicate that morphological traits can affect vertical distributions of O_2 and nitrifying microbial communities beyond the immediate burrow. By isolating various components of ventilation behaviour, we can build our mechanistic understanding of ventilation effects to produce better models and indices of this well-studied invertebrate trait. This trait assessment model can then be expanded to potentially significant, but currently under studied, invertebrate functional traits.

3. Invertebrate mucopolysaccharide effects on sediment microbial community composition and nitrogen cycling processes

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3.1. Abstract

Mucopolysaccharides (mucus) are produced by many invertebrates and have the potential to be an important source of organic carbon and nitrogen for sediment microorganisms. At present, we only have a 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, this study used sediment slurries incubated with and without *Hediste diversicolor* mucus to examine changes in dissolved inorganic nitrogen (ammonia, nitrite, and nitrate) concentrations and bacterial and archaeal community diversity. 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. *H. diversicolor* mucopolysaccharide therefore appears to enhance sediment nitrification rates by stimulating nitrifying microbial groups. We propose that invertebrate mucopolysaccharide secretion should be considered as a distinct sediment-dwelling invertebrate functional trait, to improve our mechanistic understanding of invertebrate-microbe interactions in nitrogen transformation processes.

3.2. 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 & 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 zone (Vanderborght & 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 *et al.*, 2007). Ventilation activity also increases the area of the oxic/anoxic interface in sediment, which promotes nitrification/denitrification (Sayama & Kurihara, 1983; Rysgaard *et al.*, 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 *et al.*, 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 for sediment microorganisms, and may be partly responsible for previously observed increases in microbial activity and nitrogen cycling associated with burrows (Aller & Yingst, 1985; Aller & 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 & Aller, 2016) and to stimulate nitrification rates (Kristensen *et al.*, 1985). However, at present, the extents to which nitrogen cycling microbial communities or transformations are affected by invertebrate mucus secretions are unknown.

In this study, 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^-).

3.3. Method

3.3.1. Sediment and mucopolysaccharide collection

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. *H. 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.

3.3.2. Sediment slurry incubations

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 1, 677 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.

3.3.3. Chemical analyses

Carbohydrate concentration was measured using a phenol-sulfuric acid assay (Underwood *et al.*, 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 NH_4^+, NO_2^-, and NO_3^- using standard autoanalyser protocols (Brewer & Riley, 1965; Grasshoff, 1976; Mantoura & 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, Germany).

3.3.4. Microbial analyses

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, USA) and stored at -20˚C. Q-PCR was used to determine the abundance of 16S rRNA genes, *amoA* genes, and the bacterial *nirS* gene. Ten µL reactions contained 5 µL 2x SensiFast SYBR No-ROX 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, Australia), with duplicate technical replicates for each sample. Results were converted from ng μl^{-1} to copy number.mgwwsediment^{-1}. Data are reported according to MIQE guidelines (Table 3.1, Bustin *et al.*, 2009).

Bacterial 16S rRNA gene abundance was analysed using the primers BACT1369F and PROK1492R (amplicon length 123 bp; Suzuki *et al.*, 2000). The reaction was carried out with an initial denaturation step of 95 ˚C for 30 seconds, followed by 45 cycles of 95 ˚C for 10 seconds, 59 ˚C for 15 seconds, and 72 ˚C for 20 seconds. Archaeal 16S rRNA gene abundance was analysed using the primers Ar109f and Ar915r (amplicon length 825 bp; Großkopf *et al.*, 1998). The reaction was carried out with an initial denaturation step of 94 ˚C for 10 minutes, followed by 42 cycles of 94 ˚C for 30 seconds, 52 ˚C for 30 seconds, and 72 ˚C for 60 seconds.

Bacterial *amoA* gene abundance was analysed using the primers amoA-1F and amoA-2R (amplicon length 491 bp; Rotthauwe *et al.*, 1997) with an annealing temperature of 60 ˚C, as stated in Chapter 2.3.4. Archaeal *amoA* gene abundance was analysed

using the primers ArchamoA-1F and ArchamoA-2R (amplicon length 635 bp; Francis *et al.*, 2005) with an annealing temperature of 53 ˚C, as stated in Chapter 2.3.4. Bacterial *nirS* gene abundance was analysed using the primers nirS1F and nirS3R (amplicon length 256 bp; Braker *et al.*, 1998). The reaction was carried out with an initial denaturation step of 94 ˚C for 10 minutes, followed by 42 cycles of 94 ˚C for 30 seconds, 57 ˚C for 30 seconds, and 72 ˚C for 60 seconds.

Table 3.1 Standard curve reaction efficiencies.

Summary of efficiencies, slope values and technical replicate variation for each reaction (bacterial and archaeal 16S rRNA genes, bacterial and archaeal *amoA* genes, bacterial *nirS* genes).

Gene	Efficiency	R^2	Slope	Intercept	Ct variation
Bacterial 16S rRNA	0.64	0.996	-4.643	-2.339	0.51
Archaeal 16S rRNA	0.69	0.995	-4.375	8.729	0.13
Bacterial *amoA*	0.62	0.976	-4.753	-0.156	0.2
Archaeal *amoA*	0.68	0.99	-4.459	5.039	0.24
Bacterial *nirS*	0.79	0.995	-3.966	-0.200	0.98

16S rRNA gene sequencing was performed on the Illumina MiSeq platform using V6-V8 primer sets (Comeau *et al.*, 2017), and sequences were analysed as previously published (Taylor & Cunliffe, 2015; Taylor & Cunliffe, 2017) using a combination of USEARCH v7.0.1090 (32Bit) (Edgar, 2010) and QIIME v 1.8.0 (Caporaso *et al.*, 2010). This stringent method of quality filtering and OTU picking enabled confidence in classifications to lower level taxonomic identification although it should be noted that although high quality these are still short reads. Multiplexed .fastq files had forward and reverse primer sequences removed using USEARCH python command script fastq_strip_barcode_relabel2.py. Fastq files were then quality filtered, low quality (expected error <0.5) and short sequences (<250bp) removed, length truncated (250 bp) and converted to FASTA files. The FASTA files were first dereplicated, abundance sorted and singleton sequences were removed. OTUs were clustered using the UPARSE clustering algorithm (Edgar, 2013). Chimeras were then filtered using UCHIME (Edgar *et*

al., 2011) and the Gold database (Edgar, 2010) as a reference. OTUs were then mapped back to the original reads and an OTU table produced. Taxonomy was assigned to OTUs using the UCLUST method in QIIME v1.8.0 against the SILVA database 97% representative OTUs (release 128, Quast *et al.*, 2013). Chloroplast plastid sequences and either bacterial or archaeal sequences, depending on the dataset of interest, were removed from the dataset before further analysis. Sequence data have been deposited in the European Nucleotide Archive (accession code PRJEB22034).

3.3.5. Statistical analysis

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 (Figure II.2-Figure II.7). 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 3.2), 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 & Bates, 2000). There was no violation of homogeneity of variance for carbohydrate concentration, archaeal 16S rRNA gene abundance, archaeal diversity, or bacterial *nirS* gene abundance. Diagnostic residual plots indicated heteroscedasticity in the remaining models.

The optimal variance-covariate structure for these models was determined by comparing the initial random effects LME model to an equivalent model that incorporated specific variance structures using Akaike Information Criteria (AIC) and visualisation of model residuals obtained by restricted maximum likelihood (REML) estimation. For NO_3^- concentration and *Nitrospiraceae* relative abundance, residual spread varied with mucus treatment and time. For NO_2^- concentration, Marine Group I relative abundance, bacterial and archaeal *amoA* gene abundance, and the ratio of bacterial to archaeal *amoA* gene abundance residual spread varied with mucus treatment only, while for NH_4^+ concentration, bacterial 16S rRNA gene abundance, and bacterial diversity residual spread varied with time only (Table 3.2).

The optimal fixed structure was then determined by applying manual backward selection using the likelihood ratio test and maximum likelihood (ML) estimation to sequentially remove all non-significant terms. Final optimal models were validated by assessing normality (Q-Q plots), homogeneity of variance (residuals versus fitted values), and independence of observations (residuals versus each covariate) (Zuur & Ieno, 2016). 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).

Table 3.2 Summary of the linear mixed effects models.

Models treat mucus treatment and time as fixed explanatory variables. Significant results highlighted in bold. Initial linear model: Response variable ~ Mucus*Time. (Appendix II, Statistical Information).

Model	Response variable	Mucus			Time			Mucus x Time			Variance-Covariate
		L	d.f	p	L	d.f	p	L	d.f	p	
1	**Carbohydrate**	**13.1**	**1**	**<0.001**	**10.3**	**2**	**0.006**				**-**
2	**Bacterial abundance**							**11.1**	**2**	**0.004**	**Time**
3	**Archaeal abundance**							**8.1**	**2**	**0.018**	**-**
4	**Bacterial diversity**							**27.8**	**2**	**<0.001**	**Time**
5	**Archaeal diversity**							**19.6**	**2**	**<0.001**	**-**
6	**Ammonium (NH_4^+)**							**30.5**	**2**	**<0.001**	**Time**
7	**Nitrite (NO_2^-)**							**18.3**	**2**	**<0.001**	**Mucus**
8	**Nitrate (NO_3^-)**							**26.5**	**2**	**<0.001**	**Mucus x Time**
9	**Bacterial *amoA* abundance**							**10.9**	**2**	**0.004**	**Mucus**
10	**Archaeal *amoA* abundance**							**10.3**	**2**	**0.006**	**Mucus**
11	**AOB:AOA**	**26.9**	**1**	**<0.001**							**Mucus**
12	**Bacterial *nirS* abundance**							**10.3**	**2**	**0.007**	**-**
13	***Nitrospiraceae* abundance**							**7.6**	**2**	**0.02**	**Mucus**
14	**Marine Group I abundance**							**18.5**	**2**	**0.02**	**Mucus**

3.4. Results

3.4.1. Mucopolysaccharide degradation and microbial abundance

Elemental analysis showed that the *Hediste 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 (Figure 3.1 A; Table 3.2, 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 (Figure 3.1 B; Table 3.2, Model 2) and archaeal (Figure 3.1 C; Table 3.2, Model 3) abundances, determined by quantitative PCR (Q-PCR), 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.

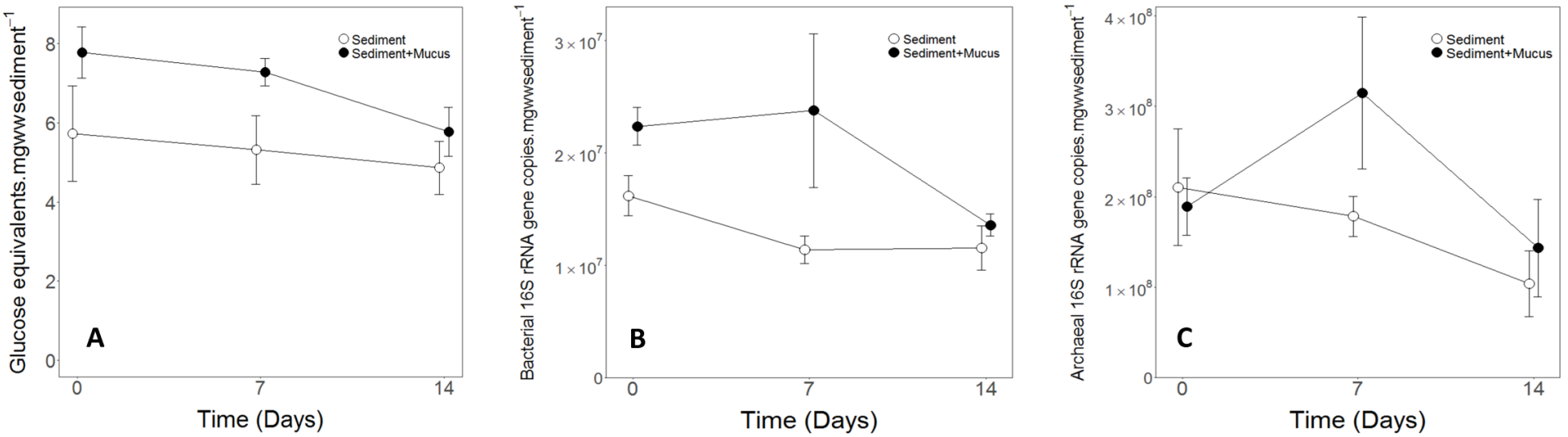

Figure 3.1 Variations in sediment carbohydrate concentration (A), and bacterial (B) and archaeal (C) 16S rRNA gene abundance in the presence of mucopolysaccharide.

Data shown are means ± SD (n = 3).

3.4.2. Microbial diversity

The diversity of bacterial (Figure 3.2 A; Table 3.2, Model 4) and archaeal (Figure 3.2 B; Table 3.2, 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 (Figure 3.2 C), 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 (Figure 3.3 A). PERMANOVA analysis of the archaeal community demonstrated no significant effect of mucus treatment or time (Figure 3.2 D). 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 (Figure 3.3 B).

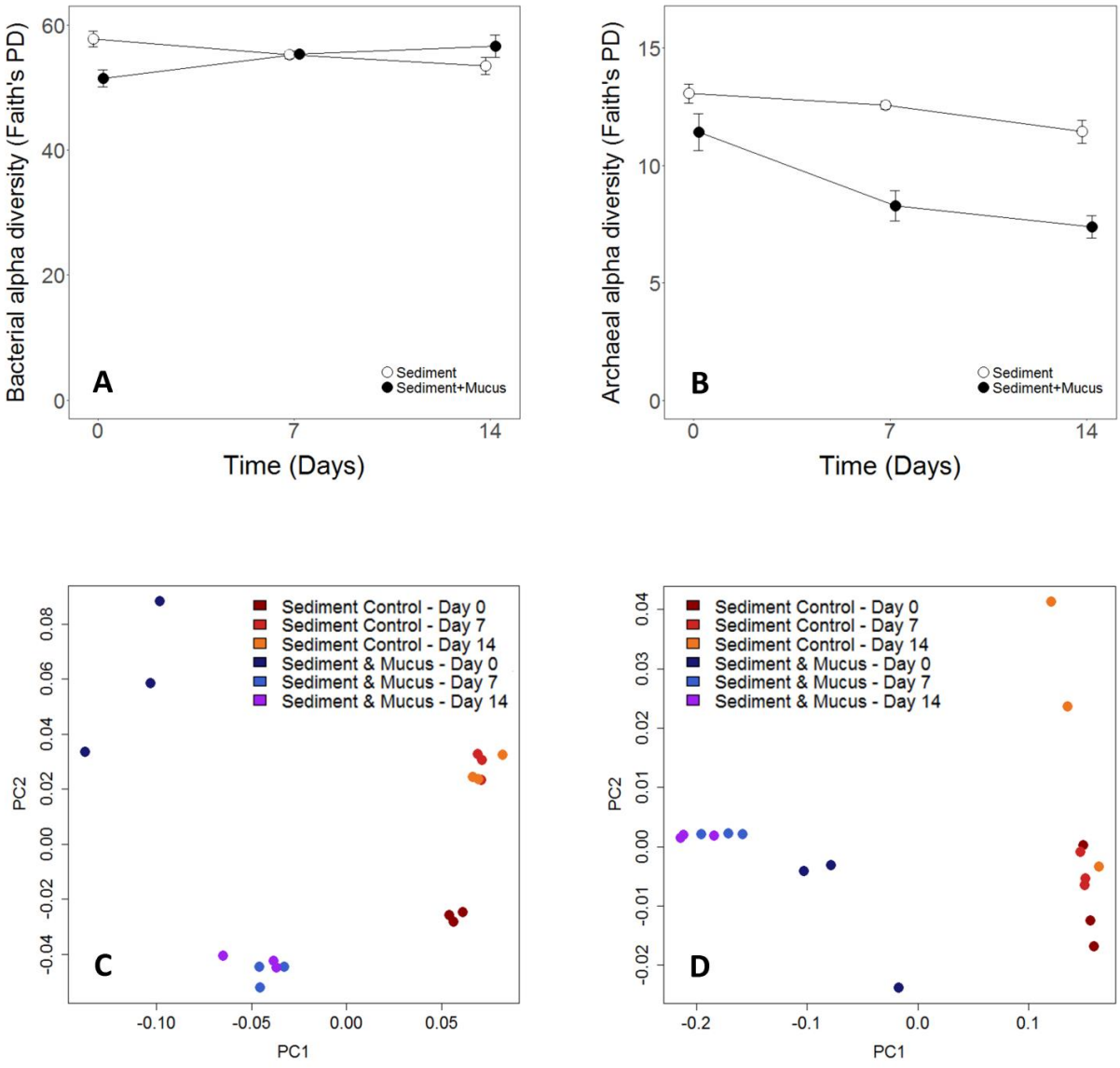

Figure 3.2 Variations in bacterial and archaeal community structure in the presence of mucopolysaccharide.

Mucopolysaccharide effects 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 (n = 3). Sediment-only incubations (Sediment Control) shown in red, sediment-mucus incubations (Sediment & Mucus) shown in blue.

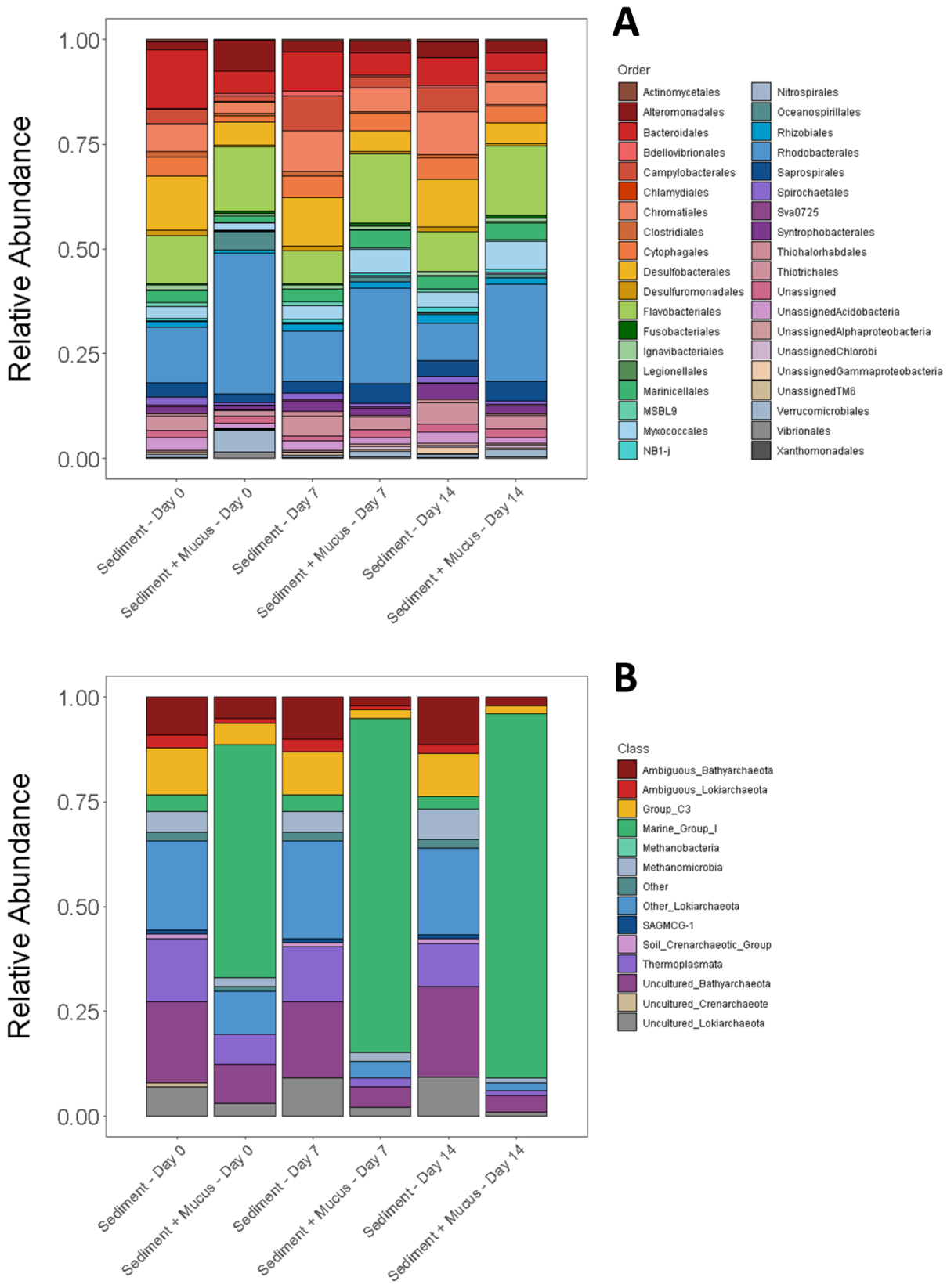

Figure 3.3 Mean relative abundance of bacterial families (A) and archaeal classes (B). Sediment-only and sediment-mucus treatment on day 0, 7, and 14 of the incubation period (n = 3) (> 1% relative abundance in at least one sample).

3.4.3. 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 3.2, Models 6-8). 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 (Figure 3.4 A). NO_2^- increased after seven days in the sediment-mucus treatment before returning to similar concentrations as the sediment-only treatment, where the concentration remained consistently low (Figure 3.4 B). NO_3^- was also consistently low in the sediment-only treatment but increased steadily throughout the incubation period in the sediment-mucus treatment (Figure 3.4 C). 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.

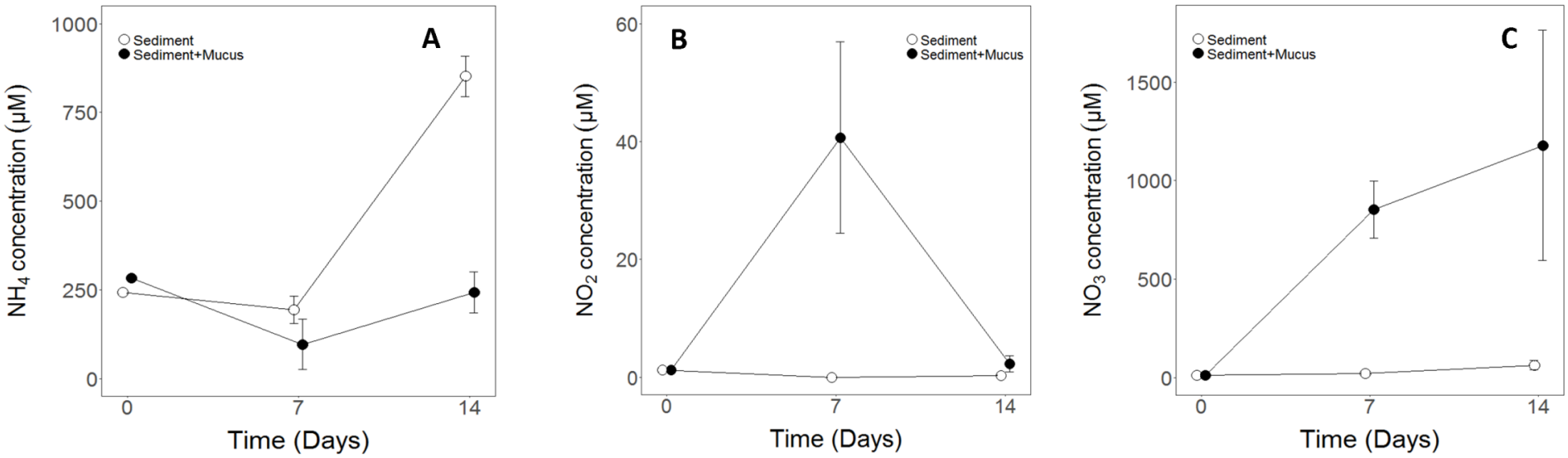

Figure 3.4 Variations in ammonium (A), nitrite (B), and nitrate (C) concentrations in the presence of mucopolysaccharide.

Data shown are means ± SD (n = 3, except sediment Day 0 and sediment + mucus Day 7 n = 2).

3.4.4. Abundance of microbial nitrogen cycling functional genes

AOB and AOA abundance, assessed by Q-PCR of *amoA* genes encoding ammonia monooxygenase, were affected by the interactive effect of mucus treatment and time (Figure 3.5 A-B; Table 3.2, 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 (Figure 3.5 C; Table 3.2, Model 11). The abundance of bacterial denitrifying groups, assessed by Q-PCR of *nirS* genes encoding nitrite reductase, was also dependent on the interactive effect of mucus treatment and time (Figure 3.5 D; Table 3.2, 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 ($p < 0.01$) (Figure 3.6).

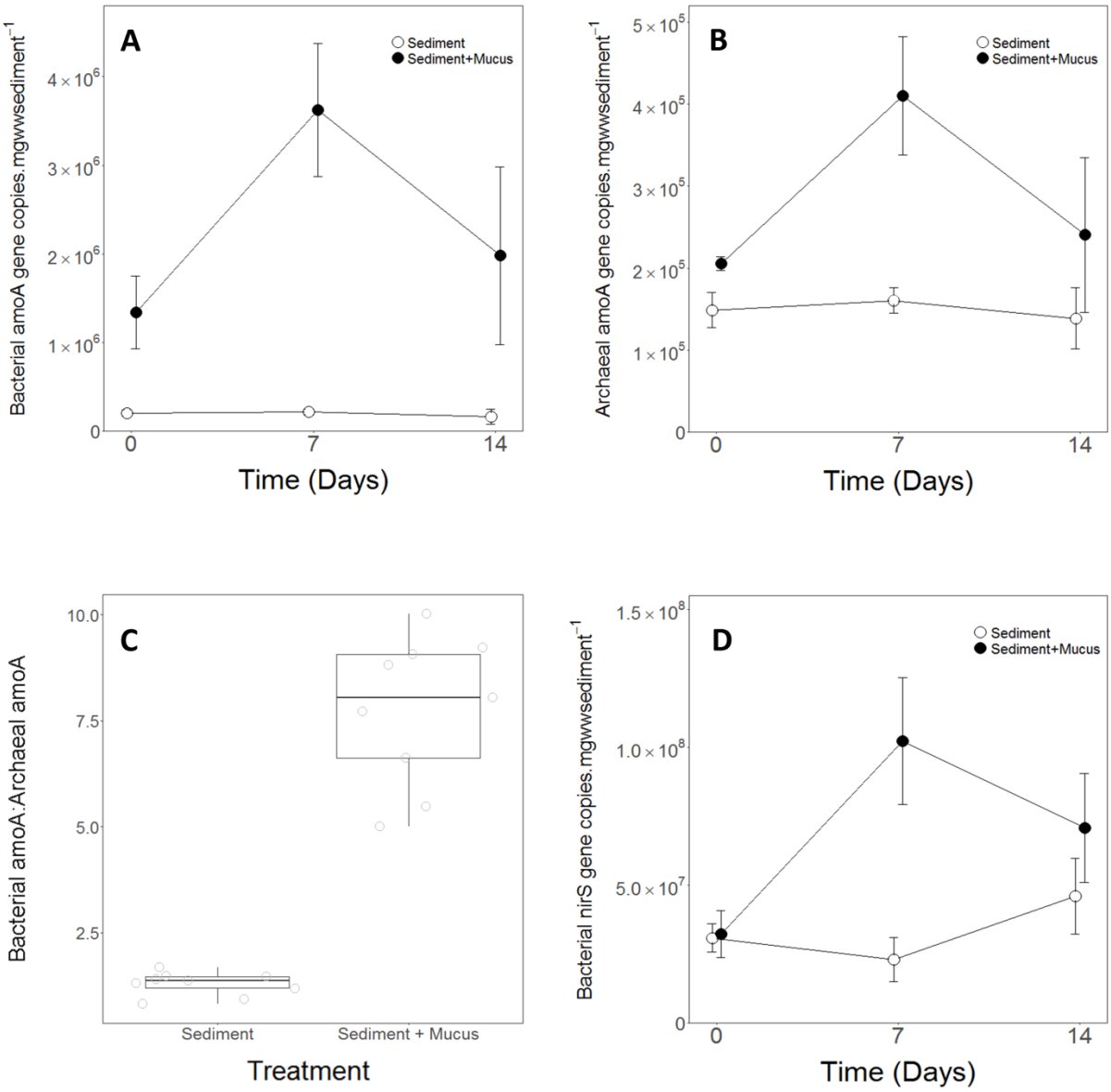

Figure 3.5 Variations in nitrogen cycling functional gene abundance in the presence of mucopolysaccharide.

Bacterial *amoA* (A), archaeal *amoA* (B), bacterial:archaeal *amoA* copy ratio (C), and bacterial *nirS* (D). Data shown are means ± SD (n = 3).

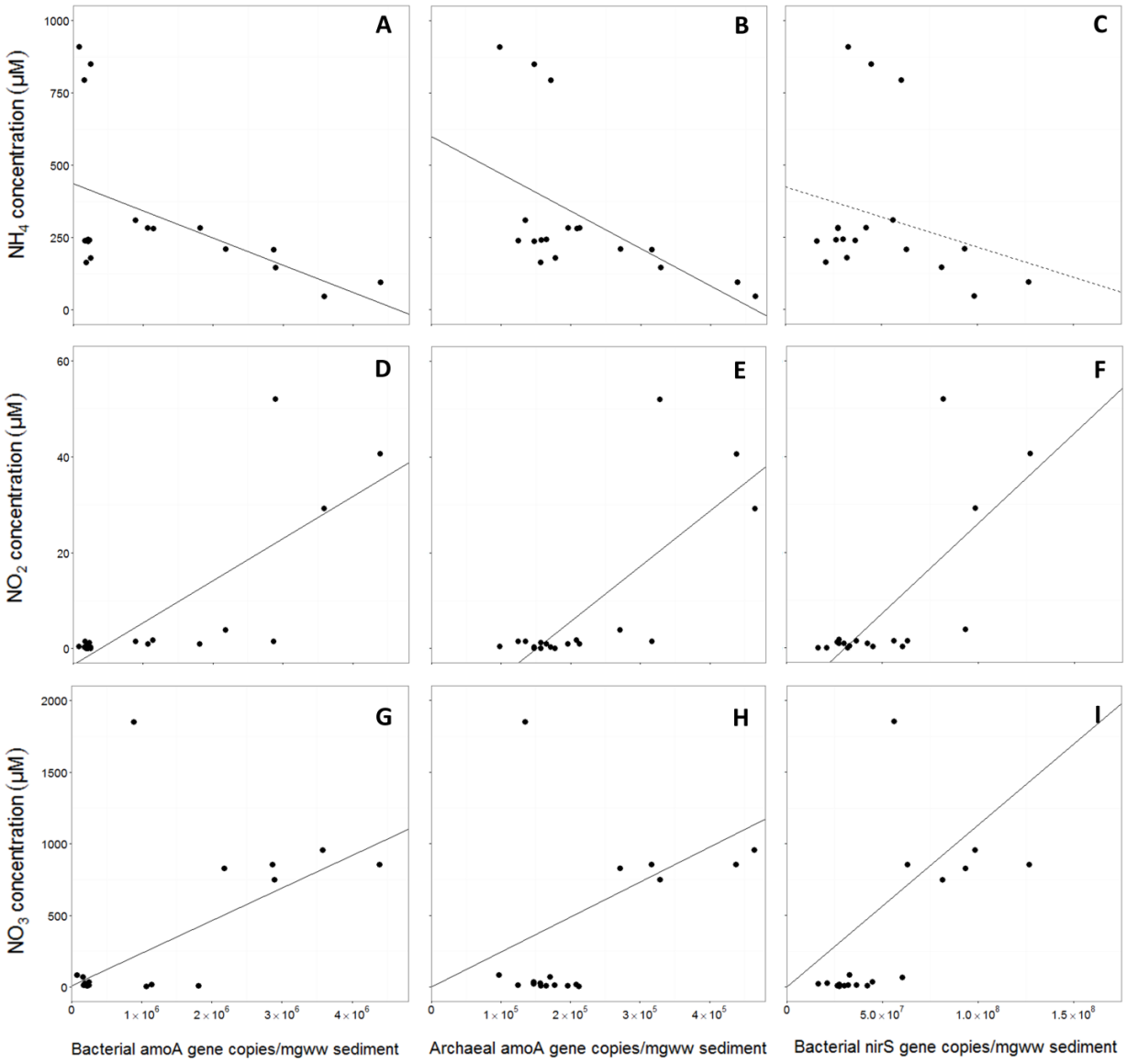

Figure 3.6 Relationship between nitrogen cycling functional gene abundances and nitrogen species concentrations (n = 18).

Correlation plots of NH_4^+ concentration against bacterial *amoA* gene copy number (R^2 = 0.257) (A), archaeal *amoA* gene copy number (R^2 = 0.284) (B), and bacterial *nirS* gene copy number (R^2 = 0.0635) (C). Correlation plots of NO_2^- concentration against bacterial *amoA* gene copy number (R^2 = 0.589) (D), archaeal *amoA* gene copy number (R^2 = 0.599) (E), and bacterial *nirS* gene copy number (R^2 = 0.546) (F). Correlation plots of NO_3^- concentration against bacterial *amoA* gene copy number (R^2 = 0.347) (G), archaeal *amoA* gene copy number (R^2 = 0.235) (H), and bacterial *nirS* gene copy number (R^2 = 0.435) (I). Dashed line represents insignificant correlations ($p > 0.05$). Missing nitrogen concentrations (n = 2) averaged from replicates.

3.4.5. 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 (Figure 3.7 A; Table 3.2, 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 (Figure 3.7 B; Table 3.2, 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).

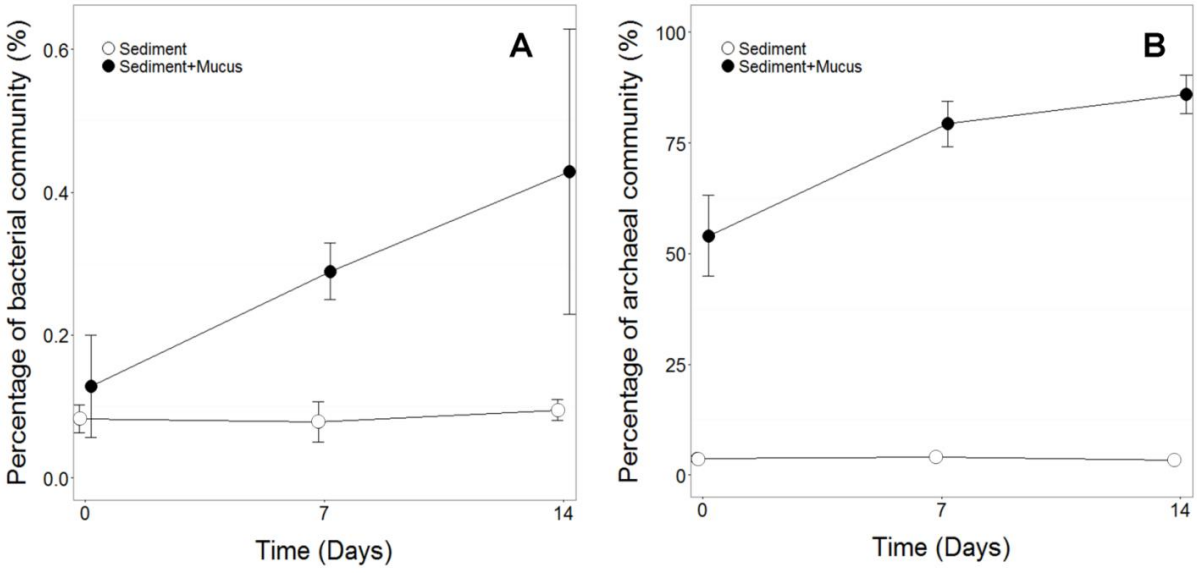

Figure 3.7 Mucopolysaccharide effects on nitrogen cycling microbial groups of interest.

Percentage of the bacterial community that belongs to the family of *Nitrospiraceae* (A), and percentage of the archaeal community that belongs to the class Marine Group I (B). Shown are the means ± standard deviations (n = 3).

3.4.6. '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, twelve bacterial families were shared exclusively by the two mucus-amended treatments (Figure 3.8 A), 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 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 (Figure 3.8 B). In the mucus-only treatment, the dominant class present was

Marine Group I (Figure 3.3 B). 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 (Figure 3.8 C - D).

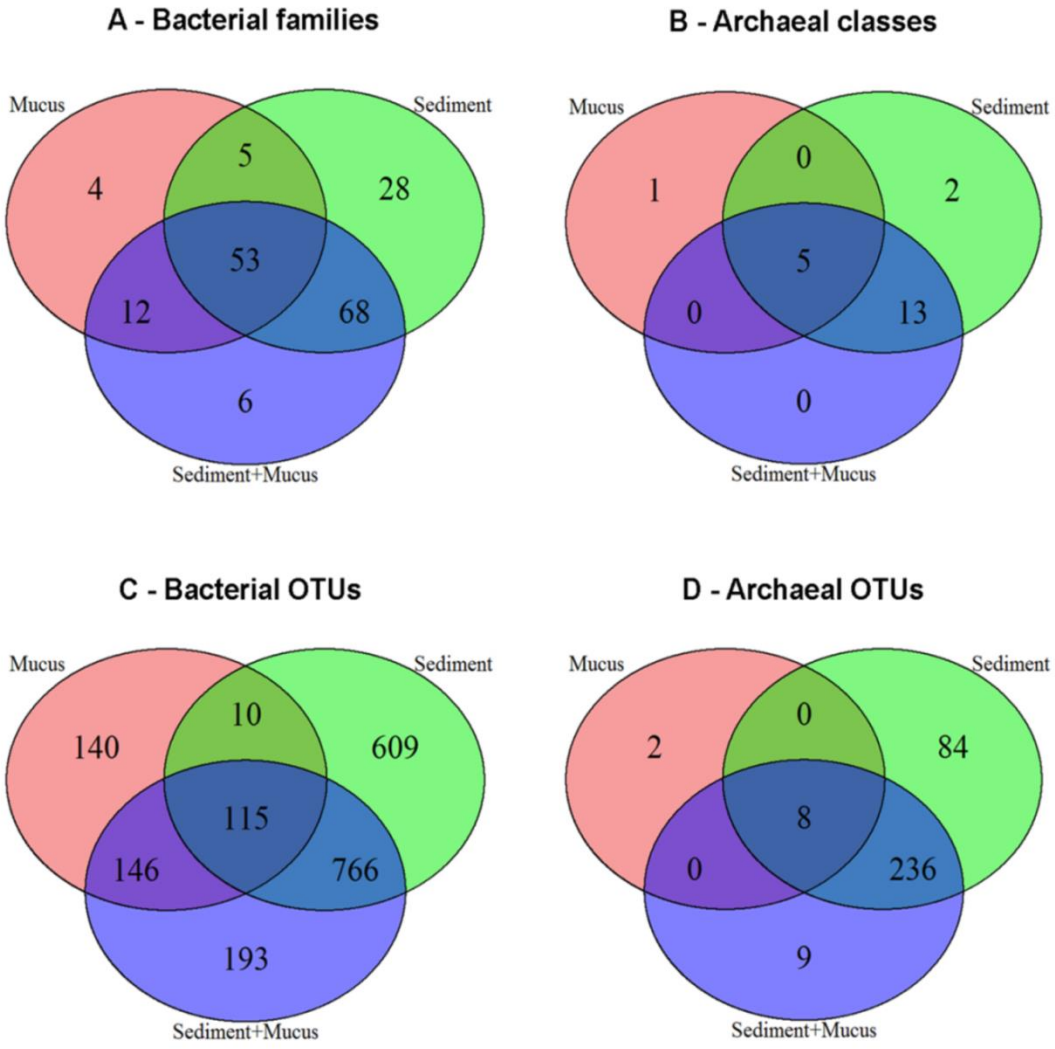

Figure 3.8 Variations in the abundance of bacterial families (A) and archaeal classes (B), and bacterial (C) and archaeal (D) OTUs.

Abundance of classes and OTUs identified in the sediment-mucus (blue) and sediment-only (green) incubations and in the seawater-mucus controls (red), 30 minutes after the start of the incubation.

3.5. 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 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 & 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 & 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 & 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 *et al.*, 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). Examination of ammonia-oxidiser

gene expression would allow better assessment of changes in nitrifying microbial activity in the presence of mucus. 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 has significantly stimulated and accelerated nitrification activity. As further support for this increase in AOB and AOA abundance, the 16S rRNA gene 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 itself simply stimulated the degradation rates of existing organic nitrogen in the sediment (Hannides & 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 and 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 and AOA. The concurrent declines in carbohydrate concentration, NO_2^- concentration, and AOB and AOA abundances suggest 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 (10^1 – 10^5 bacterial cells.ciliate^{-1} hour^{-1}) could explain the rapid decline in the abundance of nitrifying microbial groups (Starink *et al.*, 1994; Tuorto & 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 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 makes to the overall AO community, which can vary depending on environmental conditions (Duff *et al.*, 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 & 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 *et al.*, 2004; Dollhopf*, et al.*, 2005) may therefore be partially attributable to invertebrate mucopolysaccharide.

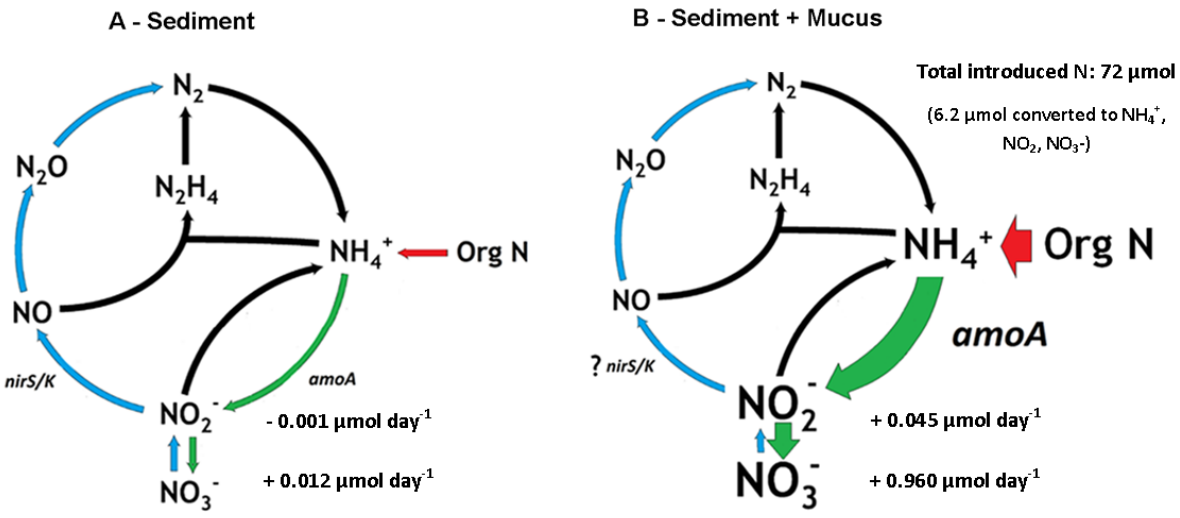

Figure 3.9 Summary of the proposed changes in nitrogen cycling processes and microbial groups.

Comparison of sediments where invertebrate derived mucopolysaccharide is absent (A) or present (B), including rates of production for NO_2^- and NO_3^- between day 0 and day 7 of the incubation (Adapted from Jetten, 2008).

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 (Figure 3.9). 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 0.05g C m^{-2} day^{-1} (Smith & 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. Additionally, it will be important to determine how these mucopolysaccharide effects are altered by unique burrow conditions, such as regular redox oscillations due to ventilation periodicity, either in the field or through further sediment slurry incubation experiments. This improved understanding will allow us to better develop nitrogen budget models and more accurately determine the effects of multiple stressors on sediment ecosystems.

4. Mucopolysaccharide secretion effects on nitrogen cycling communities under oscillating redox conditions

4.1. Abstract

Sediment-dwelling invertebrates secrete nitrogen-rich mucopolysaccharides that can support increased nitrification rates. These secretions generally form mucus linings within invertebrate burrows, and are therefore subject to rapid oscillations between oxic and anoxic conditions associated with ventilatory activity. This study used quantitative PCR/RT-PCR to assess how the abundance and activity of specific nitrogen cycling microbial functional groups are altered in sediment slurries incubated with and without mucus under continuously oxic, continuously anoxic, or oscillating oxic-anoxic conditions. Our results demonstrated that under oxic conditions, low concentrations of *Hediste diversicolor* mucopolysaccharide stimulate the activity of both ammonia-oxidising and nitrite-reducing microbial groups, which leads to enhanced coupled nitrification-denitrification within the burrow environment. Under oscillating oxic-anoxic conditions, mucus continues to stimulate nitrite-reducing bacterial groups but does not clearly stimulate ammonia-oxidiser activity. Instead the presence of mucopolysaccharide appears to support a consistent level of ammonia-oxidiser activity throughout the oscillation cycle. Our findings suggest that taxa-specific variations in mucus production and ventilation periodicity are likely to have complex and interacting effects that form an important and under-examined moderator of nitrogen cycling processes.

4.2. Introduction

In sediment ecosystems, microbially-mediated processes that play significant roles in global biogeochemical cycles can be influenced by the activities of sediment-dwelling invertebrates and by surrounding sediment redox conditions (Bertics & Ziebis, 2009; Gilbertson *et al.*, 2012; Magri *et al.*, 2017). One way in which invertebrate activity can mediate redox conditions is through burrow ventilation, which increases the supply of oxygenated water into anoxic sediment (Na *et al.*, 2008). As ventilatory activity is not continuous, with individuals ventilating between 20 and 88% of the time (Kristensen, 1984; Kristensen *et al.*, 1991; Delefosse *et al.*, 2015), invertebrate burrows are unique biogeochemical environments that regularly alter between oxic and anoxic conditions (Volkenborn *et al.*, 2010; Pischedda *et al.*, 2012; Volkenborn *et al.*, 2012). Microbial communities in burrow walls therefore experience significant redox oscillations that can affect rates of biogeochemical processes (Furukawa, 2001).

Organic matter degradation varies with changes in O_2 conditions (Aller, 1994; Timmermann *et al.*, 2008), with rates 10-fold greater under fully oxic sediment conditions than fully anoxic sediment conditions (Kristensen, 2000) while, under oscillating redox conditions, degradation rates increase as the frequency of oxic periods increases (Sun *et al.*, 2002; Caradec *et al.*, 2004). Short-term periods of anoxia therefore do not seem to negatively affect microbial groups involved in degradation (Chung & Gary, 1999). Redox oscillations can also affect sediment nitrogen cycling processes by supporting the coupling of aerobic and anaerobic pathways (Kristensen *et al.*, 1991; Howe *et al.*, 2004). The introduction of O_2 during ventilation allows burrow walls to support greater abundances of nitrifying microbial groups and increased rates of nitrification (Nielsen *et al.*, 2004; Satoh *et al.*, 2007; Laverock *et al.*, 2013; Foshtomi *et al.*, 2015). This produces nitrite (NO_2^-) and nitrate (NO_3^-) that can then fuel anaerobic removal pathways such as denitrification (Na *et al.*, 2008). Redox oscillations sustain this coupled nitrification-denitrification activity by allowing these aerobic and anaerobic processes to both occur sequentially and enhance the metabolic diversity of denitrifying microbial groups (Gilbert *et al.*, 2016; Foshtomi *et al.*, 2018). Species-specific invertebrate effects on biogeochemical processes therefore not only depend on the introduction of O_2 to sediment by ventilation, but also on the periodicity and pattern of oxic-anoxic oscillations (Volkenborn *et al.*, 2010; Pischedda *et al.*, 2012; Volkenborn *et al.*, 2012).

Sediment-dwelling invertebrate burrows are often lined with mucopolysaccharide secretions, which can be significant sources of organic carbon and nitrogen to sediment systems (Aller & Yingst, 1985; Nielsen *et al.*, 2004) and support the proliferation of

microbial communities (Aller & Aller, 1986; Herndl & Peduzzi, 1989; Papaspyrou *et al.*, 2006). Under anoxic conditions, these mucus secretions increase the production of ammonium (NH_4^+) by priming nitrogen remineralisation in sediment environments (Hannides & Aller, 2016). In oxic conditions, the presence of mucus can increase the abundance of ammonia-oxidising microbial groups, which causes a subsequent increase in nitrification rates (Dale *et al.*, 2018). Mucopolysaccharide burrow linings will be exposed to varying O_2 conditions, and yet the role that redox oscillations may play in mediating the effects of mucus secretions on nitrogen cycling processes has received little attention. By examining how nitrogen cycling microbial groups may be affected by potential interactions between mucopolysaccharide secretions and redox oscillations, we can better understand how nitrogen cycling is regulated in invertebrate burrow walls and therefore build ecological complexity for benthic system models (Graham *et al.*, 2016; Snelgrove *et al.*, 2018).

Here, we used sediment slurry incubations to assess the effects of *Hediste diversicolor* mucus on the abundance and activity of nitrogen cycling microbial groups (ammonia-oxidising bacteria, AOB; ammonia-oxidising archaea, AOA; nitrite-reducing bacteria), and porewater dissolved inorganic nitrogen compounds (ammonium; nitrite; nitrate), under oxic, anoxic, and oscillating conditions. Additionally, the concentrations of mucopolysaccharide added were significantly lower than previous incubation studies (Dale *et al.*, 2018) to examine how nitrogen cycling processes may be affected under low mucus conditions. We hypothesised that ammonia-oxidising microbial groups and nitrification activity would be stimulated under oxic conditions, but that oscillating conditions would support coupled nitrification-denitrification by stimulating both ammonia-oxidising and nitrite-reducing microbial groups.

4.3. Method

4.3.1. Sediment and mucopolysaccharide collection

Sediment (<3 cm depth) was collected in July 2018 from St Johns Lake, Cornwall (UK) (50˚21'51" N, 004˚14'08" 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* individuals were obtained from a commercial supplier (Sustainable Feeds; Newcastle upon Tyne, UK). To harvest mucopolysaccharide, 50 individuals were rinsed in UV-sterilised, filtered seawater and placed in a continuous flow seawater tank with rubber hose (internal diameter 10 mm) to act as burrow mimics in the absence of sediment. Mucus was harvested at regular intervals over a 12-day period from the outside and inner 3 cm of the hose using sterile forceps. Harvested mucus was washed in molecular grade H_2O and stored at -20 ˚C.

4.3.2. Sediment slurry incubations and sampling

Two sediment slurry treatments were set up. The sediment-only treatment was prepared by mixing 4.5 g (wet weight) sediment with 9.0 ml artificial seawater (0.2 µm filtered, 35 psu) in 120 ml serum bottles (n = 9). The sediment-mucus treatment was prepared by mixing 0.03 g (wet weight) homogenised mucus with 4.5 g sediment and 9 ml artificial seawater in 120 ml serum bottles (n = 9). Oxic, anoxic, and oscillating treatments were then applied to both sediment-only and sediment-mucus incubations. Continuously oxic incubations (Oxic, n = 6) were plugged with cotton wool and remained oxic for the duration of the incubation (Figure 4.1 A; headspace: 18.4 ± 0.32%; sediment: 218 ± 51.1 µmol/L). Continuously anoxic incubations (Anoxic, n = 6) were capped and flushed with N_2, and remained anoxic for the duration of the incubation (Figure 4.1 B; headspace: 0.43 ± 0.26%; sediment: 6.94 ± 6.52 µmol/L). Oscillating incubations (Oscillating, n = 6) were periodically switched between oxic (headspace: 18.7 ± 0.77%; sediment: 212 ± 61.7 µmol/L) and anoxic (headspace: 0.36 ± 0.18%; sediment: 3.97 ± 1.84 µmol/L) conditions every 3 days, beginning with a three day oxic period (Figure 4.1 C). The bottles were incubated in the dark at 15 ˚C for 9 days with continual shaking to maintain the sediment suspension. Bottles were regularly weighed to assess evaporation (none observed). Samples were taken at three points during the experimental period (T1 – T3): Day 3, Day 6, and Day 9. Two 0.5 ml samples were taken from each treatment and centrifuged at 1, 677 g for five minutes. The pellets were immediately stored at -80˚C for DNA/RNA extraction, while the 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 & Riley, 1965; Grasshoff, 1976; Mantoura & Woodward, 1983).

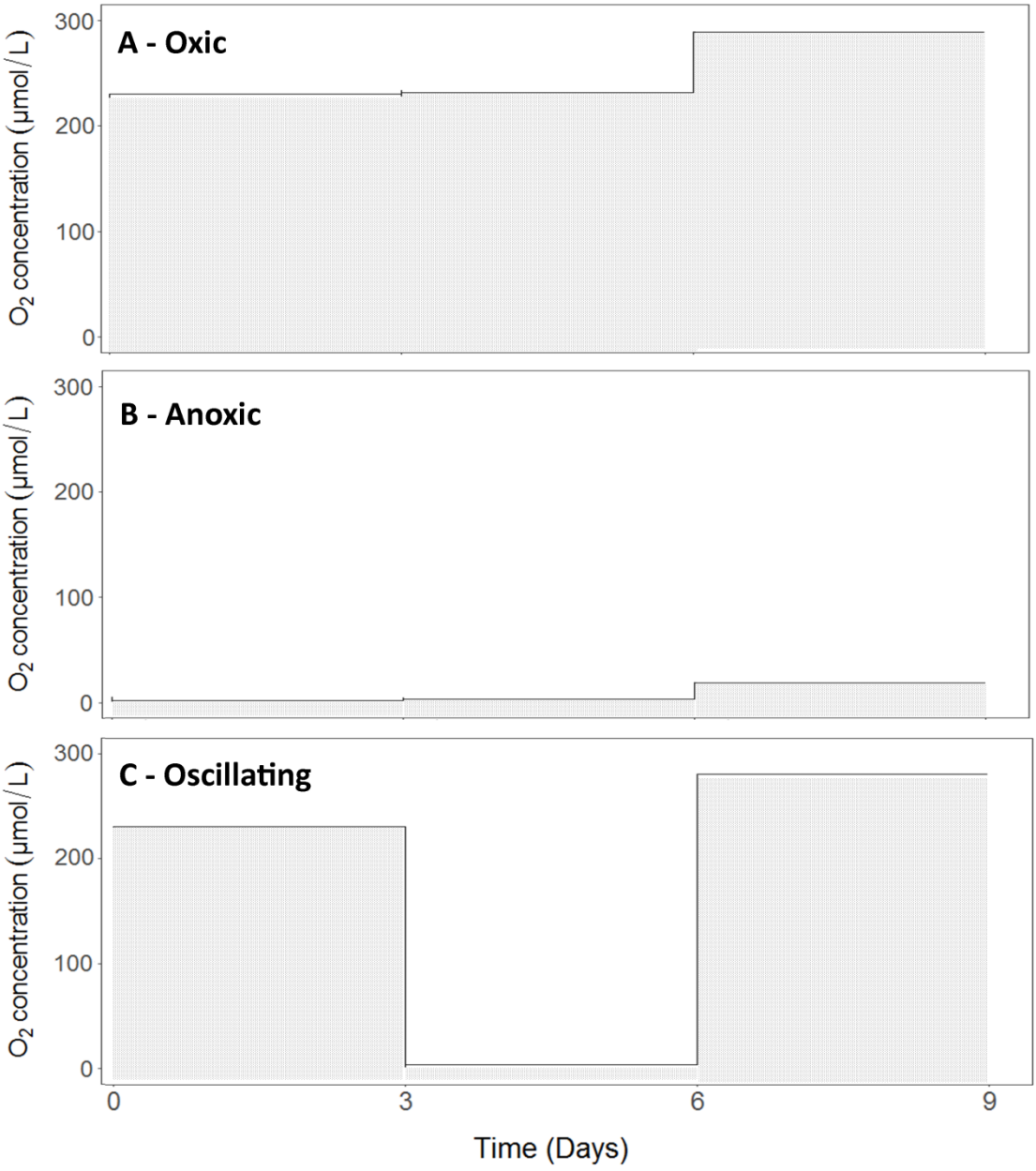

Figure 4.1 Sediment oxygen (O_2) concentrations in the oxic (A), anoxic (B) and oscillating (C) sediment slurry treatments.

Data shown are means (n = 6, except Day 0 n = 2).

4.3.3. Microbial analyses

DNA and RNA were extracted from 0.13 ± 0.017 g of sediment (wet weight) using the RNeasy PowerSoil Total RNA Kit and associated DNA Elution Kit (Qiagen, Germany). DNA and RNA yields were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80˚C. To assess the activity of the ammonia-oxidising community, bacterial and archaeal *amoA*, and bacterial *nirS* RNA transcripts were reversed transcribed to cDNA using the Omniscript Reverse Transcription Kit (Qiagen, Germany), with the Domain-specific primers amoA-2R, ArchamoA-2R, and nirS3R (Rotthauwe *et al.*, 1997; Braker *et al.*, 1998; Francis *et al.*, 2005).

The abundance and activity of ammonia-oxidisers was calculated using quantitative PCR (Q-PCR) to determine the abundance of bacterial and archaeal *amoA* DNA and cDNA respectively (Table 4.1). Bacterial *amoA* gene and transcript abundances were analysed using the primers amoA-1F and amoA-2R (amplicon length – 491bp; Rotthauwe *et al.*, 1997) with an annealing temperature of 60 ˚C. Archaeal *amoA* gene and transcript abundance was analysed using the primers ArchamoA-1F and ArchamoA-2R (amplicon length – 635 bp; Francis *et al.*, 2005) with an annealing temperature of 53 ˚C. Bacterial *nirS* gene and transcript abundance was analysed using the primers nirS1F and nirS3R (amplicon length – 256 bp; Braker *et al.*, 1998) with an annealing temperature of 57 ˚C. 10 µL reactions contained 5 µL 2x SensiFast SYBR No-ROX 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 Rotor-Gene 6000 (Corbett Life Science, Australia), with duplicate technical replicates for each sample. Reactions were carried out with an initial denaturation step of 94 ˚C for 10 minutes, followed by 42 cycles of 94 ˚C for 30 seconds, annealing temperature for 30 seconds, and 72 ˚C for 60 seconds. Samples for DNA analysis were randomised over two Q-PCR runs (Table 4.1). Normalised gene expression was calculated as the ratio of cDNA to DNA, and all results were converted from ng μl^{-1} to copy number.mgwwsediment^{-1}. Data are reported according to MIQE guidelines (Bustin *et al.*, 2009).

Table 4.1 Standard curve reaction efficiencies.

Summary of efficiencies, slope values and technical replicate variation for each Q-PCR reaction (bacterial *amoA*, archaeal *amoA*, bacterial *nirS* DNA and RNA).

Gene	Efficiency	R^2	Slope	Intercept	Ct variation
Bacterial *amoA* DNA	0.79	0.996	-3.95	-2.96	0.48
	0.75	0.998	-4.13	2.18	0.63
Archaeal *amoA* DNA	0.73	0.988	-4.19	-0.55	0.71
	0.67	0.987	-4.50	0.22	0.97
Bacterial *nirS* DNA	0.81	0.998	-3.87	2.95	0.32
	0.76	0.998	-4.06	2.89	1.13
Bacterial *amoA* RNA	0.69	0.995	-4.38	1.26	0.90
Archaeal *amoA* RNA	0.71	0.990	-4.31	-0.38	0.43
Bacterial *nirS* RNA	0.80	0.990	-3.93	2.10	1.69

4.3.4. Statistical analysis

Initial inspection of the gene abundance data showed that a subset of the samples (n = 22), which were distributed randomly across the treatment levels, had abundances two to three orders of magnitude lower than corresponding biological replicates across each of the assessed genes (bacterial *amoA*, archaeal *amoA* and bacterial *nirS*). As these data were also required to calculate normalised gene expression, these samples were excluded from any further genetic analysis. A lack of replication within the treatment levels precludes a formal statistical analysis, but data are presented so that any observable trends can be considered in the context of the nutrient data. Calculated DIN concentrations were unaffected.

For DIN concentrations, linear mixed effect (LME) models were developed for each dependent variable (Table 4.2), with mucus (2 levels: presence, absence), O_2 (3

levels: oxic, anoxic, oscillating) and time (3 levels: Day 3, 6, 19) treated as independent nominal variables. The use of LME models allowed bottle identity to be incorporated as a random effect (Zuur *et al*. 2009). Where there was evidence of heteroscedasticity, variance-covariate terms were applied to allow residual spread to vary with individual explanatory variables (Pinheiro & Bates, 2000). The optimal variance-covariate structure for these models (Table 4.2) was determined by comparing the initial random effects LME model to an equivalent model that incorporated specific variance structures, using Akaike Information Criteria (AIC) and visualisation of model residuals obtained by restricted maximum likelihood (REML) estimation. For NO_3^- concentration, residual spread varied with mucus treatment only, while for NH_4^+ and NO_2^- concentration, residual spread varied with O_2 treatment and time. The optimal fixed structure was then determined by applying manual backward selection using the likelihood ratio test and maximum likelihood (ML) estimation to sequentially remove the highest order non-significant terms. The minimal adequate models (Table 4.2) were validated by assessing normality (Q-Q plots) and by visual inspection of residuals versus fitted values (Zuur *et al.*, 2009; Zuur & Ieno, 2016). All analyses were carried out using the nlme package v. 3.1-120 (Pinheiro *et al.*, 2013) in R (version, 3.2.2, R Core Team, 2015).

4.4. Results

4.4.1. Dissolved inorganic nitrogen (DIN)

Ammonium (NH_4^+) concentration was dependent on the interactive effect of mucus and O_2 (Table 4.2, Model 1). Under continuously oxic conditions, NH_4^+ concentration was greater in the sediment-mucus incubations (7.21 ± 3.28 µM) relative to the sediment-only incubations (5.33 ± 2.81 µM) (Figure 4.2). Under continuously anoxic and oscillating O_2 conditions however, NH_4^+ concentration was greater in the sediment-only incubations (anoxic: 17.31 ± 21.55 µM; oscillating: 3.44 ± 2.22 µM) relative to the sediment-mucus incubations (anoxic: 9.68 ± 18.26 µM; oscillating: 1.12 ± 1.02 µM), but with greater variability under continuously anoxic conditions (Figure 4.2).

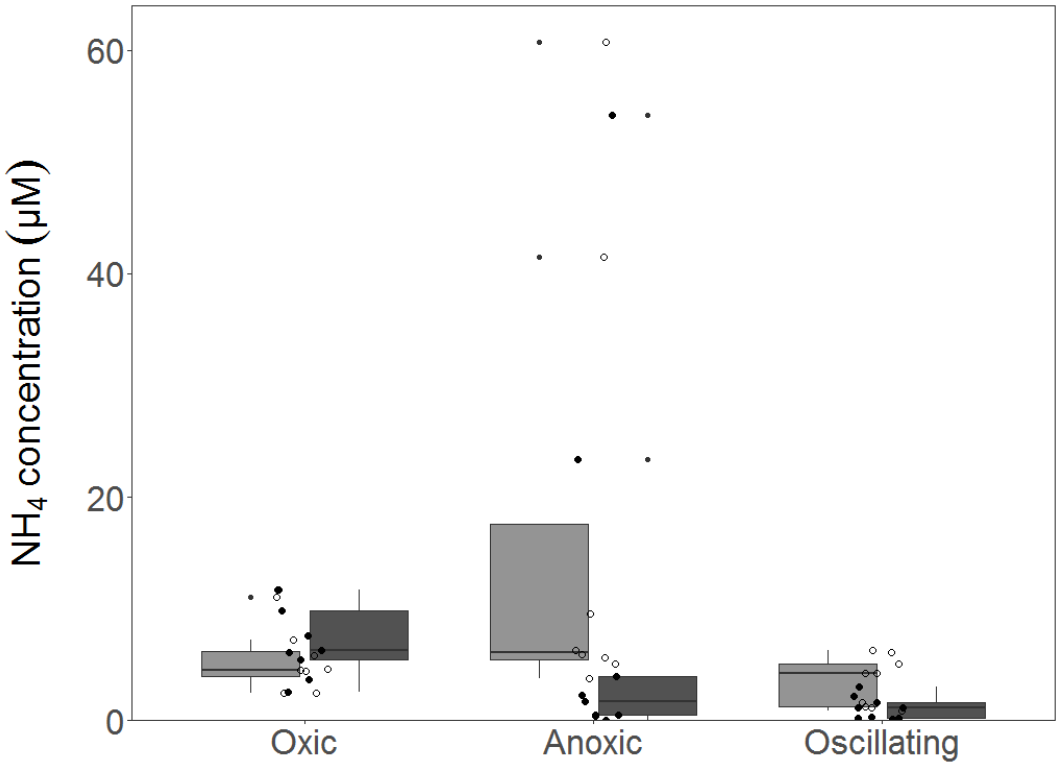

Figure 4.2 Variations in NH_4^+ concentration in the presence of mucus under varying redox conditions.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and O_2 conditions on NH_4^+ concentration (A; n = 9, except Sediment-only oxic and Sediment-only anoxic n = 8), Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

Table 4.2 Summary of the linear mixed effects models.

Models treat mucus treatment, O_2 conditions, and time as the fixed explanatory variables. Significant results highlighted in bold. Initial linear model: Response variable ~ Mucus*Time*O_2. (Appendix III, Statistical Information).

Model	Response variable	Mucus x O_2			O_2 x Time			Mucus x O_2 x Time			Variance-Covariate
		L	d.f.	p	L	d.f.	p	L	d.f.	p	
1	**NH_4^+**	**19.0**	**2**	**<0.01**							**O_2 x Time**
2	**NO_2^-**							**10.6**	**4**	**0.03**	**Mucus**
3	**NO_3^-**				**54.0**	**4**	**<0.01**				**O_2 x Time**

Nitrite (NO_2^-) concentration was dependent on the interacting effects of mucus, O_2, and time (Table 4.2, Model 2). Under continuously oxic conditions, NO_2^- concentration was greater in the sediment-mucus incubations and increased with incubation time (Figure 4.3 A) (day 3: 0.003 ± 0.002 µM; day 6: 0.053 ± 0.055 µM; day 9: 0.32 ± 0.20 µM). In the sediment-only incubations this increase was delayed, and only occurred in the final 3 days of the incubation period (day 3: 0.017 ± 0.021 µM; day 6: 0.002 ± 0.002 µM; day 9: 0.14 ± 0.073 µM). A similar trend was observed under oscillating conditions, but the concentration of NO_2^- was generally lower (sediment-only, day 9: 0.15 ± 0.05 µM; sediment-mucus, day 9: 0.17 ± 0.12 µM) and there was less variation between the mucus and non-mucus treatments (Figure 4.3 C). Conversely, under continuously anoxic conditions, NO_2^- concentrations were greater in the sediment-only incubations relative to the sediment-mucus incubations, and were generally larger and more variable than the concentrations observed under the other O_2 conditions (Figure 4.3 B). The exception to this is day 6 which follows a similar trend to the continuously oxic and oscillating treatments.

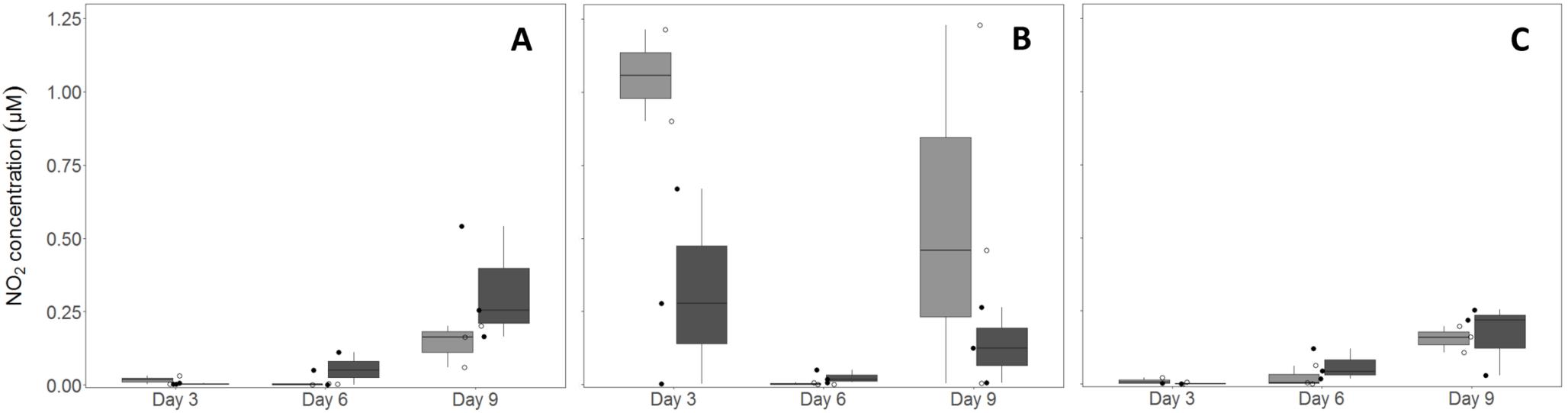

Figure 4.3 Variations in NO_2^- concentration in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on NO_2^- concentration under oxic (A; n = 3, except Sediment-only Day 3 n = 2), anoxic (B; n = 3 except Sediment-only Day 3 n = 2), and oscillating (C; n = 3 except Sediment-mucus Day 3 n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

Nitrate (NO_3^-) concentration was dependent on the interactive effect of O_2 and time (Table 4.2, Model 3). Over the first 6 days of the incubation, there was little observable difference between the O_2 treatments (Oxic: 3.39 ± 1.27 µM; Anoxic: 3.39 ± 2.65 µM; Oscillating: 4.08 ± 2.35 µM) (Figure 4.4). Between day 6 and day 9 however, though the anoxic concentration remained low (2.48 ± 0.53 µM), there were significant increases in NO_3^- concentration in the oscillating incubations (39.6 ± 19.9 µM) and, to a greater extent, in the continuously oxic incubations (74.6 ± 29.4 µM).

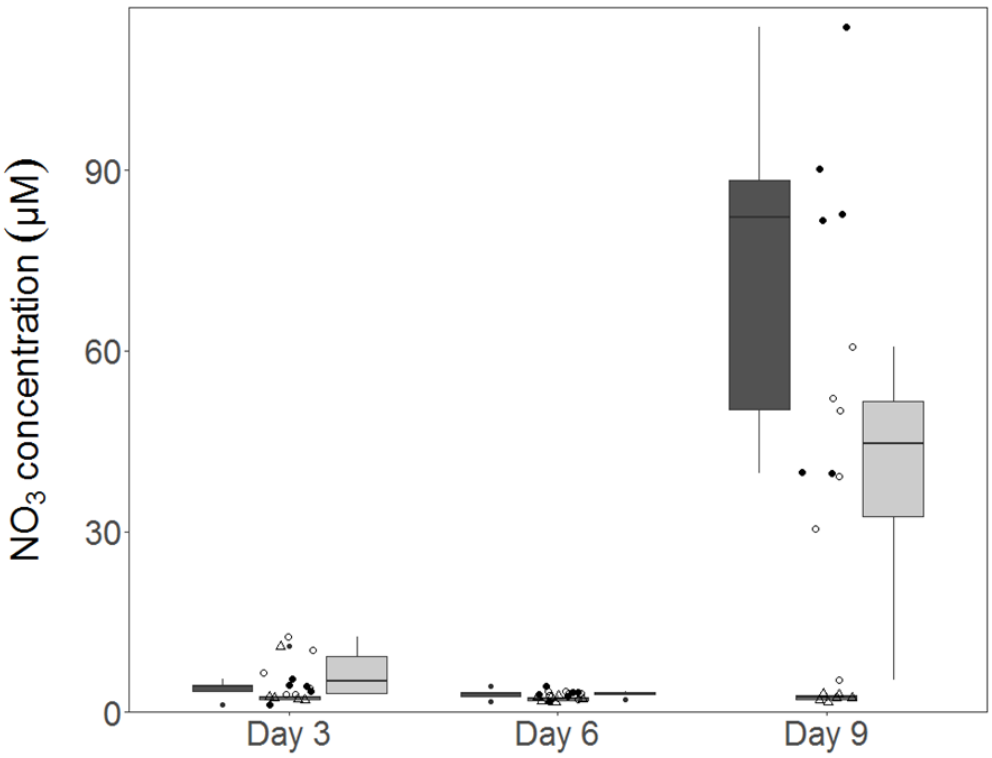

Figure 4.4 Variations in NO_3^- concentration under varying redox conditions over time.

The effect of O_2 conditions (Oxic:dark grey, closed circles; Anoxic: medium grey, open triangles; Oscillating: light grey, open circles) and time on NO_3^- concentration (n = 6, except Oxic Day 3 and Anoxic Day 3 n = 5). Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

4.4.2. Abundance of nitrogen cycling microbial groups

The abundance of ammonia-oxidising bacteria (AOB) and archaea (AOA), determined by Q-PCR, did not display any clear trends with mucopolysaccharide presence, O_2 conditions or time (Figure 4.5, Figure 4.6), with any potential trends seemingly driven by outliers. In general, however, under continuously oxic and continuously anoxic conditions, ammonia-oxidiser abundance appeared greater in the sediment-only incubations relative to the sediment-mucus incubations (Figure 4.5 A-B, Figure 4.6 A-B). Meanwhile, under oscillating oxic-anoxic conditions, there seemed to be a general increase in abundance with incubation time and the difference between the sediment-only and sediment-mucus incubations was less pronounced (Figure 4.5 C, Figure 4.6 C). The ratio of AOB to AOA abundance did not appear to be dependent on any of the assessed variables (Figure 4.7).

Bacterial *nirS* gene abundance also displayed no clear trends with mucopolysaccharide presence, O_2 conditions or time (Figure 4.8), particularly under continuously anoxic conditions (Figure 4.8 B). Under continuously oxic conditions, abundance appeared to be greater in the sediment-only incubations relative to the sediment-mucus incubations (Figure 4.8 A). Under oscillating oxic-anoxic conditions, however, bacterial *nirS* gene abundance in the sediment-only incubations seemed to be lower than that observed under continuously oxic conditions. As this decline was not observed in the sediment-mucus incubations (Figure 4.8 C), abundance appeared to be greater in the presence of mucus than in the absence of mucus. There also seems to have been an increase with incubation time under oscillating oxic-anoxic conditions in both the sediment-only and sediment-mucus treatments.

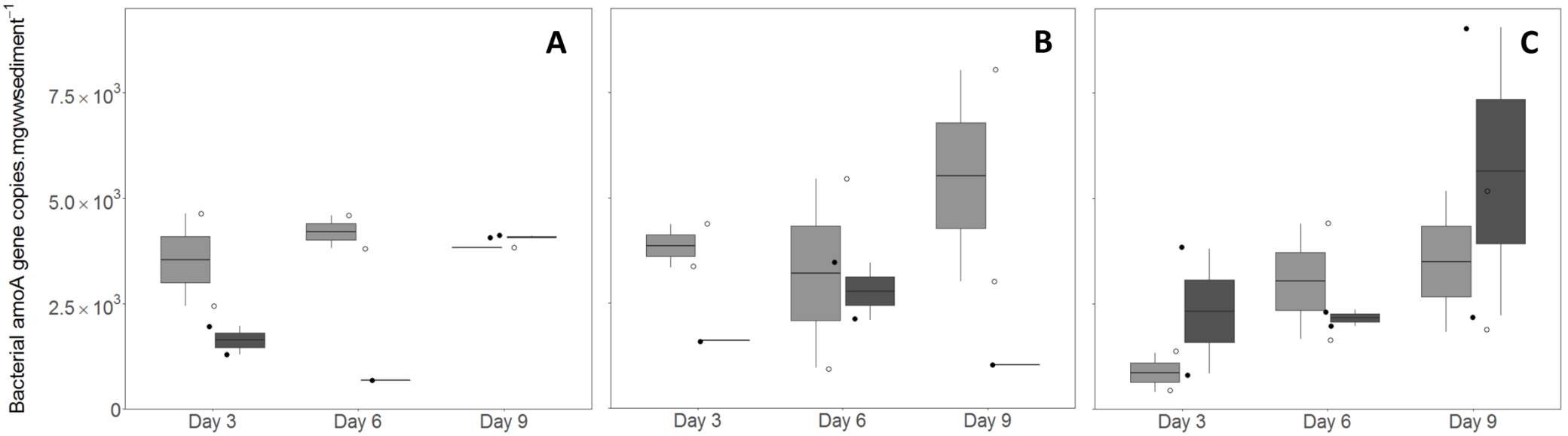

Figure 4.5 Variations in bacterial *amoA* gene copy number in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on bacterial *amoA* gene abundance under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

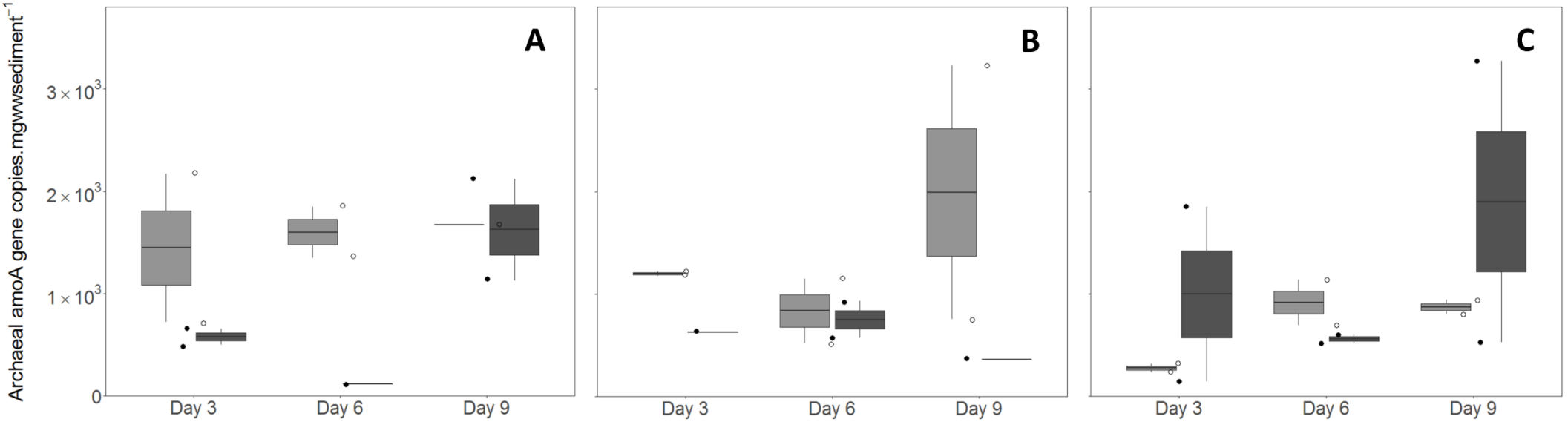

Figure 4.6 Variations in archaeal *amoA* gene copy number in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on archaeal *amoA* gene abundance under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

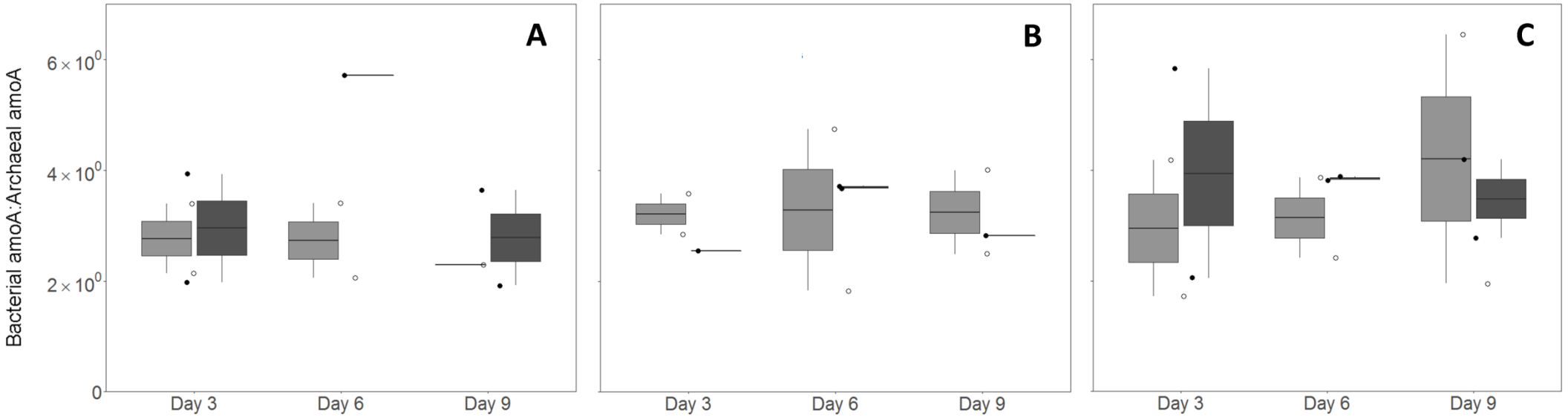

Figure 4.7 Variations in bacterial:archaeal *amoA* gene copy number in the presence of mucus under varying redox conditions over time. The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on bacterial:archaeal *amoA* gene abundance under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

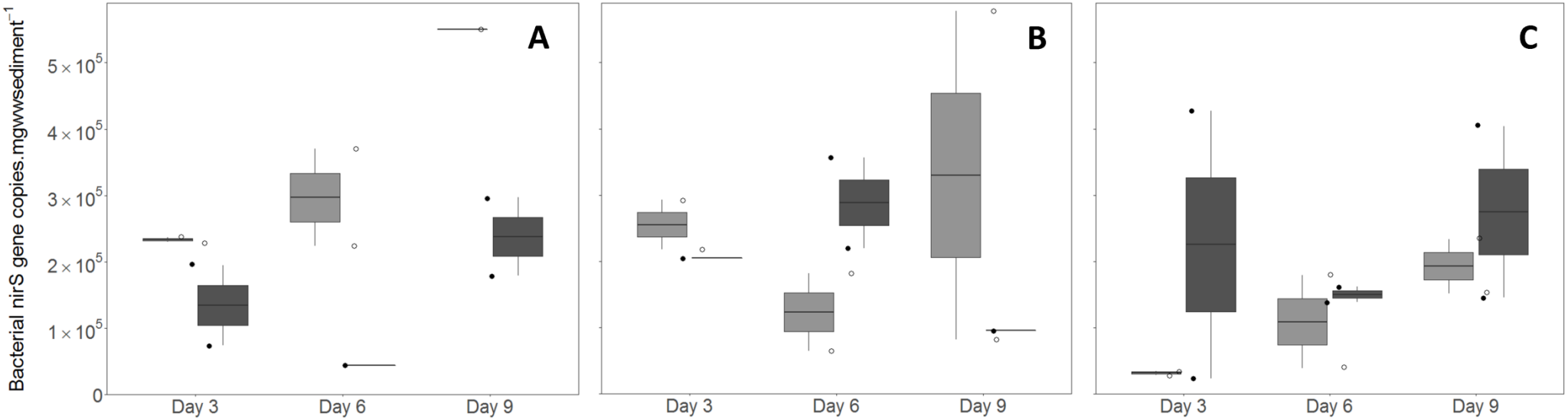

Figure 4.8 Variations in bacterial *nirS* gene copy number in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on bacterial *nirS* gene abundance under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

4.4.3. Activity of nitrogen cycling microbial groups

Normalised bacterial and archaeal *amoA* gene expression did seem to show some clearer trends with changing mucopolysaccharide and O_2 conditions (Figure 4.9, Figure 4.10), though limited replication may mean the data presented do not fully reflect activity trends. Under continuously oxic conditions, bacterial and archaeal *amoA* gene expression seemed generally greater in the sediment-mucus incubations relative to the sediment-only incubations (Figure 4.9 A, Figure 4.10 A). Bacterial *amoA* gene expression, however, declined throughout the experimental period in both treatments, while archaeal *amoA* gene expression only declined in the sediment-mucus incubations. Under continuously anoxic conditions, the trends were less clear. Bacterial and archaeal *amoA* gene expression does seem to have been initially greater in the sediment-only incubations relative to the sediment-mucus incubations, and to generally have declined over the incubation period (Figure 4.9 B, Figure 4.10 B). Conversely, in the sediment-mucus incubations, gene expression may have increased with time. Under oscillating O_2 conditions, there was also a decline in expression in both treatments over time (Figure 4.9 C, Figure 4.10 C), with gene expression generally unaffected by mucus treatment at day 3 (oxic). At day 6 (anoxic), gene expression had declined more in the sediment-only incubations relative to the sediment-mucus incubations and was therefore greater in the presence of mucus. At day 9 (oxic) however, gene expression in the sediment-only incubation remained relatively constant but had declined substantially in the sediment-mucus incubations, though the extent of the archaeal *amoA* expression decline varied greatly.

Normalised bacterial *nirS* gene expression seemed to be dependent on both mucopolysaccharide and O_2 conditions but did not vary with time (Figure 4.11). Under continuously oxic and oscillating conditions, gene expression was generally greater in the sediment-mucus incubations relative to the sediment-only incubations (Figure 4.11 A, Figure 4.11 C). Under anoxic conditions however, the presence or absence of mucopolysaccharide seemed to have little effect on bacterial *nirS* gene expression (Figure 4.11 B). Relative to the continuously oxic incubations, redox oscillations appeared to increase gene expression in the sediment-only incubations but not in the sediment-mucus incubations.

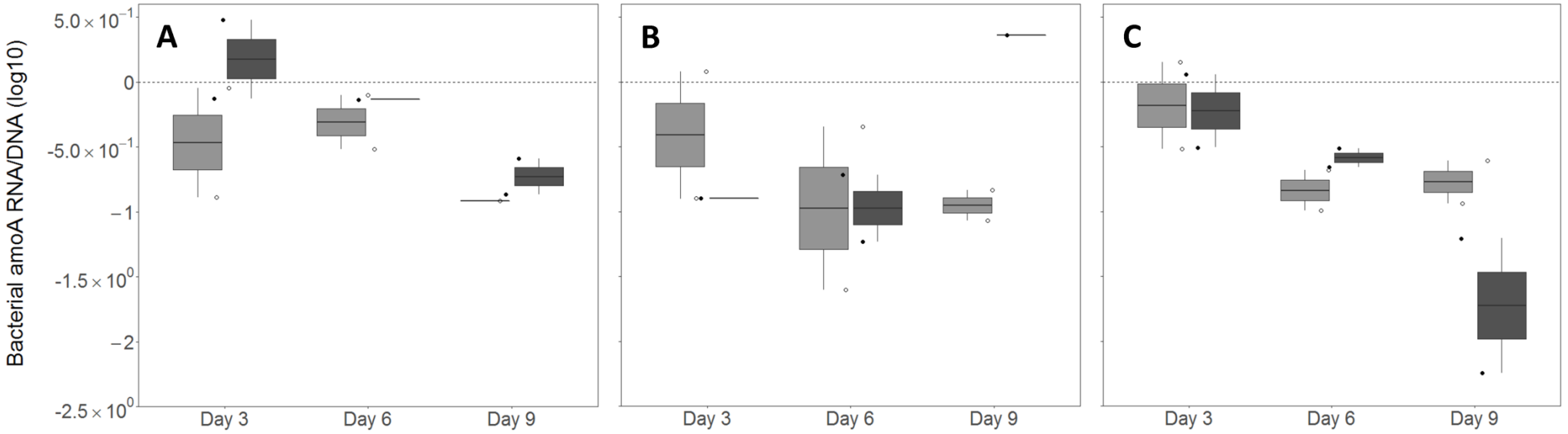

Figure 4.9 Variations in normalised bacterial *amoA* gene expression in the presence of mucus under varying redox conditions over time. The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on normalised bacterial *amoA* gene expression under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Data are log transformed (log_{10}) for ecological clarity. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

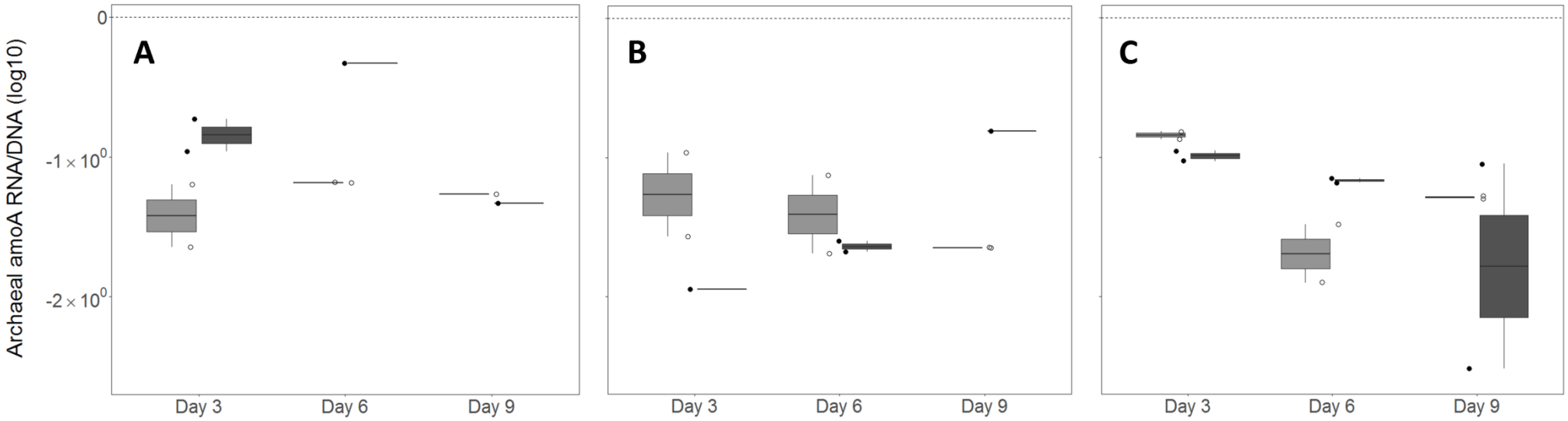

Figure 4.10 Variations in normalised archaeal *amoA* gene expression in the presence of mucus under varying redox conditions over time. The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on normalised archaeal *amoA* gene expression under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Data are log transformed (log_{10}) for ecological clarity. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

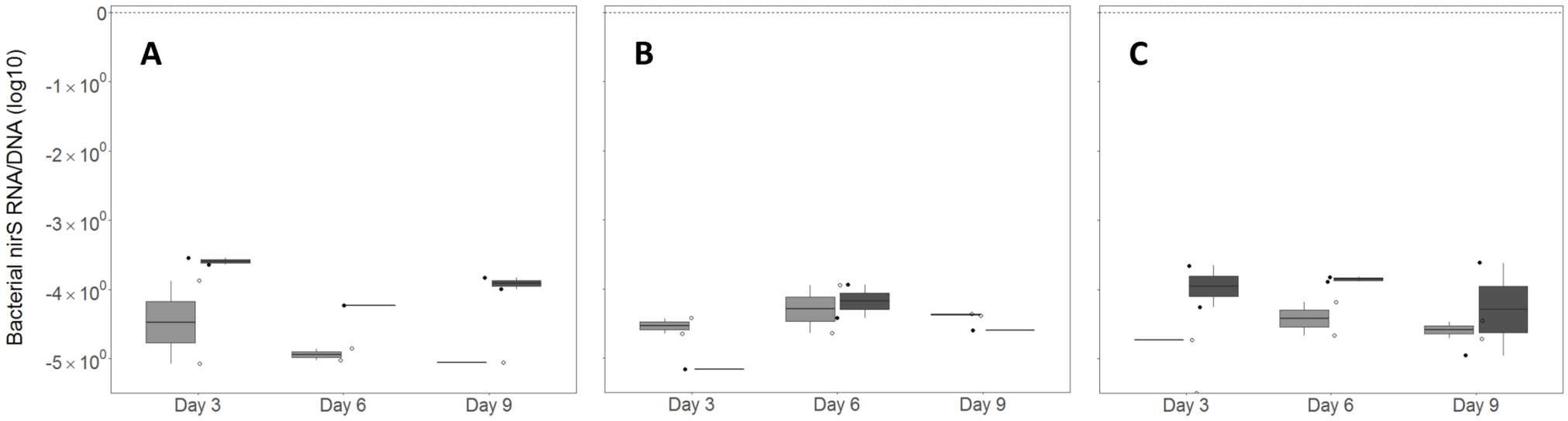

Figure 4.11 Variations in normalised bacterial *nirS* gene expression in the presence of mucus under varying redox conditions over time. The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on normalised bacterial *nirS* gene expression under oxic (A; n = 2, except Sediment-mucus Day 6 and Sediment-only Day 9 n = 1), anoxic (B; n = 2 except Sediment-mucus Day 3 and Day 9 n = 1), and oscillating (C; n = 2) O_2 conditions. Data are log transformed (log_{10}) for ecological clarity. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

4.5. Discussion

Invertebrate mucopolysaccharide secretions have long been suggested as a labile nitrogen source that can increase NH_4^+ production and stimulate nitrification (Aller & Yingst, 1985; Kristensen *et al.*, 1985; Bonaglia *et al.*, 2014). We have previously demonstrated that, under continuously oxic conditions, incubating sediment with *Hediste diversicolor* mucus (9:1 w/w) can stimulate ammonia-oxidiser proliferation and increase nitrification rates (Dale *et al.*, 2018). Yet here, under continuously oxic and continuously anoxic conditions, the abundance of ammonia-oxidising bacteria (AOB) and archaea (AOA) decreased in the presence of mucus. As proportionally less mucopolysaccharide (150:1 w/w) was added to these sediment incubations, low levels of NH_4^+ production could have allowed heterotrophs to outcompete ammonia-oxidisers for NH_4^+, which would cause ammonia-oxidiser abundance to decline despite the apparent increase in organic matter (Verhagen *et al.*, 1992). Ammonia-oxidiser proliferation could therefore be mediated by mucopolysaccharide concentration, and may be more prevalent in mucus-rich niches like burrow walls (Satoh *et al.*, 2007). Consequently, mucus production rates may need to be considered in invertebrate trait assessments rather than the simple presence or absence of mucopolysaccharide.

Oscillating oxic-anoxic conditions seemed to support ammonia-oxidiser proliferation as abundance generally increased over time and trends in the data suggest populations were uninhibited by mucopolysaccharide. Redox oscillations may therefore be partially responsible for observed increases in ammonia-oxidation and ammonia-oxidiser abundance in burrow walls (Satoh *et al.*, 2007; Laverock *et al.*, 2013), where oscillating oxic-anoxic conditions are common (Volkenborn *et al.*, 2010; Pischedda *et al.*, 2012). Comammox bacteria were not examined here, but these taxa have been identified in sediment systems (Pjevac *et al.*, 2017; Yu *et al.*, 2018) and, as comammox *Nitrospira* possess genes that may allow growth in low O_2 environments (Palomo *et al.*, 2018), these communities may respond differently to oscillating oxic-anoxic conditions than AOB and AOA.

Variations in nitrification processes are not always matched by variations in ammonia-oxidiser abundance (Gilbertson *et al.*, 2012), and can instead be related to gene expression (Bowen *et al.*, 2014). Here, under certain O_2 conditions, ammonia-oxidiser activity and NO_2^- concentrations indicate that *H. diversicolor* mucopolysaccharide can positively affect sediment nitrification processes, as observed in previous research (Dale *et al.*, 2018). Under continuously oxic conditions, greater concentrations of NH_4^+ were observed in the presence of mucus which may be due to efficient aerobic degradation of

the nitrogen-rich mucopolysaccharide (Aller, 1994; Kristensen, 2000). Increased NH_4^+ production subsequently stimulated ammonia-oxidiser activity which increased NO_2^- production rates. In the absence of mucus, ammonia-oxidiser activity and NO_2^- concentration also increased but at a lower rate. The overall increase in NO_3^- concentration suggests that oxic conditions promoted nitrite-oxidising bacteria (NOB), but mucopolysaccharide had no observable effect despite previous research showing a significant increase in NO_3^- concentration with mucus (Dale *et al.*, 2018). It is possible that ammonia-oxidation was not stimulated to a great enough extent by the limited mucopolysaccharide present to affect the NOB community. By the end of the incubation period, ammonia-oxidiser activity had declined suggesting that degradation of the finite mucus source meant there was no longer sufficient NH_4^+ production to support ammonia-oxidation (Bowen *et al.*, 2014; Foshtomi *et al.*, 2015). NH_4^+ concentrations were unaffected by time however, so this gene expression decline may be an artefact of the minimal replication or may represent a loss of activity at the very end of the incubation. Continuous production of invertebrate mucopolysaccharide in inhabited burrows would likely mitigate this issue.

Under oscillating oxic-anoxic conditions, organic matter degradation rates generally fall between those observed under oxic and anoxic conditions (Sun *et al.*, 2002; Caradec *et al.*, 2004). In this study, without mucopolysaccharide, NH_4^+ production was relatively similar under both oscillating and continuously oxic conditions. Yet, with mucopolysaccharide, NH_4^+ concentrations declined. As oscillating conditions can produce more lysable forms of NH_4^+ (Aller, 1994), it is possible that NH_4^+ production rates in the presence of mucopolysaccharide were masked by high rates of ammonia-oxidation or general heterotrophic microbial uptake for assimilation (Reyes *et al.*, 2017). Intermittent oxygen introduction should allow episodic nitrification to occur (Gilbert *et al.*, 2016). During the initial three days however, when O_2 conditions were identical to the continuously oxic treatments, mucopolysaccharide had no observable effect on ammonia-oxidiser activity. After three days under anoxic conditions, ammonia-oxidation activity declined but to a lesser extent in the presence of mucopolysaccharide. This is supported by the generally greater rates of NO_2^- production in the presence of mucus. Invertebrate mucopolysaccharide may therefore have a stabilising effect on ammonia-oxidiser activity, possibly by promoting NH_4^+ production under anoxic conditions (Hannides & Aller, 2016).

Aerobic activity was expected to recover after the reintroduction of O_2 (Aller, 1994) but, in the absence of mucus, activity remained low while, in the presence of mucus, there was a further decline. Activity was only assessed at the end of this period however, so there may have been an unobserved increase in ammonia-oxidation that declined as

available nitrogen was consumed. By facilitating remineralisation under anoxic conditions (Hannides & Aller, 2016), mucopolysaccharide may have caused available nitrogen to be consumed at a greater rate which could explain the potentially greater decline in activity. The increase in NO_2^- concentrations in the final oxic experimental period supports this recovery theory. Overall though, NO_2^- and NO_3^- production was lower under oscillating conditions than continuously oxic conditions, which could reflect some inhibition of these aerobic processes during the anoxic period.

Under continuously anoxic conditions, the rate and efficiency of organic matter degradation can be lower than when O_2 is present (Aller, 1994; Sun *et al.*, 2002; Caradec *et al.*, 2004), though other studies have demonstrated higher degradation rates or more effective degradation of complex organic matter under anoxic conditions (Kristensen & Blackburn, 1987; McKew *et al.*, 2013). Here, trends in the data suggest that ammonia-oxidiser activity was initially greater in the absence of mucopolysaccharide under anoxic conditions, and this was supported by greater concentrations of NH_4^+ and NO_2^- Over time however, this activity seemed to decline possibly because organic nitrogen was rapidly broken down to NH_4^+ and therefore rapidly utilised. Meanwhile, in the presence of mucopolysaccharide, the initial degradation of labile mucus components could have facilitated competition with heterotrophic organisms and therefore limited ammonia-oxidation (Verhagen *et al.*, 1992). As the incubation progressed however, anoxic conditions may have allowed the degradation of complex mucopolysaccharide components that could then have stimulated ammonia-oxidising activity (McKew *et al.*, 2013). The lack of a concurrent increase in NO_2^- concentration suggests that this increase in activity may only have occurred at the very end of the incubation, or may also represent an artefact of the lack of replication.

Counterintuitively, ammonia-oxidising microbes appear more active under anoxic conditions than oxic conditions as anoxic conditions produced greater concentrations of NO_2^-. Yet, ammonia-oxidisers have a higher affinity for O_2 than nitrite oxidisers, which means that, under low O_2 conditions, NO_2^- will accumulate as it is not being converted to NO_3^- (Bernet *et al.*, 2001). As NO_3^- was not produced, and NO_2^- concentrations were low throughout the incubations, ammonia-oxidation rates under anoxic conditions were likely low despite the apparent increase in ammonia-oxidiser gene expression. Overall, there seems to have been adequate O_2 present in these anoxic incubations to support low levels of aerobic ammonia-oxidising activity (Zhu *et al.*, 2013), but not enough to support full nitrification.

Denitrifying bacterial abundance has previously been shown to vary in relation to concentrations of bioavailable nitrogen (Foshtomi *et al.*, 2015; Smith *et al.*, 2015; Lee & Francis, 2017). In this study, however, mucopolysaccharide had a generally positive effect on NO_2^- production and yet denitrifying bacterial abundance was only greater with mucus under oscillating O_2 conditions. It is possible that, as with the ammonia-oxidising community, the additional organic nitrogen supplied by the mucopolysaccharide was insufficient to stimulate proliferation. Similarly, under oscillating O_2 conditions, mucopolysaccharide does not appear to have promoted the proliferation of denitrifying bacteria. Instead, the presence of mucopolysaccharide seems to have stabilised the abundance of denitrifying bacteria across the changing O_2 environments, while, in the absence of mucopolysaccharide, abundance declined between oxic and oscillating conditions. This decline is unexpected as previous work indicates that oxic-anoxic oscillations allow for continuous denitrification (Gilbert *et al.*, 2016). In that case however, oscillating conditions only had an impact after 10 days. Different results may therefore have been observed under a longer incubation period, as redox oscillations supported proliferation of denitrifying bacterial groups over time.

Mucopolysaccharide did seem to promote the activity of denitrifying bacterial groups under continuously oxic and oscillating O_2 conditions. As the increase in denitrification activity appears to relate to increases in NO_2^- production, mucopolysaccharide may facilitate coupled nitrification-denitrification activity and could be partially responsible for observed increases in this coupling in bioturbated sediment (Howe *et al.*, 2004; Dollhopf *et al.*, 2005). Interestingly, mucopolysaccharide promotion of *nirS* expression occurs before NO_2^- concentrations increase, which suggests mucopolysaccharide stimulated ammonia-oxidation at the beginning of the incubation but NO_2^- did not accumulate because of denitrification activity. Additionally, while NO_2^- concentration increased over time, the activity of denitrifiers remained relatively stable. This could be explained by the considerable increase in NO_3^- in the final three days of the incubation, as denitrifiers could have been outcompeted for NO_2^- by nitrite-oxidising bacteria. It could also represent competition with other denitrification pathways that use NO_2^-, such as dissimilatory nitrite-reduction to ammonium (DNRA) (Jetten, 2008). Generally though, *nirS* gene expression does not track denitrification rates as well as *amoA* gene expression tracks nitrification rates (Bowen *et al.*, 2014), and so denitrification activity may have increased throughout.

O_2 conditions did not seem to consistently mediate mucopolysaccharide effects on denitrifying activity but, under continuously anoxic conditions, activity was unaffected by mucus. As mucopolysaccharide appears to enhance denitrification through increased

nitrification, the lack of stimulation under anoxic conditions supports previous research that suggests a lack of nitrification activity can limit denitrification (Gilbert *et al.*, 2016). Interestingly, in the absence of mucopolysaccharide, trends in the data suggest that redox oscillations enhanced denitrification activity relative to continuously oxic conditions as expected (Gilbert *et al.*, 2016), but this increase in activity was not observed with mucopolysaccharide. This may indicate that O_2 conditions and mucopolysaccharide have a synergistic effect on denitrifying activity, with mucopolysaccharide again having a potentially stabilising role in regulating nitrogen cycling processes across changing environmental conditions.

Clearly, the lack of statistical justification for the microbial trends observed in this study makes it difficult to precisely assess how nitrogen cycling communities will be affected by both redox oscillations and mucopolysaccharide secretions. By combining these observed trends with robust DIN measurements, however, this study demonstrates that invertebrate mucopolysaccharide secretions can not only stimulate ammonia-oxidation activity but may also stimulate coupled nitrification-denitrification, and therefore contribute to nitrogen removal processes. Variations in the O_2 environment subsequently alter how mucopolysaccharide affects the activity of ammonia-oxidising functional groups and the production rates of bioavailable nitrogen. Meanwhile, the presence of mucopolysaccharide could provide a stabilising effect that supports a consistent level of microbial activity despite changing O_2 conditions. These results also indicate that a relatively small amount of mucopolysaccharide (0.03 g containing approximately 0.0001 g N; Dale *et al.*, 2018) may be enough to stimulate ammonia-oxidation and nitrite-reduction activity, though this was not enough to affect nitrite-oxidation or the proliferation of nitrogen cycling microbial groups. Future effort should therefore establish how both the abundance and activity of nitrogen cycling microbial groups alter with changing concentrations of mucopolysaccharide and varying periodicities of invertebrate ventilation. Further examination of how mucus concentration, mucus turnover rates, mucus composition, redox oscillation periodicity, and ventilation intensity can interact to regulate nitrogen cycling within inhabited invertebrate burrows will help develop the mechanistic understanding required to effectively model nitrogen turnover in sediment systems.

5. Exploring the effect of marine polychaete gut passage on sediment microbial assemblage structure

Chapter published in full : Dale H, Solan M, Lam P, Cunliffe M (2019) Sediment microbial assemblage structure is modified by marine polychaete gut passage. FEMS Microbiology Ecology ***95****: fiz047.*

5.1. Abstract

Invertebrate activities in sediments, such as the redistribution of particles and porewater, are known to regulate the structure and function of associated microbial assemblages. Yet, the effects of sediment ingestion, gut passage, and subsequent excretion by deposit-feeding invertebrates have received little attention. This study used high-throughput sequencing and quantitative PCR to examine how passage through the gut of the marine polychaete *Hediste diversicolor* affects the structure of bacterial and archaeal assemblages and the abundance of nitrogen cycling taxa. Our findings demonstrate that the digestive tract of *H. diversicolor* contains unique transitory microbial assemblages that, during gut passage, become more like the surrounding sediment assemblages. The hindgut of *H. diversicolor* also forms a reservoir for unique ammonia-oxidising archaeal taxa. Furthermore, distinct microbial assemblages on external polychaete surfaces suggest that deposit-feeding invertebrates may act as vectors that transport microbes between sediment patches. Collectively, these results suggest that the passage of sediment through the gut of deposit feeding invertebrates could play a significant role in regulating sediment microbial assemblages.

5.2. Introduction

Coastal sediment environments are globally important sites for organic matter decomposition and remineralisation (Middelburg *et al.*, 1997). The biological complexity associated with sediment ecosystems remains, however, insufficiently constrained in biogeochemical models to generate accurate projections (Snelgrove *et al.*, 2018). In particular, the representation of microbial processes underpinning biogeochemical transformations requires more thorough consideration because sediment-dwelling invertebrates directly and indirectly alter the structure and activity of microbial communities (Mermillod-Blondin *et al.*, 2005; Gilbertson *et al.*, 2012).

Invertebrate burrows are sites of steep chemical gradients, high levels of organic matter cycling, and increased oxygen penetration (Kristensen, 2000; Nielsen *et al.*, 2004; Jovanovic *et al.*, 2014), that vary in relation to the feeding, burrowing, and ventilatory activity of the invertebrate inhabitant. Consequently, burrow walls support distinct microbial communities that display both spatial and inter-specific variation (Bertics & Ziebis, 2009; Laverock *et al.*, 2010; Pischedda *et al.*, 2011; Taylor & Cunliffe, 2015). Whilst it is known that changes in the structure and diversity of microbial assemblages related to invertebrate activities (e.g. mucopolysaccharide production) can have substantive effects on biogeochemical cycles (Satoh *et al.*, 2007; Foshtomi *et al.*, 2015; Dale *et al.*, 2018; Foshtomi *et al.*, 2018), studies to date have largely focused on the effects of particle and fluid displacement by infauna and do not consider the roles of other significant animal-environment-microbial interactions.

As marine sediment ecosystems are dominated by deposit-feeding invertebrates, transit of sediment through the digestive tract of these communities is likely to be particularly important in determining the benthic contribution to biogeochemical cycling (Thorsen, 1998; Biron *et al.*, 2014; Troussellier *et al.*, 2017). In terrestrial systems, the specific organic matter, pH, and redox conditions of the earthworm (*Lumbricus rubellus*) gut means that ingestion of soil significantly alters the abundance of certain microbial taxa in the transitory substrate assemblage (Furlong *et al.*, 2002; Pass *et al.*, 2015). In marine deposit-feeders, abundance-based techniques have demonstrated a general loss of bacteria in the foregut, followed by regrowth towards the hindgut (Plante *et al.*, 1989; Hymel & Plante, 2000). Bacteriolytic activity and digestion are centred in the stomach and decline towards the gut posterior (Plante & Mayer, 1994; Mayer *et al.*, 1997), where bacterial growth can be stimulated in the absence of competitors and the presence of elevated levels of organic matter (Andresen & Kristensen, 2002). As the digestion and subsequent regrowth of bacterial assemblages can be species-specific and vary between

individuals within an invertebrate population (Plante *et al.*, 1989; Plante & Mayer, 1994; Mayer *et al.*, 1997), it follows that the nature of invertebrate-microbial gut interactions within a community will be of functional importance to ecosystem processes within the sediment profile. Clone library studies have also shown that gut passage can alter assemblage diversity and the ratio of aerobic to anaerobic taxa (Lau *et al.*, 2002; Li *et al.*, 2009), but the extent to which the surrounding sediment assemblages are influenced by these changes remains unclear (King, 2018).

Invertebrate guts may introduce functionally important taxa into the surrounding sediment or they may act as a vector, which transports a subset of microbes between sediment patches (Troussellier *et al.*, 2017). Earthworm guts have been shown to contain active nitrate-reducing populations (Furlong *et al.*, 2002; Wüst *et al.*, 2011) at abundances that are orders of magnitude higher than the surrounding substrate (Karsten & Drake, 1997). The presence of these ingested denitrifying taxa means that earthworm guts are sources of nitrous oxide (N_2O) (Horn *et al.*, 2006a; Horn *et al.*, 2006b). In marine sediments, gut emissions from deposit feeding invertebrates also form a significant contribution to N_2O fluxes because of incomplete denitrification occurring in the gut (Heisterkamp *et al.*, 2010). The significance of these emissions is constrained by a lack of mechanistic understanding of microbe-invertebrate interactions during gut passage, including consideration of whether gut conditions encourage the growth of microbial functional groups that contribute to sediment nitrogen cycling.

Here, we assessed the variation between bacterial and archaeal assemblage structure in the surrounding sediment environment, the external body surface, and the internal gut of the sediment-dwelling polychaete *Hediste diversicolor*, using both 16S rRNA gene amplicon sequencing and quantitative PCR (Q-PCR). Our aim was to determine whether the transitory microbial assemblages within the gut or on the external surfaces of the polychaete are distinct from the assemblages located in the local burrow wall or surrounding peripheral sediment, and to establish the extent to which these assemblages contribute to the wider sediment microbial community and potential nitrogen cycling. We hypothesised that there would be distinct transitory gut tract assemblages that would likely display high levels of individual variation, and that may be excreted into the surrounding sediment.

5.3. Method

5.3.1. Sample collection and processing

Samples were collected from three mud flat sites (~15 m apart) at St Johns Lake, Cornwall, UK (50˚21'51" N, 004˚14'08" W) in September 2017 (Figure 5.1 A). A previous survey at this location showed that the sediment in this area is predominantly silt (16 - 63 µm) with an organic carbon content of 6.9% (Ecospan, 2010). For each site, three surficial sediment samples (surface, upper 0.5 cm) were obtained using a sterile syringe and six burrows occupied by *Hediste diversicolor* were identified (Figure 5.1 B-C). Individual *H. diversicolor* were removed from each burrow, swabbed (Fisherbrand swabs, Fisher Scientific) and then anaesthetised in 40 ml of $MgCl_2$ in seawater (2.5% $MgCl_2$) to prevent gut evacuation (Rouse, 2004). Sediment from the burrow wall ('burrow', 3 cm depth from the sediment-water interface, 0.5 cm of burrow wall thickness sampled) and from the surrounding, non-bioturbated area ('deep', 3 cm depth from the sediment-water interface, 4 cm from the burrow) was obtained with a sterile syringe. All sediment samples and swabs were snap frozen using a liquid N_2 dry shipper, and stored at -80 °C. Individual *H. diversicolor* were washed in distilled water, measured (10 ± 4 cm, n = 18), and dissected on the day of collection. An incision was made after the foregut apparatus (foregut, ~31 ± 6.9% body length, n = 13) with a separate incision towards the end of the hindgut (hindgut, ~79 ± 5.6% body length, n = 16) to facilitate the removal of two 1 cm sections of gut contents using sterile tweezers. Dissection tools were washed with ethanol between each incision. All samples were stored at -80 °C.

5.3.2. DNA extraction and Q-PCR

DNA was extracted (sediment samples, 0.25 g wet weight; swab samples, whole swab; gut samples, available sediment content) from 11 burrow systems (Site 1, n = 4; Site 2, n = 3; Site 3, n = 4) using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA yield was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and the extracted DNA stored at -20 ˚C.

Figure 5.1 St John's Lake.
Sample site at St John's Lake, Cornwall (A), with a vertical cross-section of the sediment profile displaying visible *Hediste diversicolor* burrow systems with oxidised interfaces (B) and an individual worm within burrow (C). Photographs - Davis Laundon (MBA).

Q-PCR was used to assess the abundance of 16S rRNA genes, *amoA* genes, and the bacterial *nirS* gene in 4 of the burrow systems. Ten µL reactions contained 5 µL 2x SensiFast SYBR No-ROX 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, Australia), with duplicate technical replicates for each sample. Swab samples were excluded from Q-PCR analyses because of difficulties in obtaining accurate sample weights. Results were converted from ng μl^{-1} to copy number mgww.sediment^{-1}. Data are reported according to MIQE guidelines (Table 5.1, Bustin *et al.*, 2009).

Table 5.1 Standard curve reaction efficiencies.
Summary of efficiencies, slope values and technical replicate variation for each reaction (bacterial and archaeal 16S rRNA genes, bacterial and archaeal *amoA* genes, bacterial *nirS* genes).

Gene	Efficiency	R^2	Slope	Intercept	Ct variation
Bacterial 16S rRNA	0.53	0.992	-5.397	4.582	0.54
Archaeal 16S rRNA	1.08	0.976	-3.145	1.154	0.22
Bacterial *amoA*	0.70	0.999	-4.322	0.616	0.31
Archaeal *amoA*	0.71	0.997	-4.311	0.081	0.40
Bacterial *nirS*	0.74	0.997	-4.179	1.136	0.14

Bacterial 16S rRNA gene abundance was analysed using the primers BACT1369F and PROK1492R (amplicon length 123 bp; Suzuki *et al.*, 2000) with an annealing temperature of 59 ˚C, as stated in Chapter 3.3.4. Archaeal 16S rRNA gene abundance was analysed using the primers Ar109f and Ar915r (amplicon length 825 bp; Großkopf *et al.*, 1998) with an annealing temperature of 52 ˚C, as stated in Chapter 3.3.4. Bacterial *amoA* gene abundance was analysed using the primers amoA-1F and amoA-2R (amplicon length 491 bp; Rotthauwe *et al.*, 1997) with an annealing temperature of 60 ˚C, as stated in Chapter 2.3.4. Archaeal *amoA* gene abundance was analysed using the primers ArchamoA-1F and ArchamoA-2R (amplicon length 635 bp; Francis *et al.*, 2005) with an annealing temperature of 53 ˚C, as stated in Chapter 2.3.4. Bacterial *nirS* gene abundance was analysed using the primers nirS1F and nirS3R (amplicon length 256 bp; Braker *et al.*, 1998), as stated in Chapter 3.3.4. The reaction was carried out with an initial denaturation step of 94 ˚C for 10 minutes, followed by 42 cycles of 94 ˚C for 30 seconds, 57 ˚C for 30 seconds, and 72 ˚C for 60 seconds.

5.3.3. 16S rRNA gene sequencing and analysis

Bacterial and archaeal 16S rRNA gene PCR was used to establish sequencing viability for the surface (n = 9), burrow (n = 11), deep (n = 11), swab (n = 11), foregut (n = 11), and

hindgut (n = 11) samples. Fifty µL reactions contained 10 µL 5x GoTaq Flexi Buffer (Promega, USA), 4 µL DNTP's, 4 µl $MgCl_2$, 1 µL 10 pM forward and reverse primers, 0.25 µL goTaq G2 Flexi DNA Polymerase (Promega, USA) and 28.75 µL molecular grade H_2O, using the bacterial and archaeal primers stated in Chapter 5.3.2. Reactions were run in a Mastercycler nexus gradient PCR machine (Eppendorf, Germany). The bacterial 16S rRNA gene reaction was carried out with an initial denaturation step of 94 ˚C for 5 minutes, followed by 35 cycles of 94 ˚C for 30 seconds, 59 ˚C for 30 seconds, and 72 ˚C for 30 seconds, with a final elongation step of 72 ˚C for 7 minutes. The archaeal 16S rRNA gene reaction was carried out with an initial denaturation step of 94 ˚C for 5 minutes, followed by 35 cycles of 94 ˚C for 30 seconds, 52 ˚C for 30 seconds, and 72 ˚C for 60 seconds, with a final elongation step of 72 ˚C for 7 minutes. Based on these results, the swab samples from site 2 (n = 3) were excluded from both bacterial and archaeal sequencing analyses, with additional samples excluded from the archaeal sequencing analysis (Table 5.2). 16S rRNA gene sequencing was performed on the Illumina MiSeq platform using V6-V8 primer sets (Comeau *et al.*, 2017). Poor quality sequencing runs excluded five archaeal samples from further analysis (Table 5.2).

Sequences were analysed using the R package DADA2 (Callahan *et al.*, 2016). Based on the forward and reverse read error profiles, reads were truncated at position 100 to account for the poor read quality of some of the gut and swab samples. The resulting read length was not enough for acceptable overlap between the forward and reverse reads, therefore only the forward reads were used for the remainder of the analysis. Forward primer sequences were removed, and then reads were filtered to allow a maximum of 4 errors per read to obtain the best fit between the expected and estimated error rates. The sequence data were dereplicated to remove redundancy, sequence variance was inferred, and a sequence table produced. Taxonomy was assigned to operational taxonomic units (OTUs) using the SILVA database (Quast *et al.*, 2013) and loaded into the phyloseq package (McMurdie & Holmes, 2013). Chloroplast plastid sequences, other eukaryotic sequences, and either bacterial or archaeal sequences, depending on the dataset of interest, were removed before further analysis. Sequence data have been deposited in the European Nucleotide Archive (accession code PRJEB29031).

Table 5.2 Summary of samples excluded from bacterial and archaeal 16S rRNA gene sequencing analysis.

Excluded from Bacterial 16S sequencing run	Excluded from Archaeal 16S sequencing run	Excluded after Archaeal 16S sequencing
Site 2 - Swab 1	Site 1 - Foregut 1	Site 1 - Burrow 4
Site 2 - Swab 2	Site 1 - Foregut 2	Site 3 - Foregut 2
Site 2 - Swab 3	Site 1 - Foregut 3	Site 3 - Hindgut 1
	Site 1 - Foregut 4	Site 1 - Swab 1
	Site 2 - Foregut 1	Site 1 - Swab 4
	Site 2 - Foregut 2	
	Site 2 - Foregut 3	
	Site 3 - Foregut 3	
	Site 3 - Foregut 4	
	Site 1 - Hindgut 2	
	Site 2 - Hindgut 1	
	Site 2 - Hindgut 3	
	Site 3 - Hindgut 2	
	Site 3 - Hindgut 3	
	Site 3 - Hindgut 4	
	Site 2 - Swab 1	
	Site 2 - Swab 2	
	Site 2 - Swab 3	

5.3.4. Statistical analysis

The single archaeal foregut sample was excluded from the statistical analyses because it was not replicated. Diversity was calculated with the phyloseq package using the Chao1 diversity index and ANOVA to determine statistical significance. Variations in assemblage composition between environmental DNA sources (surface, burrow, deep, swab, foregut, hindgut) and sample sites (1, 2, 3) were calculated from weighted Bray-Curtis dissimilarity matrices rarefied to the minimum number of reads using PERMANOVA (999 permutation), and visualised using Nonmetric Multidimensional Scaling (nMDS) (vegan; v2.4-6;

Oksanen *et al.*, 2016) and Minimum Spanning Trees (phyloseqGraphTest). As sample site had no significant effect on assemblage structure, the three sites were pooled and site effects were disregarded. Pairwise PERMANOVA analyses (999 permutations) were also carried out between each sample type. Relative abundance plots were produced for the abundant bacterial orders and archaeal classes (>5% relative abundance). This high cut-off value was selected to minimise any potential bias introduced by the low read numbers obtained for some samples. Differential abundances of taxa (Log2-FoldChange) between pairs of sample types relevant to the aims of this study were calculated for the bacterial assemblages in the DESEQ2 package (Love *et al.*, 2014) using the Wald parametric test ($p < 0.05$). Changes in the abundance of nitrifying taxa were assessed using ANOVA once the key taxa were identified from the sequence table and sequence identity confirmed using online BLAST searches. Differences in bacterial and archaeal 16S rRNA genes, bacterial and archaeal *amoA* genes, the ratio of bacterial and archaeal *amoA* (AOB:AOA) genes, and bacterial *nirS* genes between sample types were calculated using ANOVA, and multiple comparisons to identify the source of any significant differences were performed using Tukey's post hoc tests. Bacterial and archaeal 16S rRNA, archaeal *amoA*, and AOB:AOA were log transformed before analysis. For ecological clarity, we present untransformed data in the figures. All statistical analyses were performed with R (version, 3.2.2, R Core Team, 2015).

5.4. Results

For the bacterial community, sequencing analysis generated a total of 1,847,603 reads which, after filtering, grouped into 4,917 unique OTUs. Read depth was slightly higher and less variable in the sediment samples (burrow, 33,726 ± 5,238; deep, 41,375 ± 6,020; surface, 34,189 ± 4,928) relative to the worm-associated samples (foregut, 9,602 ± 10,144; hindgut, 27,687 ± 25,380; swab, 37,952 ± 22,679) (Table IV.1). For the archaeal community, sequencing analysis generated a substantially lower total of 307,073 reads which, after filtering, grouped into 268 unique OTUs. This low read depth, along with a more uneven distribution of reads between samples (burrow, 8,924 ± 5,187; deep, 13,269 ± 4,303; surface, 7,246 ± 3,647; hindgut ,105 ± 129; swab, 1,035 ± 397) (Table IV.2), means that the archaeal community cannot be assessed with the same degree of certainty but is included here to support the trends observed within the bacterial community.

5.4.1. Microbial abundance and alpha diversity

Bacterial abundance, determined by 16S rRNA gene Q-PCR, was dependent on the sample type (Log_{10}, $F_{4, 14} = 3.7$, $p = 0.0295$), with a greater abundance of bacteria present in the hindgut (1,192,363 ± 293,596 copies mgww.sediment^{-1}) than the foregut (87,145 ± 61,045 copies mgww.sediment^{-1}) (Log_{10}, Tukey's, $p = 0.039$, Table 5.3) (Figure 5.2 A). Archaeal abundance also differed between sample types (Log_{10}, $F_{4, 14} = 59.82$, $p < 0.001$), with the greatest mean abundance in the burrow and deep sediment assemblages (burrow, 55,671 ± 29,315 copies mgww.sediment^{-1}; deep, 116,472 ± 51,386 copies mgww.sediment^{-1}; Log_{10}, Tukey's, $p < 0.001$, Table 5.4), and the lowest mean abundance in the foregut assemblage (203 ± 94 copies mgww.sediment^{-1}; Log_{10}, Tukey's, $p < 0.05$, Table 5.4) (Figure 5.2 B, Figure IV.3). Overall, archaeal abundance was lower than bacterial abundance, but followed a generally similar trend despite the low abundance in the hindgut.

Bacterial diversity, determined using the Chao1 index, was also dependent on sample type ($F_{5, 55} = 17.79$, $p < 0.001$), with greater diversity in the sediment and swab assemblages (surface, 590 ± 55; burrow, 620 ± 50; deep, 674 ± 72; swab, 657 ± 372) than the gut assemblages (foregut, 144 ± 97; hindgut, 286± 206) (Figure 5.3 A). Archaeal diversity was generally lower, but was still dependent on sample type ($F_{5, 35} = 15.59$, $p < 0.001$) and showed greater diversity in the sediment assemblages (burrow, 65 ± 24; deep, 80 ± 15; surface, 68 ± 24) than in the worm-associated (swab, 26 ± 24; hindgut, 6 ± 3) assemblages (Figure 5.3 B).

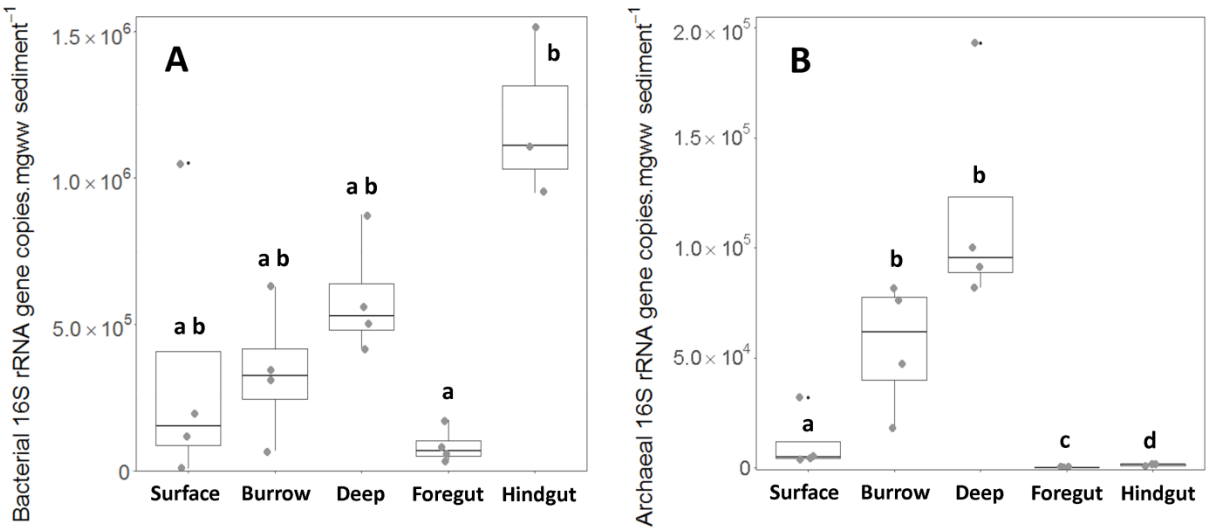

Figure 5.2 Variations in bacterial (A) and archaeal (B) 16S rRNA gene abundance. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (n = 4). Untransformed data presented. Letters (a, b, c, d) indicate significant difference between samples ($p < 0.05$; Table 5.3, Table 5.4).

Table 5.3 Tukey's pairwise comparisons of bacterial 16S rRNA gene abundance (log_{10}).
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	ns	ns	ns	ns
Burrow	0.823	-	ns	ns	ns
Deep	0.296	0.859	-	ns	ns
Foregut	0.980	0.513	0.125	-	*
Hindgut	0.097	0.430	0.909	0.039	-

Table 5.4 Tukey's pairwise comparisons of archaeal 16S rRNA gene abundance (log_{10}).
*** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	**	***	***	**
Burrow	0.01	-	ns	***	***
Deep	<0.001	0.440	-	***	***
Foregut	<0.001	<0.001	<0.001	-	*
Hindgut	0.019	<0.001	<0.001	0.025	-

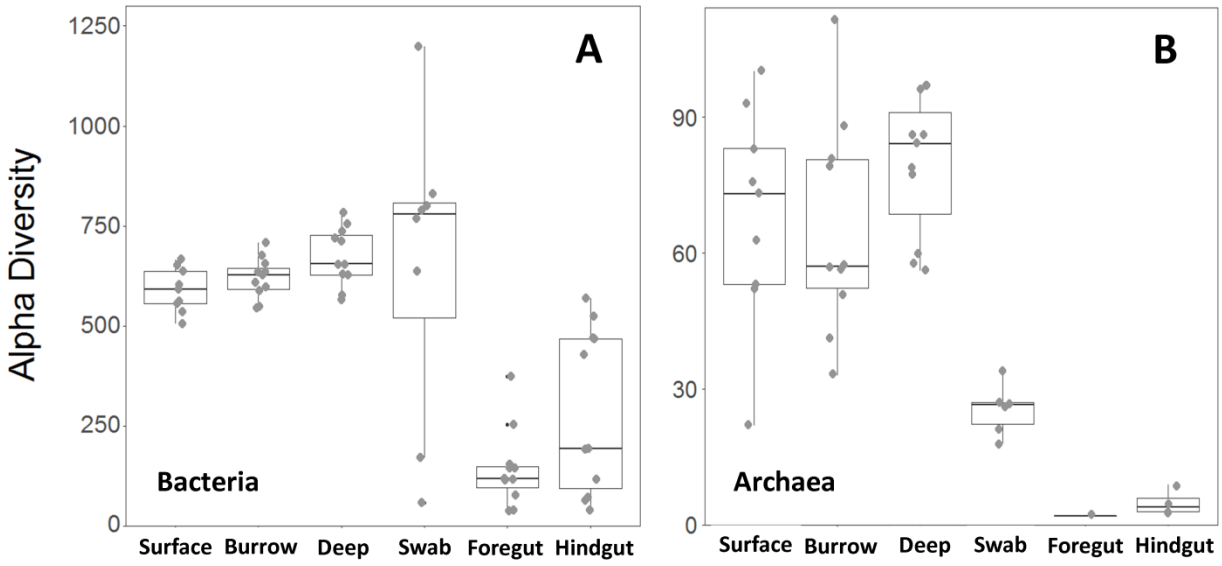

Figure 5.3 Variations in bacterial (A) and archaeal (B) alpha diversity.
Calculated using Chao1 diversity index. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (Bacteria: n = 11, except surface n = 9; Archaea: surface n = 9, burrow n = 10, deep n = 11, swab n = 6, foregut n = 1, hindgut n = 4).

5.4.2. Microbial assemblage structure

Bacterial assemblage structure, determined by 16S rRNA gene sequencing, was dependent on sample type ($F_{5, 55} = 10.27$, $p = 0.001$), with all sources being distinguishable from one another (Table 5.5). Overall, the sediment samples clustered separately from the gut samples. Within the sediment cluster, the burrow samples formed an intermediate cluster between the surface and deep sediment assemblage clusters (Figure 5.4 A-B), although the majority of the burrow samples were more affiliated to the surface assemblages (Figure 5.4 B). The gut assemblages clustered together, though in more variable and less distinct groups, with hindgut and sediment assemblages more closely affiliated with one another than the foregut and sediment assemblages (Figure 5.4 A-B). The external swab samples formed a distinct bacterial group between the sediment and gut clusters, being most closely affiliated to the surface and hindgut assemblages (Figure 5.4 A-B).

Sample type also affected archaeal assemblage structure ($F_{4, 35} = 9.62$, $p = 0.001$), with each source forming an independent cluster (Table 5.6). As with the bacterial assemblages, sediment samples clustered together with the burrow samples forming an intermediate cluster between the deep and surface assemblages (Figure 5.4 C). Unlike the bacterial assemblages, however, the archaeal burrow assemblages appeared to be more closely affiliated with the deep sediment assemblages (Figure 5.4 D). Similarly, distinct hindgut assemblages showed greatest affiliation with the external swab assemblages, which formed an intermediate group between the gut and sediment assemblages (Figure 5.4 C-D).

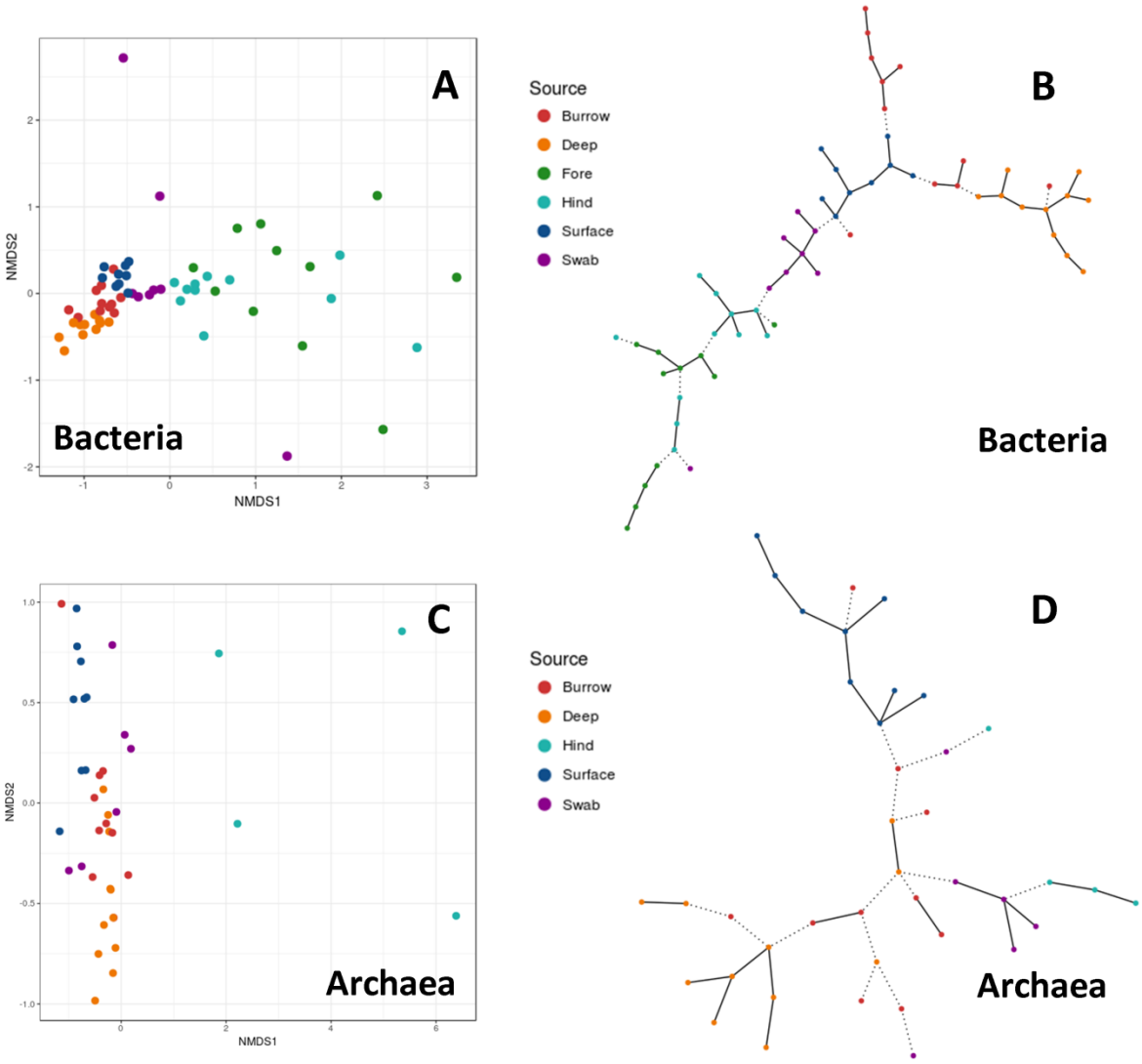

Figure 5.4 Bacterial and archaeal community structure.

Nonmetric Multidimensional Scaling (nMDS) (Bray-Curtis) and network analysis (Minimum Spanning Tree, Bray-Curtis, p = 0.002) plots of bacteria (A, B) and archaeal (C, D) community structure.

Table 5.5 Summary of bacterial community pairwise PERMANOVA comparisons. Calculated from weighted Bray-Curtis dissimilarity matrices scaled to the minimum number of reads (999 permutations, p-value in parentheses, bold typeface indicates pairwise comparison ($p < 0.05$)).

	Burrow	Deep	Foregut	Hindgut	Swab	Surface
Burrow	-	F = 5.19 **(0.001)**	F = 15.68 **(0.001)**	F = 16.1 **(0.001)**	F = 4.42 **(0.001)**	F = 5.9 **(0.001)**
Deep		-	F = 16.91 **(0.001)**	F = 18.31 **(0.001)**	F = 6.6 **(0.001)**	F = 19.59 **(0.001)**
Foregut			-	F = 2.75 **(0.007)**	F = 4.09 **(0.001)**	F = 13.37 **(0.001)**
Hindgut				-	F = 3.38 **(0.004)**	F = 13.14 **(0.001)**
Swab					-	F = 3.74 **(0.001)**
Surface						-

Table 5.6 Summary of archaeal community pairwise PERMANOVA comparisons. Calculated from weighted Bray-Curtis dissimilarity matrices scaled to the minimum number of reads (999 permutations, p-value in parentheses, bold typeface indicates pairwise comparison ($p < 0.05$)). Foregut sample excluded from analysis (Table 5.2).

	Burrow	Deep	Hindgut	Swab	Surface
Burrow	-	F = 2.93 **(0.03)**	F = 11.71 **(0.006)**	F = 4.24 **(0.001)**	F = 10.24 **(0.001)**
Deep		-	F = 15.26 **(0.001)**	F = 8.38 **(0.001)**	F = 26.60 **(0.001)**
Hindgut			-	F = 6.75 **(0.003)**	F = 11.10 **(0.002)**
Swab				-	F = 6.04 **(0.001)**
Surface					-

5.4.3. Summary of abundant microbial taxa

The abundant bacterial assemblage (>5% relative abundance), determined by 16S rRNA gene sequencing, was made up of 18 orders that accounted for 54.6 ± 12.9% of the total OTU abundance (Figure 5.5 A). This abundant subset differed between the sediment (surface, burrow, deep) and gut environments (foregut, hindgut), with the sediment assemblages dominated by the orders *Desulfobacterales*, *Flavobacterales*, and *Xanthomonadales*. Comparing the surface and deep sediment assemblages, the surface samples had greater abundances of *Cytophagales* and *Rhodobacterales*, while the deep samples had greater abundances of *Desulfobacterales* and *Myxococcales*. The burrow assemblages were a combination of both the surface and deep assemblages, with abundant populations of *Desulfobacterales*, *Myxococcales*, and *Rhodobacterales*. In the gut, the majority of the samples were dominated by *Flavobacterales* and *Rhodobacterales*. Additionally, as observed previously in the community structure analyses (Figure 5.4), there seems to be greater variation in the overall assemblage composition between the individual *Hediste diversicolor* gut tracts than between the individual burrow systems. The external swab assemblages were also more varied in composition relative to the burrow systems, with some individuals showing high abundances of *Xanthomonadales*, as observed in the sediment assemblages, while others showed high abundances of *Rhodobacterales*, similar to the gut assemblages.

The abundant archaeal assemblage (>5% relative abundance) was made up of five classes that accounted for 94.6 ± 3.5% of the total OTU abundance (Figure 5.5 B). This abundant assemblage also differed between the sediment and gut environments, with the external swab and sediment assemblages dominated by Group C3 and *Thermoplasmata* (Figure 5.5 B). *Methanomicrobia* was also abundant in the surface and swab assemblages, with some surface sediment assemblages also enriched in Marine Group I. As with bacteria, the burrow archaea assemblage was a combination of the surface and deep assemblages, which were dominated by Group C3 and *Thermoplasmata*, with high abundances of Marine Group I and *Methanomicrobia* in some individual burrows. In contrast, the gut assemblages were dominated by the Soil Crenarchaeotic Group, with *Methanomicrobia* only present in high abundances in the hindgut assemblages.

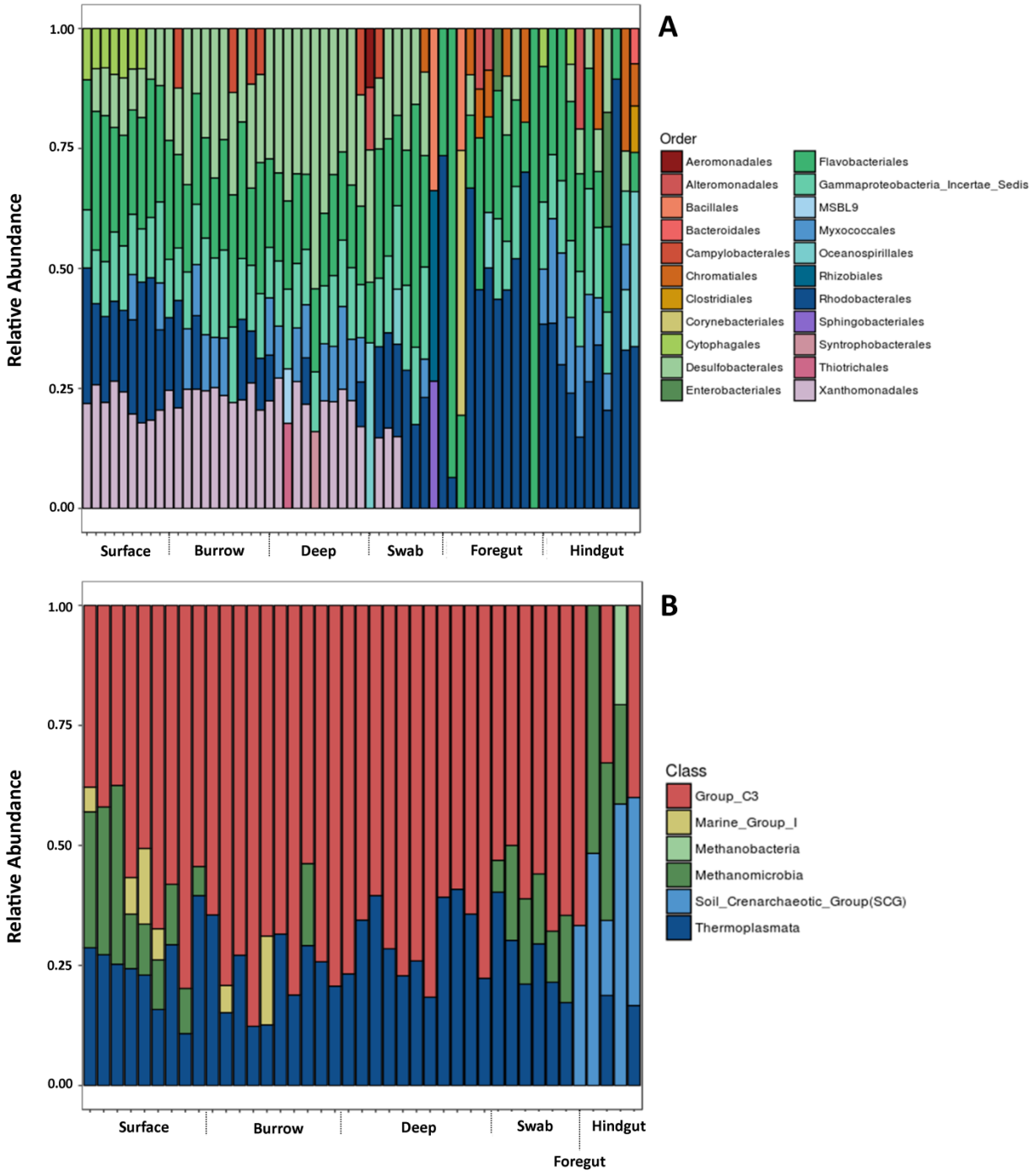

Figure 5.5 Relative abundance of bacterial orders (A) and archaeal classes (B) (> 5% relative abundance) between each of the samples.

5.4.4. Taxonomic variations between sample type assemblages

Pairwise comparisons of the abundance of taxonomic groups between sample types (plotted as log2 fold changes) revealed that bacterial burrow assemblages were significantly enriched in *Desulfobacterales* and *Myxococcales* relative to surface assemblages, which were enriched in *Flavobacteriales* and *Rhodobacterales* (Figure 5.6 A). In contrast, comparison between burrow and deep sediment assemblages revealed the reverse trend (Figure 5.6 B), which indicates that burrow assemblages were enriched in both surface and deep sediment taxa. Generally fewer taxa varied between the foregut and hindgut, but greater abundances of *Cytophagales*, *Flavobacteriales*, and *Myxococcales* were present in the hindgut relative to the foregut (Figure 5.6 C). Between the hindgut and the burrow assemblages, these three orders were generally enriched in the burrow environment, although some representatives were more abundant in the hindgut (e.g. *Ekhidna sp*., *Actibacter sp*.) (Figure 5.6 D).

Between the swab and surface assemblages, only one Cyanobacteria representative differed between the two sample types (Figure IV.2 A). Swab assemblages were enriched in some *Desulfobacterales* taxa relative to burrow and hindgut assemblages and in *Flavobacterales* when compared to the hindgut assemblage alone (Figure 5.6 E-F). Bacterial orders enriched during gut passage (i.e. *Cytophagales*, *Flavobacteriales*, *Myxococcales*) were also more abundant in the external swab assemblage relative to the deep anoxic sediment (Figure IV.2 B).

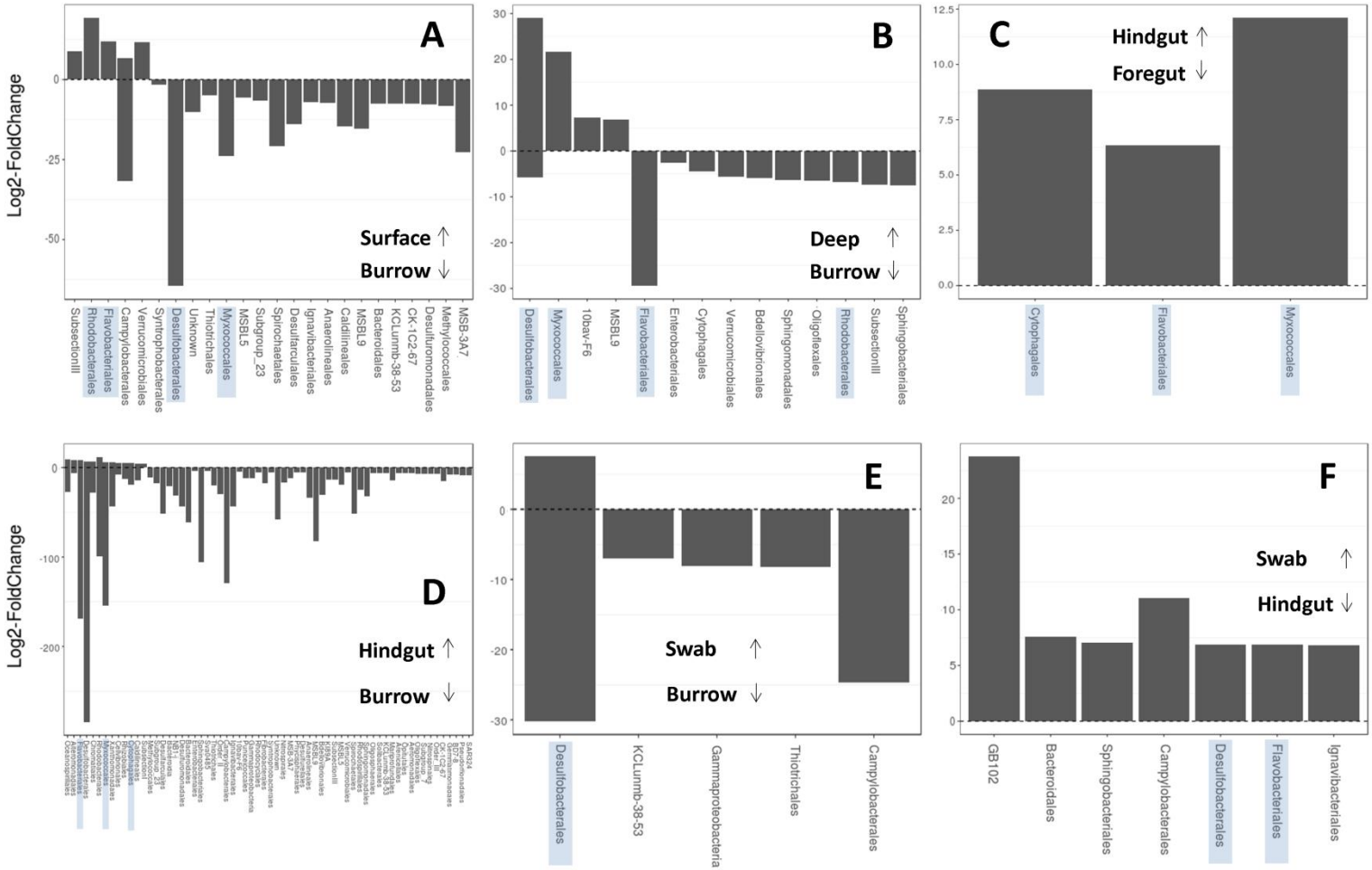

Figure 5.6 Change in abundance of taxa (Log2 fold; p <0.05) between ecologically relevant combinations of sources. (A – Burrow vs Surface; B – Burrow vs Deep; C – Foregut vs Hindgut; D – Burrow vs Hindgut; E – Burrow vs Swab; F – Hindgut vs Swab). For each combination, taxa below 0 are more abundant in first stated sample and taxa above 0 are more abundant in second stated sample. Key taxa are highlighted.

5.4.5. Abundance of nitrogen cycling taxa

Abundance of ammonia-oxidising bacteria (AOB) was dependent on sample type ($F_{4, 14}$ = 5.935, p = 0.005), although the significance of this term was driven by a greater abundance in the burrow and deep sediment assemblages (burrow, 629 ± 210 copies mgww.sediment^{-1}; deep, 789 ± 365 copies mgww.sediment^{-1}) relative to the foregut assemblage (69 ± 32 copies mgww.sediment^{-1}; Tukey's, p < 0.05, Table 5.7; Figure 5.7 A). Concomitantly, the sequencing analysis showed that the majority of ammonia-oxidising and nitrite-oxidising (NOB) bacterial taxa (*Nitrosomonadaceae*, *Nitrosococcus*, *Nitrobacter*, *Nitrospina*) were either absent or did not differ with sample type. Nitrospira (NOB) was the exception ($F_{5, 55}$ = 2.97, p = 0.02) and had a lower relative abundance in the hindgut (0.02 ± 0.03% relative abundance) compared to the deep sediment assemblage (0.15 ± 0.10% relative abundance; Tukey's, p <0.05).

Ammonia-oxidising archaea (AOA) abundance was dependent on sample type (Log_{10}, $F_{4, 14}$ = 10.65, p <0.001), with greater abundances in sediment and hindgut assemblages (burrow, 363 ± 66 copies mgww.sediment^{-1}; deep, 517 ± 121 copies mgww.sediment^{-1}; hindgut, 1,951 ± 1,129 copies mgww.sediment^{-1}) relative to the foregut assemblage (90 ± 148 copies mgww.sediment^{-1}; Log_{10}, Tukey's, p <0.05, Table 5.8; Figure 5.7 B). The ratio of AOB to AOA abundance (AOB:AOA) was also influenced by sample type (Log_{10}, $F_{4, 14}$ = 4.08, p = 0.021), with the lowest mean ratio present in the hindgut (0.267 ± 0.323), intermediate ratios in the sediment (surface, 1.11 ± 0.630; burrow, 1.70 ± 0.291; deep, 1.48 ± 0.406), and the highest mean ratio in the foregut (4.43 ± 2.86; Log_{10}, Tukey's, p <0.05, Table 5.9; Figure 5.7 C). A ratio less than one indicates an AOA dominated community, while a ratio greater than one indicates an AOB dominated community. Interestingly, the dominant AOA in most of the hindgut samples (Nitrosocosmicus) was from the Soil Crenarchaeotic Group, while the dominant group in the sediment samples (Nitrosopumilus) was from Marine Group I which was only observed in some individual surface and burrow samples.

Bacterial *nirS* gene copy numbers, calculated as a proxy for the denitrifying bacterial assemblage, differed in abundance with sample type ($F_{4, 14}$ = 10.26, p <0.001). Denitrifying bacterial abundance was generally lower in the gut assemblages (foregut, 671 ± 484 copies mgww.sediment^{-1}; hindgut, 27,442 ± 7,546 copies mgww.sediment^{-1}) relative to the sediment assemblages (surface, 82,577 ± 40,786 copies mgww.sediment^{-1}, 112,541 ± 37,716 copies mgww.sediment^{-1}; deep, 106,859 ± 34,420 copies mgww.sediment^{-1}; Tukey's, p < 0.05, Table 5.10), with the exception of the hindgut and surface sediment assemblage (Figure 5.7 D).

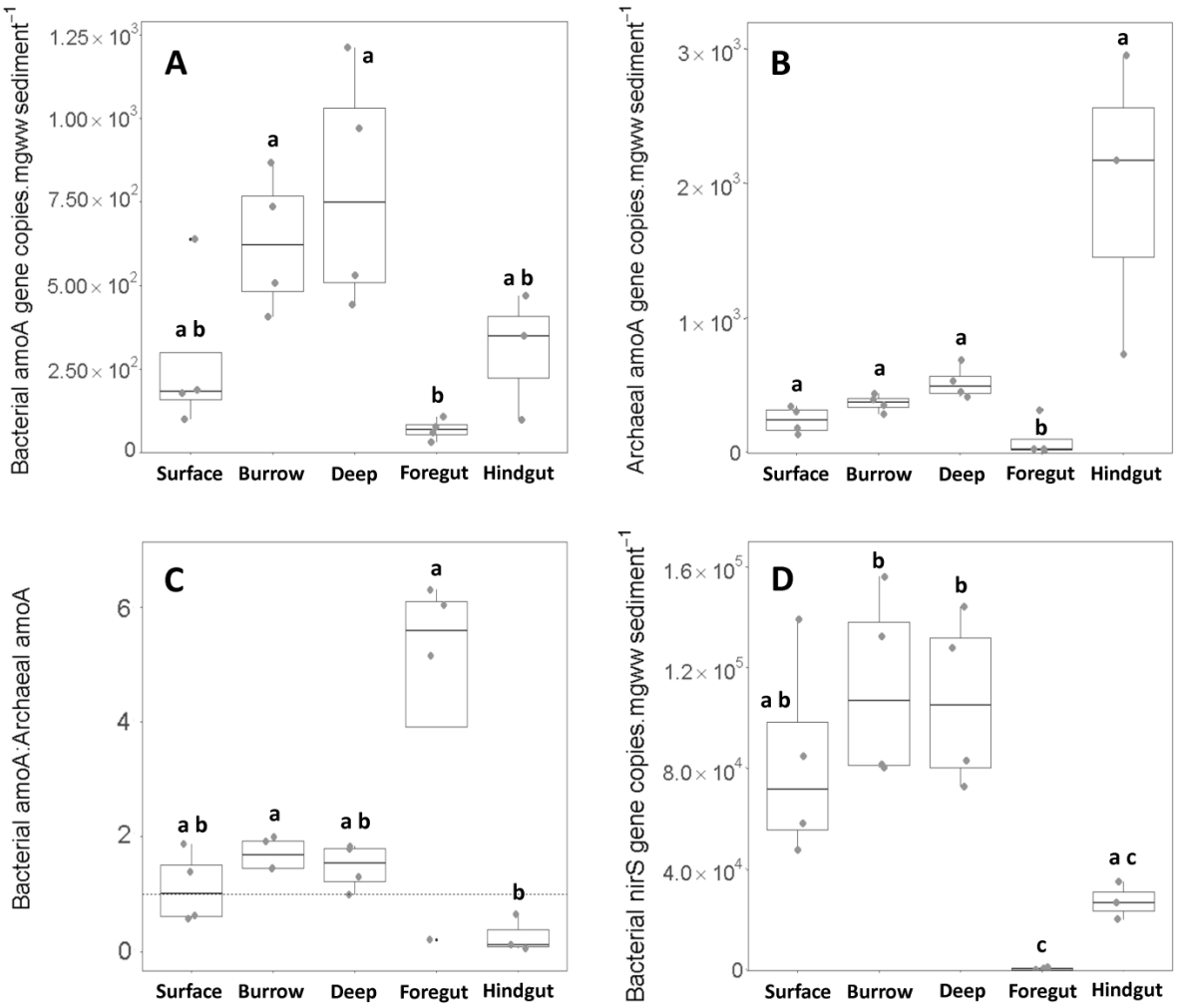

Figure 5.7 Variations in nitrogen cycling functional gene abundance.
Bacterial (A) and archaeal (B) *amoA* gene copy number, bacterial:archaeal *amoA* copy ratio (C), and bacterial *nirS* gene copy number (D). Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (n = 4). Untransformed data presented. Letters (a, b, c) indicate significant difference between samples ($p < 0.05$; Table 5.7 - 5.10).

Table 5.7 Tukey's pairwise comparisons of bacterial *amoA* gene abundance.

*** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	ns	ns	ns	ns
Burrow	0.271	-	ns	*	ns
Deep	0.056	0.871	-	**	ns
Foregut	0.731	0.033	0.006	-	ns
Hindgut	0.999	0.418	0.110	0.692	-

Table 5.8 Tukey's pairwise comparisons of archaeal *amoA* gene abundance (log_{10}).

*** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	ns	ns	*	ns
Burrow	0.924	-	ns	**	ns
Deep	0.65	0.977	-	**	ns
Foregut	0.037	0.008	0.003	-	***
Hindgut	0.052	0.187	0.400	<0.001	-

Table 5.9 Tukey's pairwise comparisons of AOB:AOA.

*** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	ns	ns	ns	ns
Burrow	0.932	-	ns	ns	*
Deep	0.981	0.999	-	ns	ns
Foregut	0.661	0.976	0.922	-	*
Hindgut	0.144	0.041	0.060	0.015	-

Table 5.10 Tukey's pairwise comparisons of bacterial *nirS* gene abundance.

*** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant.

	Surface	Burrow	Deep	Foregut	Hindgut
Surface	-	ns	ns	*	ns
Burrow	0.641	-	ns	***	*
Deep	0.788	0.999	-	**	*
Foregut	0.014	0.001	0.002	-	ns
Hindgut	0.179	0.018	0.029	0.776	-

5.5. Discussion

The passage of sediment through the gut of an invertebrate has the potential to be a key mechanistic ecological process that helps explain the influence of deposit feeding invertebrates on sediment microbial assemblages and subsequent biogeochemical cycling. Using high-throughput sequencing, we demonstrate that a common sediment-dwelling invertebrate (*Hediste diversicolor*) has a distinct transitory gut assemblage, with regrowth of both bacterial and archaeal taxa at the posterior end of the digestive tract. Hindguts of *H. diversicolor* also appear to be 'incubators' for distinct ammonia-oxidising archaeal assemblages. This specific transitory assemblage, and the distinct assemblage on the external surface of the polychaete, has the potential to introduce higher abundances of specific taxa to the surrounding sediment, and therefore modify the sediment assemblage structure and facilitate the transport of microbial taxa between sediment patches (Godbold *et al.*, 2011).

Bacterial burrow assemblages have been shown to be similar to both surface (Laverock *et al.*, 2010; Pischedda *et al.*, 2011) and deep sediment assemblages (Papaspyrou *et al.*, 2005; Papaspyrou *et al.*, 2006). Here, we show that bacterial and archaeal abundances were generally similar between all surface, burrow, and surrounding sediment, and abundances observed were consistent with those in other bioturbated sediments (Laverock *et al.*, 2013). Assemblage composition analysis, however, indicated that the burrow assemblages were a combination of both the surface and deep sediment assemblages, with the bacterial assemblage most similar to the surface sediment assemblage and the archaeal assemblage seemingly most similar to the deep sediment assemblage. Overall, this fits with the paradigm that intermittent irrigation of burrows by invertebrates creates an oscillating oxic-anoxic environment (Volkenborn *et al.*, 2012), which switches the habitat between 'surface-like' and 'deep-like' available O_2 conditions and allows the coexistence of aerobic and anaerobic microbial taxa in the burrow. Other invertebrate activities occurring within burrows, such as mucopolysaccharide production (Dale *et al.*, 2018) or the secretion of biocides (King, 1988), are also likely to contribute to burrow assemblage structure.

The gut tracts of *H. diversicolor* contained distinct bacterial and archaeal assemblages that were less diverse than the surrounding sediment (King, 2018) and included taxa, such as *Thermoplasmatales* and *Methanomicrobiales*, which have been previously observed in polychaete digestive tracts (Li *et al.*, 2009). Gut assemblage structure also demonstrated much greater inter-individual variation than the burrow assemblages, which suggests that *H. diversicolor* individuals may have distinct effects on

their transitory assemblages. Overall, the transitory assemblage was most distinct from the surrounding sediment after passage through the foregut, where both bacterial and archaeal abundance and alpha diversity declined. This suggests that archaea could also be a food source for *H. diversicolor*, which does not seem to have been considered in previous research. Within the hindgut, the assemblages remained distinct but seemed to increase in abundance and became more similar to the sediment, though this was most likely not due to the addition of taxa as there was no significant increase in bacterial or archaeal diversity. These observations follow the abundance patterns observed in other marine deposit feeders, where bacteriolytic activity is highest in the fore and midgut (Plante *et al.*, 1989; Plante & Mayer, 1994; Mayer *et al.*, 1997), but are not consistent with previous studies of *H. diversicolor* where abundance-based techniques using epifluorescence microscopy have shown higher lytic activity and limited regrowth in the hindgut (Lucas & Bertru, 1997; Lucas *et al.*, 2003). Here, the use of molecular tools with increased resolution has shown that *Cytophagales*, *Flavobacteriales*, and *Myxococcales* increase in abundance during *H. diversicolor* gut transit. Both *Cytophagales* and *Flavobacteriales* have previously been observed in the guts of deposit-feeding shrimp (Lau *et al.*, 2002) and are known to degrade complex macromolecules (Reichenbach & Dworkin, 1981; McBride, 2014) that are likely to be abundant in hindgut environments. As these taxa are also present in the surrounding sediment bacterial assemblages, gut conditions appear to affect assemblage composition by altering the abundance of existing transitory sediment taxa (Furlong *et al.*, 2002).

As both bacterial and archaeal assemblages increased in abundance in the hindgut, it is possible that this microbial ‘incubator’ could contribute to the wider sediment assemblage once excreted. Because the distinct hindgut assemblage was more similar to the surrounding sediment than the foregut assemblage was, and greater abundances of the hindgut-enriched taxa were observed in the burrow environment relative to the surrounding deep anoxic sediment, it is possible that there was some introduction of hindgut enriched taxa to the sediment. Organic enrichment within the burrow environment, however, could have encouraged the proliferation of *Cytophagales* and *Flavobacterales* (Aller & Aller, 1986; Papaspyrou *et al.*, 2006), while *Flavobacterales* may also have been translocated from surficial sediments. Overall, the majority of taxa were more abundant in the sediment than the hindgut but the hindgut may act as a reservoir for at least some representatives of specific taxa (King, 2018). Hence, to fully characterise how sediment functioning may be affected by gut passage and the introduction of specific taxa, it will be beneficial to consider the activity levels of microbial functional groups in both gut, faecal cast, and sediment environments.

Invertebrates have been suggested as potential transporters of microbial groups within sediment (Troussellier *et al.*, 2017), but the significance of such transport has not been assessed. In this study, the composition of the external microbial assemblages of *H. diversicolor* reflected a combination of both hindgut and sediment assemblages, particularly the surface sediment assemblage. This observation supports the view that a proportion of the hindgut assemblage may be excreted into the sediment. The close relationship with the surficial sediment also suggests that either *H. diversicolor* spends a portion of time at the surface (e.g. foraging, Vedel & Andersen, 1994), or that the very inner burrow sediment immediately in contact with the invertebrate is more closely related to the surface sediment than the other burrow wall assemblages (Bertics & Ziebis, 2009). As the *H. diversicolor* external assemblages had greater abundances of some *Desulfobacterales* taxa compared to both the burrow and hindgut assemblages, specific taxa may be concentrated on the external surfaces of individuals and therefore be redistributed within the burrow system. Additionally, the taxonomic groups that increased in abundance during gut passage were more abundant on the external surfaces of the polychaete than they were in the deep sediment, and so *H. diversicolor* individuals could transport taxa into anoxic sediment during burrow construction and extension (Davey, 1994).

Nitrification is a significant process in benthic nitrogen cycling as it converts NH_4^+ released from organic matter back into NO_3^- (Herbert, 1999) that can then support primary productivity in the overlying water column (Boynton & Kemp, 1985). Although the abundances of ammonia-oxidising bacteria (AOB) and archaea (AOA) in the sediment were consistent with other sediment environments (Laverock *et al.*, 2013; Bowen *et al.*, 2014), they were also consistent between the surface, burrow, and surrounding sediment which suggests that these burrow systems were not hotspots for nitrifying taxa. In the foregut of *H. diversicolor*, there seemed to be an overall reduction of both AOB and AOA in the transitory assemblage, though the high AOB:AOA ratio suggests that AOA taxa were generally more susceptible to potential digestive foregut loss processes.

Conversely, passage through the hindgut supported a substantial increase of AOA to abundances comparable with the surrounding sediment so that AOA dominated the nitrifying community within the hindgut environment. This suggests that the organic content differed between the surrounding sediment and the hindgut, most likely because of the digestion of organic matter in the foregut and the release of NH_4^+ in the hindgut, and indicates that hindguts have the potential to contribute AOA to the surrounding sediment through excretion. Sequencing analysis, however, demonstrated that the dominant AOA taxon in the *H. diversicolor* hindguts was *Nitrosocosmicus sp*., which has a higher

tolerance for NH_4^+ and NO_2^- than other AOA (Lehtovirta-Morley *et al.*, 2016), while the dominant AOA in the sediment samples was instead Nitrosopumilus sp. (Reyes *et al.*, 2017), which tends to dominate under low NH_4^+ conditions (Martens-Habbena *et al.*, 2009). It is therefore possible that the high organic matter content in deposit feeder gut passages supports a unique AOA assemblage, which is then outcompeted when introduced to sediment. Whether the AOA are actively undertaking ammonia-oxidation in these low O_2 environments (Plante & Jumars, 1992) to the extent that they significantly contribute to sediment nitrogen cycling remains to be determined.

Earthworm gut passages and faecal casts can contain larger and more active populations of denitrifying microbial groups than the surrounding soil (Karsten & Drake, 1997; Furlong *et al.*, 2002). Here, denitrifying bacteria were reduced during foregut passage and, although certain nitrite-reducing taxa did form a significant portion of the transitory assemblage, there was no significant regrowth in the hindgut. *H. diversicolor* guts are therefore unlikely to contribute denitrifiers to sediment assemblages, but the presence of these taxa will still have functional value. Complete and incomplete denitrification by ingested soil taxa means that earthworms are sources of both N_2 and N_2O (Horn *et al.*, 2006a; Horn *et al.*, 2006b). Release of N_2O from deposit feeder guts has been shown to contribute to the overall flux from sediment systems (Stief *et al.*, 2009; Heisterkamp *et al.*, 2010), though this study also indicated that *H. diversicolor* has a slightly lower N_2O release rate than other deposit feeders (Heisterkamp*, et al.*, 2010). This may be due to a generally lower abundance of denitrifiers in *H. diversicolor* guts, though this would need to be confirmed by comparisons with other deposit-feeding invertebrate taxa. It also cannot be ruled out that complete denitrification (i.e. N_2 release instead of N_2O accumulation) may occur within *H. diversicolor* guts, but that remains to be determined via either direct activity measurements or identification of genes responsible for the final denitrification step (*nosZ*). Understanding how denitrifying assemblages differ between deposit feeding taxa, and whether this is related to subsequent variations in N_2O and N_2 release, will be beneficial to efforts seeking to improve current estimates of sediment N budgets.

Collectively, our findings indicate that the internal and external transport of microbial assemblages by deposit feeders has the potential to regulate sediment microbial assemblages. By accumulating both sediment and gut-associated taxa on external surfaces, burrowing invertebrates may alter local sediment microbial distributions. The transitory sediment assemblage in gut passages also has the potential to contribute to sediment nitrogen cycling, either by introducing key microbial functional groups or by supporting these taxa within the gut and excreting products. More thorough conclusions

could potentially have been drawn from a more extensive dataset, with fewer excluded samples and greater read depth, by pooling individual gut and swab samples to improve DNA yield. Yet, by pooling these samples we were able to explore the inter-individual effects that are crucial to widening our understanding of spatiotemporal variations in invertebrate regulation of biogeochemical cycling, as the majority of invertebrate trait assessments assume that trait effects do not vary within or between populations (Wohlgemuth *et al.*, 2017).

H. diversicolor often dominates the biomass of intertidal mudflats and is known to reach densities of 3700 ind. m^{-2} (Scaps, 2002). As related polychaete populations have been estimated to ingest 5 kg (dry weight) sediment m^{-2} $year^{-1}$ (Cammen, 1980), sediment microbial assemblages will be regularly and consistently exposed to gut conditions. Yet, the wider ecological consequences of this process are understudied, especially in the context of expanding polychaete fisheries (Watson *et al.*, 2017; Cole *et al.*, 2018). Future effort should assess transitory taxa activity and expand the scope of this current research by establishing whether the effects of gut passage vary between alternative invertebrate groups, between populations of the same species, and spatiotemporally across seasons or environmental gradients. Additionally, based on previous assessments of *H. diversicolor* emissions (Heisterkamp *et al.*, 2010), these populations have the potential to release 8880 nmol N_2O m^{-2} day^{-1} with significant implications for sediment denitrification and nitrogen fluxes. Further examination of gut passage and external transport as invertebrate functional traits will improve our understanding of invertebrate-microbe interactions, and the role this ecological process plays in regulating sediment ecosystem functioning.

6. General Discussion

6.1. Introduction

Sediments are often poorly represented in benthic-pelagic models used to predict changes in ecosystem function because of a lack of spatiotemporal resolution and limited inclusion of complex biological processes (Lessin *et al.*, 2018; Snelgrove *et al.*, 2018). Effectively incorporating the necessary biological complexity into sediment system models requires an understanding of how invertebrate-microbe interactions can regulate sediment microbial ecology and functioning. The work presented in this thesis used molecular ecology tools to isolate the effects of specific invertebrate functional traits on general sediment microbial community composition and nitrogen cycling microbial functional groups, while highlighting the importance of assessing how these interactions may vary with changing abiotic conditions. In this discussion, I will examine how these findings have advanced our understanding of invertebrate-microbe-functioning interactions, before considering the wider impacts, limitations and potential future directions of this research.

6.2. Role of invertebrate burrow morphology

Invertebrate burrows are typically grouped into open ("U-shaped") and closed ("J-shaped) morphologies, with water exiting the burrow through a sediment opening or through percolation. As burrow ventilation plays an important role in regulating bacterial activity, including nitrogen cycling process rates and sediment nitrogen fluxes (Mermillod-Blondin *et al.*, 2005; Na *et al.*, 2008), attempts have been made to produce an easily applicable bioirrigation index that uses various invertebrate traits to characterise ventilation for a particular area (Renz *et al.*, 2018; Wrede *et al.*, 2018). Despite research indicating that burrow morphology can affect sediment-water exchange and bacterial activity (Heron & Ridd, 2008; Renz & Forster, 2013; Vasquez-Cardenas *et al.*, 2016), current indices do not consider burrow morphology because of interdependency with other traits (Renz *et al.*, 2018), or assume that open burrows have a lesser effect on sediment systems than closed burrows (Wrede *et al.*, 2018). Yet, there have been no genetic molecular assessments to determine how burrow morphology affects key microbial functional groups.

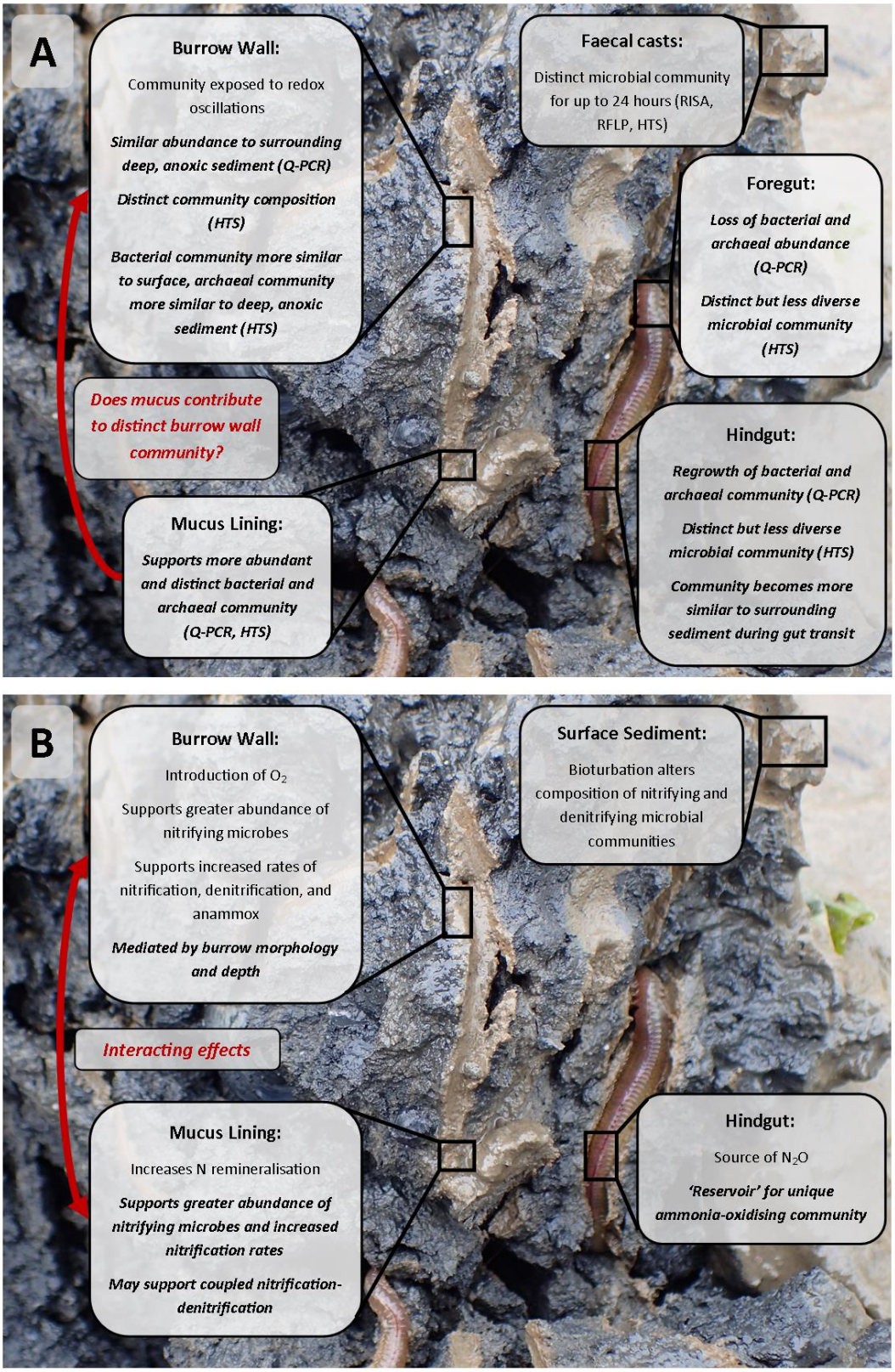

Figure 6.1 Thesis contributions (bold, italic) to current understanding of how sediment-dwelling invertebrate activities regulate whole sediment microbial communities (A) and nitrogen cycling microbial communities (B).

Text in parentheses represents microbial ecology technique used to observe stated trend (Photograph - Davis Laundon, MBA).

Overall, the abundance data presented in Chapter 2 supports the current bioirrigation index morphology scoring system (Wrede *et al.*, 2018) because, at mid-mixing depths, closed burrows did support greater abundances of nitrifying microbial groups (Figure 6.1 B). Based on visual observations of the cores, this seems to be related to variations in O_2 diffusion and sediment-water exchange between the two morphologies (Nielsen *et al.*, 2004; Santos *et al.*, 2012; Jovanovic *et al.*, 2014). Open burrows, however, appeared to support greater nitrification activity. Although these activity levels may be biased by the lack of temporal resolution, it does indicate that assessing effects on functioning using various parameters is essential in effectively examining the importance of invertebrate trait variations. Importantly, this study demonstrates that burrow morphology can play a role in regulating nitrogen cycling and therefore further study is required to determine how this can be incorporated into future irrigation indices and natural sediment systems.

Chapter 2 also highlights important future methodological considerations for trait-based assessments of invertebrate-microbe interactions. First, the need for experimental systems that isolate specific traits of interest. Differing effects of closed and open burrows on sediment microbial activity have previously been suggested (Vasquez-Cardenas *et al.*, 2016), but the use of live invertebrates means morphological effects cannot be separated from other varying traits e.g. ventilation rate or particle reworking. Here, by isolating the effect of burrow morphology and combining this with modern molecular techniques, a better assessment of this invertebrate trait can be made to support models. This could be expanded to other traits, such as ventilation periodicity variations (Kristensen *et al.*, 1991; Volkenborn *et al.*, 2010; Volkenborn *et al.*, 2012), or used to expand understanding further by combining trait variations to assess interaction effects. Second, vertical variations in trait effects on microbial communities require greater consideration. Despite research demonstrating clear differences between burrow wall and surface sediment communities (Pischedda *et al.*, 2011; King, 2018), and the importance of depth distribution when examining sediment properties (e.g. particle reworking, O_2 distribution) (Na *et al.*, 2008; Braeckman *et al.*, 2010; Stauffert *et al.*, 2013), invertebrate-microbe interaction studies often only examine surface sediment communities (Gilbertson *et al.*, 2012; Stauffert *et al.*, 2013; Sciberras *et al.*, 2017). In this study, the analysis of only the surface or deep sediment would have led to the conclusion that burrow morphology does not affect ammonia-oxidising assemblages. Incorporating vertical variation could improve the accuracy of invertebrate trait assessments and help build biological complexity for sediment system models.

6.3. Role of invertebrate secretions in shaping burrow communities

Building realistic biological complexity also requires the identification and isolation of previously under considered invertebrate traits. Nitrogen-rich mucopolysaccharide secretions (i.e. mucus) have been suggested to mediate microbial activity and coupled nitrification-denitrification processes around invertebrate burrows (Aller & Aller, 1986; Bonaglia *et al.*, 2014). Isolated assessments, however, have been limited to measurements of nitrogen remineralisation and bacterial abundance through epifluorescence microscopy (Herndl & Peduzzi, 1989; Peduzzi & Herndl, 1991; Hannides & Aller, 2016). Mucus secretions have therefore not been examined as a potential invertebrate trait that could affect sediment ecosystem functioning.

The study presented in Chapter 3 demonstrates that, under continuously oxic conditions, *Hediste diversicolor* mucus increases the proliferation of ammonia-oxidising microbial groups and NO_2^- and NO_3^- production rates (Figure 6.1 B). This supports previous suggestions about mucus and indicates that increases in nitrification activity around invertebrate burrows (Sayama & Kurihara, 1983; Nielsen *et al.*, 2004; Satoh *et al.*, 2007) could be attributable to the presence of mucus linings rather than just increased oxygenation. Chapter 4 appears to confirm this, as significantly lower concentrations of mucopolysaccharide were still able to increase ammonia-oxidiser activity. Interestingly however, at this lower mucus concentration, ammonia-oxidiser proliferation was not stimulated, most likely due to competition with heterotrophs (Verhagen *et al.*, 1992), and NO_3^- production was unaffected. Mucus concentration effects should therefore be examined in more detail, particularly as mucus lining thickness can vary between invertebrate taxa and alter sediment-water exchange (Hannides *et al.*, 2005; Zorn *et al.*, 2006).

The two mucus incubation studies (Chapter 3 & 4) also demonstrate that secretions support coupled nitrification-denitrification, with increases in both denitrifying bacterial abundance and activity most likely due to increases in NO_2^-/NO_3^- production from nitrification (Figure 6.1 B). Invertebrate mucus secretions may therefore also be partially responsible for previously observed increases in coupled nitrification-denitrification in bioturbated sediments (Kristensen *et al.*, 1991; Gilbert *et al.*, 1998; Howe *et al.*, 2004). Overall, mucus is an invertebrate trait that can significantly affect multiple sediment nitrogen cycling processes. To further validate this, finite amounts of mucus were added to each incubation with the expectation that mucopolysaccharide would stimulate nitrogen

cycling until the supply was exhausted, at which point the lack of NH_4^+ production would cause these processes to decline as observed in Chapter 3 (Bowen *et al.*, 2014; Foshtomi *et al.*, 2015). Continuous addition of mucus to incubations could provide a better estimate of nitrogen cycling processes in burrow environments, though this would first require accurate quantification of mucus production rates which have typically only been assessed for rocky intertidal gastropod species (Davies *et al.*, 1990; Peck *et al.*, 1993).

Mucus linings in burrow environments are naturally exposed to redox oscillations (Furukawa, 2001). Results from Chapter 2 suggest that changes in O_2 distribution can affect AOB and AOA proliferation, while previous research shows that sediment redox oscillations can affect organic matter degradation rates (Aller, 1994; Sun *et al.*, 2002; Caradec *et al.*, 2004) and support sustained denitrification activity (Gilbert *et al.*, 2016). From Chapter 4 (Figure 6.1 B), it seems that under the oxic-anoxic oscillations common in burrow environments, mucus secretions have a stabilising effect on ammonia-oxidiser activity possibly by increasing rates of nitrogen remineralisation during the anoxic period (Hannides & Aller, 2016). These anoxic periods did result in lower overall production of NO_2^- and NO_3^-, however, and could therefore affect nitrogen fluxes between benthic and pelagic systems (Ieno *et al.*, 2006; Ekeroth *et al.*, 2016). Redox oscillations also supported increased coupling of nitrification-denitrification (Gilbert *et al.*, 2016), but only in the absence of mucus which suggests mucopolysaccharide and O_2 oscillations could have a synergistic rather than additive effect.

Mucus secretions can also affect the abundance and structure of total sediment microbial communities (Figure 6.1 A). The increased proliferation of bacteria and archaea observed in Chapter 3 validates previous observations of increased abundance on mucus linings with epifluorescence microscopy (Papaspyrou *et al.*, 2006). Additionally, high-throughput sequencing demonstrated that the presence of mucus in sediment produces distinct bacterial and archaeal communities (Chapter 3). Mucus secretions could subsequently affect additional biogeochemical processes not assessed in this thesis, such as carbon cycling, and may help to structure the unique burrow communities generally observed in natural sediment systems (Chapter 5) (Taylor & Cunliffe, 2015). Previous sequencing work has suggested that, on the whole, sediment invertebrates affect the structure of sediment microbial communities by altering the distribution and abundance of the existing sediment taxa (King, 2018). The unique mucus community observed in Chapter 3 supports this, though there was some evidence of the introduction of specific functional groups. Mucus roles in structuring distinct burrow communities therefore require further attention to determine the extent of these potential functional impacts beyond nitrogen cycling.

6.4. Role of internal and external deposit feeder transport in shaping burrow microbial communities

Chapter 5 also examined two additional invertebrate activities that could play a role in structuring burrow communities: internal gut transit and external surface transport. The findings indicated that there was a distinct transitory community within *Hediste diversicolor* gut tracts, with more variation observed between gut communities than burrow communities (Figure 6.1 A). Individual *H. diversicolor* may therefore have individual-specific effects on their own transitory community. This highlights the importance of examining intra-population effects, as invertebrate trait assessments often assume that traits do not vary within a population (Wohlgemuth *et al.*, 2017), which could limit our ability to fully predict and understand variations in trait effects.

As expected from previous epifluorescent microscopy work, bacterial and archaeal abundance declined between the sediment and the end of the foregut (Figure 6.1 A) which was most likely caused by digestion (Plante & Mayer, 1994; Hymel & Plante, 2000). This was followed by an increase in abundance in the hindgut (Figure 6.1 A), which has again been observed in other deposit-feeding taxa due to high organic matter availability and relatively lower digestion (Mayer *et al.*, 1997; Hymel & Plante, 2000; Andresen & Kristensen, 2002). In particular, the hindgut encouraged the growth of microbial taxa capable of degrading complex macromolecules, which supports the idea that transitory gut communities respond to changing organic matter concentrations. Additionally, in the hindgut, transitory sediment communities became similar again to the surrounding sediment (Figure 6.1 A). This suggests that there is potential for hindgut communities to be introduced to the surrounding sediment through excretion. Faecal casts produced by terrestrial earthworms and deposit-feeding invertebrates can contain phenotypically and genotypically distinct communities compared to the surrounding sediment, but this difference is often lost within 24 hours (Furlong *et al.*, 2002; King, 2018). The data presented in Chapter 5 therefore confirms that excretion effects require further assessment to determine the roles that they can play in shaping surrounding sediment communities. Finally, gut transit, like mucus secretions, did not appear to alter community composition by introducing novel taxa, but instead altered abundances within the existing community. This seems to be an important emerging theme from high-throughput sequencing assessments of sediment invertebrate-microbe interactions (King, 2018), possibly because advances like high-throughput sequencing are allowing greater capture of total diversity than previously used techniques (e.g. DGGE) (Figure II.1, Figure IV.1).

Understanding excretion effects may be particularly important in the context of nitrogen cycling, as *H. diversicolor* hindguts could be incubators of distinct ammonia-oxidising archaeal (AOA) assemblages (Figure 6.1 B). It is still unclear, however, whether these taxa are excreted into the sediment, whether they are active within the gut passage which should be a low O_2 niche (Plante & Jumars, 1992), and whether excreted nitrification products could contribute to wider sediment nitrogen cycling processes. Equally, the limited regrowth of denitrifying bacteria in *H. diversicolor* guts could be responsible for the lower excretion of N_2O from *H. diversicolor* (0.398 ± 0.319 nmol g^{-1} $hour^{-1}$) than other invertebrate taxa (e.g. *Corophium volutator*: 0.955 ± 0.664 nmol g^{-1} $hour^{-1}$; Heisterkamp*, et al.*, 2010). Denitrifying bacterial abundances in deposit feeder guts may be an appropriate proxy for understanding invertebrate N_2O production. Both examples indicate that further examination of invertebrate gut transit effects on biogeochemical processes could be important in improving understanding of sediment systems.

In addition to the transitory gut community, there was a distinct community on the external polychaete surface that seemed to be predominantly accumulated from foraging and excretion (Figure 6.1 A). When individuals create new burrow systems in unbioturbated sediment (Davey, 1994), there is potential for the individual to act as a vector to transport this external community that could then form the basis of the new burrow community. Invertebrate external transport between sediment patches could therefore be an under-assessed mechanism for microbial redistribution in benthic environments.

6.5. Opening the sediment “black box” with molecular methods

Overall, the research presented in this thesis has contributed to our understanding of sediment invertebrate-microbe interactions and their associated effects on microbial nitrogen cycling. It has also demonstrated the need for greater use of contemporary molecular methods in bioturbation studies. Epifluorescent microscopy has previously shown limited regrowth and high potential for digestion in the hindguts of *Hediste diversicolor* (Lucas *et al.*, 2003), but the use of Q-PCR and high-throughput sequencing in Chapter 5 showed the opposite trend and supported what has been observed in the majority of other deposit-feeding invertebrate taxa. Additionally, the use of DGGE and T-RFLP led to debate over whether burrow communities were an extension of surface or deep sediment communities (Papaspyrou *et al.*, 2005; Laverock *et al.*, 2010; Pischedda *et*

al., 2011). Using high-throughput sequencing (Chapter 5), this study demonstrated that *H. diversicolor* burrows are a combination of both surface and deep communities and similarities depend on the taxa involved (i.e. bacteria or archaea).

Accurate assessments of invertebrate-microbe interactions can also be improved by the use of multiple molecular techniques in combination. Sediment ecology studies have previously used Q-PCR to examine nitrogen cycling functional group abundance (Satoh *et al.*, 2007; Gilbertson *et al.*, 2012; Laverock *et al.*, 2014; Smith *et al.*, 2015), but few studies have confirmed changes in gene transcription using RT-QPCR (Bowen *et al.*, 2014). In this thesis, the use of both Q-PCR and RT-QPCR has allowed better characterisation of the role of burrow morphology in mediating nitrification (Chapter 2) and demonstrated that mucopolysaccharide can increase ammonia-oxidiser activity (Chapter 4), which confirmed the abundance-based theory formed in Chapter 3. Similarly, the combined use of Q-PCR and high-throughput sequencing in Chapter 5 allowed the identification of the proliferation of a unique ammonia-oxidising archaeal community in the hindgut of *H. diversicolor*.

Invertebrate-microbe interactions have largely been a 'black box' in sediment ecology studies, where invertebrate effects on ecosystem functioning are typically determined by measuring changes in nutrient concentrations (Ieno *et al.*, 2006; Solan *et al.*, 2008). Building ecological complexity therefore requires analysis of this missing microbial link, but it is still important to combine this with biogeochemical measures. For example, the nutrient data presented in Chapters 3 & 4 confirmed the functional relevance of observed increases in ammonia-oxidiser abundance and activity because of the concomitant increase in NO_2^- and NO_3^- production. The key to opening the microbial 'black box' associated with sediment nitrogen cycling is to examine each step of the functional link, from invertebrate activity, to altered microbial community composition and activity, and the subsequent impact on functioning.

6.6. Wider implications

This thesis aimed to improve understanding of sediment invertebrate-microbe interactions, with a particular focus on *Hediste diversicolor* as a model sediment organism that can reach densities of 3700 individuals m^{-2} (Scaps, 2002). Populations of this density could ingest 5 kg dw sediment m^{-2} $year^{-1}$ (Cammen, 1980), release over 2.7 µmol N_2O m^{-2} day^{-1} (Heisterkamp *et al.*, 2010) and secrete 15 g m^{-2} day^{-1} mucopolysaccharide that will contribute 50 mg N m^{-2} day^{-1} (Chapter 3). The scale of these activities coupled with the

data presented throughout this thesis demonstrate the substantial effects that gut transit and mucus secretion could have on sediment biogeochemistry, and that excluding these traits from sediment models could lead to misinterpretations of nitrogen processing regulation. This is particularly important when considering that polychaete bait fisheries, which account for 121,000 tonnes globally each year, are largely unregulated (Watson *et al.*, 2017). More knowledge of polychaete populations is required to support effective management (Cole *et al.*, 2018), and to understand how the removal of wild populations or cultivation of aquaculture populations may alter sediment functioning.

Understanding factors that regulate sediment nitrogen cycling is also important because of the effects that this process can have on key ecosystem services. Chapters 3 & 4 indicate that NO_2^- and NO_3^- production rates are affected by the presence of mucopolysaccharide, with potential implications for nitrogen fluxes between the sediment and the overlying water column (Ieno *et al.*, 2006; Bulling *et al.*, 2010). As primary productivity in marine environments is often nitrogen limited (Elser *et al.*, 2007), this recycling of nitrogen compounds through benthic-pelagic coupling back into the water column can support water column productivity with subsequent effects on marine food webs (Boynton & Kemp, 1985; Ekeroth *et al.*, 2016). Conversely, excess reactive nitrogen introduction could be one of the most significant pollution problems in coastal areas (Howarth & Marino, 2006), and can lead to increased microbial activity and hypoxic conditions that alter sediment communities and functions (Herbert, 1999). It is possible that invertebrate activities that support nitrogen removal could help mitigate this effect (Ma *et al.*, 2015), but accurately predicting this requires a greater understanding of all invertebrate interactions with nitrogen cycling microbial communities.

The findings reported in this thesis could have additional implications for how we consider invertebrate communities. Sediment-dwelling invertebrates have species-specific effects on nutrient fluxes and bacterial communities, which have generally been attributed to changes in bioturbation activity (Biles *et al.*, 2002; Mermillod-Blondin *et al.*, 2004; Ieno *et al.*, 2006). To more easily examine invertebrate effects on sediment processes, invertebrate taxa have been divided into functional groups based on particle reworking or ventilation traits that are assumed to have similar effects (François-Carcaillet & Poggiale, 1997; Hooper *et al.*, 2005; Kristensen *et al.*, 2012). This has culminated in the production of indices that aim to simplify community effects into a single number (Renz *et al.*, 2018; Wrede *et al.*, 2018). Data presented in this thesis, however, have demonstrated that invertebrate traits beyond bioturbation activity may play a role in regulating ecosystem functioning, and may differ between invertebrate taxa or even within populations (Chapter 5). Variations in burrow morphology can affect nitrogen cycling communities (Chapter 2)

despite this trait rarely being included in bioirrigation indices. Mucus concentration (Chapter 3 & Chapter 4) could affect the nitrification processes that are stimulated in burrow environments and so form the basis of a new set of functional groupings. As future changes to benthic communities could lead to the loss or gain of various invertebrate activities (Waters *et al.*, 2016; Kauppi *et al.*, 2018), understanding how all inter- and intra-specific variations can affect ecosystem functioning will be vital in predicting how future invertebrate communities may regulate nitrogen cycling processes.

Although this research focused primarily on nitrogen cycling, both mucus secretion and gut transit activities (Chapter 3 & Chapter 5) had whole bacterial and archaeal community effects, with implications for a broad range of ecosystem functions that are rarely considered in invertebrate trait assessments. Chapter 5 demonstrated that relative abundances of sulfate-reducing bacteria (SRB) can vary between bioturbated and non-bioturbated sediment and between internal and external polychaete assemblages (Figure 5.6). Sulfate reduction is an important function that can account for 90% of anaerobic organic matter remineralisation (Hansen *et al.*, 1996), with bioturbation activity known to alter SRB distribution and sulfate reduction rates in sediment. Yet, this has mainly been assessed through the molecular techniques discussed previously (MPN, PLFA, clone libraries) (Hines & Jones, 1985; Hansen *et al.*, 1996; Ashforth *et al.*, 2011; Vasquez-Cardenas *et al.*, 2016), and only a single study has examined species-specific invertebrate effects on SRB communities (Mermillod-Blondin *et al.*, 2005). Therefore, the trait assessments that have been used throughout this thesis could be applied to sulfate cycling to understand how this process may vary in the future and examine whether traits important in regulating nitrogen cycling regulate other biogeochemical processes.

This research could also be applied to bioremediation studies, to understand how variations in invertebrate traits may affect, or be affected by, hydrocarbon or heavy metal pollution. Hydrocarbons are widespread pollutants that accumulate in sediments (Christensen *et al.*, 2002a; Christensen *et al.*, 2002b; Peng *et al.*, 2008). Sediment-dwelling invertebrate activity can stimulate the activity of hydrocarbon-degrading bacteria and increase the removal or burial of hydrocarbons (Cuny *et al.*, 2007; Stauffert *et al.*, 2014; Taylor & Cunliffe, 2015). Few studies have examined differences in bioremediation stimulation between invertebrate taxa, and those that have did not isolate the traits responsible for promoting hydrocarbon removal or identify affected microbial groups (Christensen *et al.*, 2002b). Similarly, sediments can be significant reservoirs of heavy metals (Ciutat & Boudou, 2003; Atkinson *et al.*, 2007), with concentrations able to alter sediment invertebrate community composition due to differing tolerances between taxa (Somerfield *et al.*, 1994; Ward & Hutchings, 1996; Cronin-O'Reilly *et al.*, 2018).

Invertebrate activity can also alter the bioavailability of metals by altering sediment redox conditions, and therefore alter exposure of sediment communities to heavy metals (Amato *et al.*, 2016; Remaili *et al.*, 2016). Yet, there has been no assessment of the functional link between invertebrate activity, heavy metals, and sediment microbial communities. Bioremediation processes should therefore be examined using the techniques set out in this thesis, namely trait effect isolation, combinations of contemporary molecular techniques, and depth variations, to continue building the ecological complexity required for realistic sediment system models.

6.7. Overall limitations

In addition to those already mentioned in the data chapters of this thesis, there were some overarching limitations to this research. First, model sediment systems were used to isolate individual trait effects on microbial communities as they allow high levels of environmental control (Benton *et al.*, 2007; Papaspyrou *et al.*, 2007). As with other model systems, it is not clear whether these controlled experiments can be effectively scaled up to complex, natural sediment systems (Duffy, 2009) because they generally mimic average environmental conditions and cannot examine all possible interactions between organisms (Snelgrove *et al.*, 2014). This reduces the total applicability of results to real world situations. Model sediment systems also require sieved sediments to ensure homogenous initial conditions, which can subsequently affect grain size distribution and lead to some loss of microbial groups (Gilbert *et al.*, 1995; Bonaglia *et al.*, 2013). All of these factors should be considered when examining these results, but the overall aim was not to mimic natural systems but to improve mechanistic understanding of sediment biogeochemical cycling (Bowen *et al.*, 2014).

The same logic can be applied to the use of artificial irrigation systems. Burrow ventilation activity is formed of complex variations in irrigation periodicity, rate, and direction (Solan *et al.*, 2019), while artificial irrigation (Chapter 2) tends to be a simplified continuous flow that is controllable and reproducible (Matsui *et al.*, 2004). These systems also separate burrow ventilation from other invertebrate activities (Quintana *et al.*, 2007; Solan *et al.*, 2008), which may form complex synergistic interactions with irrigation that are complicated to predict (Chapter 4). Microbial communities may respond differently to this complexity, and so future research should focus on ground-truthing these burrow morphology findings to make applicable conclusions that can be extrapolated to natural systems. Trait isolation is necessary, however, to determine whether individual trait

variations are important regulators of functioning and whether they should be investigated further or included in sediment models.

This thesis has focused predominantly on *Hediste diversicolor*, with no assessment of how differences in mucus composition or gut transit between invertebrate taxa may help to shape observed differences in burrow communities (Papaspyrou *et al.*, 2006; King, 2018). The examination of inter-species or inter-population effects is needed to effectively understand natural invertebrate communities and the importance of different traits. There was also limited spatiotemporal resolution, with sediment communities collected exclusively from the area surrounding Plymouth (UK), which makes it difficult to examine how location can mediate trait effects. For instance, temperature changes can affect nutrient release from bioturbation activity, and alter the structure of denitrifying communities (Bulling *et al.*, 2010; Lee & Francis, 2017). Similarly, as mentioned previously, hydrocarbon and heavy metal concentrations can alter the composition of invertebrate and microbial communities (Somerfield *et al.*, 1994; Ward & Hutchings, 1996; Taylor & Cunliffe, 2015), necessitating comparisons between both less and more polluted areas. Finally, *H. diversicolor* behaviour is plastic and will vary with context. Individuals are able to switch between deposit and suspension feeding depending on phytoplankton concentrations in the overlying water column (Riisgard, 1991). This means that transitory gut communities, mucus production rates and potentially mucus composition could vary with changing environmental conditions and seasons.

As this thesis generally focused on nitrification, nitrogen cycling measurements could be considered limited. Measurements of N_2 or N_2O to examine denitrification rates (Gilbert *et al.*, 1995; Na *et al.*, 2008), and ^{15}N tracers to examine the extent of coupled nitrification-denitrification (Sørensen, 1978; Jenkins & Kemp, 1984), would have provided a more complete picture of changing nitrogen cycling processes. Additionally, a subset of nitrogen cycling functional genes (*amoA* and *nirS*) were focused on throughout this research for consistency. This misses potential effects on other significant nitrogen cycling microbial groups that could be affected by invertebrate processes, such as comammox bacteria (Pjevac *et al.*, 2017; Yu *et al.*, 2018) or anammox bacteria (Strous *et al.*, 1999; Thamdrup & Dalsgaard, 2002; Laverock *et al.*, 2014). Changes in bacterial *nirK* gene abundance, which is functionally equivalent but structurally distinct from the bacterial *nirS* gene (Braker *et al.*, 2000), were also not assessed as several studies have shown that bacterial *nirS* genes are typically more abundant and diverse than *nirK* genes (Abell *et al.*, 2010: Smith *et al.*, 2015; Lee & Francis, 2017). Further insight into bacterial denitrifying community structure may have been obtained by examining both genes.

Finally, the results presented in this thesis are predominantly based on Q-PCR and high-throughput sequencing techniques. Although each Q-PCR was performed and reported according to MIQE guidelines (Bustin *et al.*, 2009), the technique is inherently subjective and accuracy can largely depend on sample preparation, standard quality, and gene choice (Klein, 2002). This is highlighted by the issues encountered in Chapter 4 that resulted in an incomplete dataset in an attempt to avoid potentially misleading results. To mitigate these limitations, I selected primers that had been used consistently within the sediment ecology literature and predominantly focused on differences in relative abundance between sample groups, rather than on actual measurements. Meanwhile, high-throughput sequencing can provide large amounts of data that allow for whole community assessment. Yet, this is reliant on incomplete sequence databases, especially for archaea, and there is a risk of simply examining everything rather than remaining hypothesis-driven (Prosser *et al.*, 2007).

6.8. Future directions

6.8.1. Increased links to natural sediment communities

One future step for this research would be to further link these findings to natural sediment ecosystems. As stated previously, the use of continuous ventilation in burrow morphology assessments (Chapter 2) could reduce applicability. Invertebrate ventilation behaviour is intermittent, with both inter- and intra- specific variations in ventilation periodicity, rate and direction (Volkenborn *et al.*, 2012; Jovanovic *et al.*, 2014; Delefosse *et al.*, 2015). Using artificial burrow experiments to examine how these additional variations mediate burrow morphology effects on nitrifying microbial communities could help build the ecological complexity necessary for effective bioirrigation indices. Incorporating these realistic ventilation periodicities into further mucopolysaccharide isolation experiments (Chapter 4) would also provide better estimates of how specific invertebrate taxa affect degradation and nitrogen cycling within burrow environments.

This thesis also established a clear functional link between invertebrate mucopolysaccharide secretions, nitrifying microbial groups, and nitrification activity, and so further research should assess whether any specific factors of this trait contribute to variations between invertebrate taxa. Chapters 3 & 4 indicate that mucus concentration may be important, but mucus production rates and composition remain poorly characterised. Initial research should therefore quantitatively establish variations in mucus production rates, mucus lining thickness (Zorn *et al.*, 2006), and mucus composition

(Talmont & Fournet, 1990: Santos *et al.*, 2009; Stabili *et al.*, 2014) between invertebrate taxa, which are likely to alter the amount and type of organic matter entering sediment systems and potentially affect nitrifying microbial communities. These factors can then be incorporated into sediment slurry incubations, both in isolation and combination, to again add complexity to our understanding of this functional trait. Finally, to examine the significance of mucus secretion traits in relation to larger scale traits like burrow ventilation, mesocosm experiments with live *Hediste diversicolor* could be set up. By comparing changes in microbial community composition to measurements of mucus secretions, ventilation activity (Quintana *et al.*, 2007; Renz & Forster, 2013), and particle reworking (Gilbert *et al.*, 1995; Braeckman *et al.*, 2010), we could begin to determine what proportion of community variation is attributable to each trait.

The gut passage and external transport effects identified in Chapter 5 also represent understudied traits with limited understanding of inter-specific variation. Previous work has indicated that different deposit-feeding taxa can have different gut enzyme activities (Mayer *et al.*, 1997) and distinct effects on organic matter and amino acid degradation during gut passage (Woulds *et al.*, 2012). There are likely to be variations in transitory communities between invertebrate species that need to be examined by future sequencing projects before we can determine whether this trait should be included in sediment system models (King, 2018). Similarly, the community data presented in Chapter 5 demonstrate inter-individual variation, which has previously been identified in terrestrial land snails where ingested communities varied based on habitat and physiological state (Nicolai *et al.*, 2015). Investigations should therefore also examine, within a single species, how temporal and spatial variations can mediate gut passage effects, including investigations of the same invertebrate population over multiple seasons and multiple years and differing populations located across the UK and Europe (Wohlgemuth *et al.*, 2017). Similarly, although Chapter 5 demonstrates the presence of a unique transitory gut community, the functional impacts of this are difficult to determine without understanding what proportion of the community is introduced to the sediment. This could be done by simply assessing faecal cast community composition (King, 2018), though significantly more insight could be achieved by examining the activity of faecal cast communities. By determining which taxonomic and functional groups remain active during gut transit, we can begin to predict which products may be exported to the sediment during excretion (e.g. NO_2^-, NO_3^-, N_2O).

6.8.2. Expanding biogeochemical and molecular microbial assessments

In this thesis, trait assessments were examined in the context of a specific set of nitrogen cycling processes, but trait effects may vary between different nitrogen cycling pathways and this should be addressed in future work. As mentioned previously, additional nitrogen cycling functional genes could be used to examine abundance and activity changes in comammox, anammox and DNRA communities. Trait effects could also be better linked to changes in sediment process rates by analysing a greater range of nitrogen compounds, such as N_2 release as a proxy for denitrification or by using acetylene to inhibit nitrification or denitrification (Nielsen *et al.*, 2004; Gilbert *et al.*, 2016). Both of these methods would provide a more complete understanding of changing nitrogen pathways in the presence of different invertebrate taxa. Previous work has also used isotopic tracers (^{15}N) to examine partitioning between denitrification, DNRA, and anammox in sediment systems (Sørensen, 1978; Thamdrup & Dalsgaard, 2002; Gilbert *et al.*, 2016), and to assess coupling between nitrogen cycling processes (Gongol & Savage, 2016). Combining this with recent advances, like nanoSIP (nanometre-scale stable isotope probing) or nanoSIMS (nanometre-scale secondary ion mass spectrometry), would allow us to examine how mucopolysaccharide production or burrow morphology affect metabolic interactions between organisms and track nitrogen compound uptake (Musat *et al.*, 2016; Berthelot *et al.*, 2018). In this way, coupled nitrification-denitrification could be examined in detail in relation to invertebrate traits. These techniques could also help answer questions about how organic matter (carbon and nitrogen) released from mucopolysaccharide breakdown is used by sediment microbial communities: is mucus a source of nitrogen to fuel nitrification and/or a 'stimulator' for degradation of existing sediment nitrogen?

Further targeted molecular techniques could also improve understanding of trait effects. In sediment environments, sequencing of specific functional genes (e.g. *amoA* and *nirS*) has been used to look at spatiotemporal changes in nitrogen cycling group diversity in relation to various environmental factors (Abell *et al.*, 2010; Foshtomi *et al.*, 2015; Lee & Francis, 2017; Alves *et al.*, 2018). These techniques have rarely been applied to sediment invertebrate studies (Stauffert *et al.*, 2014) to assess nitrogen cycling community responses to invertebrate traits and determine whether these responses are uniform across microbial functional groups. These assessments could be further expanded by the use of metatranscriptomics, to examine whole community activity through gene regulation. There is a general need for quantitative data assessing relationships between biogeochemical cycling and sediment microbial community structure (Bowen *et al.*, 2014; Gilbert *et al.*, 2016). Targeted, hypothesis-driven '-omics'

research may allow us to develop this functional understanding of microbial roles in sediment nitrogen cycling processes.

Finally, future research could expand the findings presented in this thesis by combining molecular techniques with the latest sediment ecology methods. CT (computed tomography) and PET/CT (positron emission tomography/computed tomography) scans are now being used to non-invasively and precisely analyse variations in burrow morphology (Hale *et al.*, 2014), and to examine high-resolution spatiotemporal variations in burrow ventilation (Delefosse *et al.*, 2015). Similarly, planar optodes can be used to visualise the vertical and horizontal distribution of O_2 throughout a sediment system over time, to assess changing sediment-water exchange (Volkenborn *et al.*, 2010; Pischedda *et al.*, 2012; Volkenborn *et al.*, 2012). These high-resolution snapshots of invertebrate burrow systems could allow us to target sampling at specific areas of interest, and therefore more directly examine the role of ventilation or burrow morphology on specific microbial groups of interest or whole community composition. Overall, combining these latest advances in molecular and sediment ecology measurements into invertebrate trait assessments will further develop our mechanistic understanding of invertebrate-microbe interactions and their role in biogeochemical processes.

Appendix I: Chapter 2 Supplementary Information

Statistical Information

Summary of statistical models (Model I.1 to I.15), for bacterial (Models I.1-3) and archaeal (Models I.4-6) *amoA* DNA abundance, bacterial (Models I.7-9) and archaeal (Models I.10-12) *amoA* gene expression, and AOB:AOA (Models I.13-15). For each model, we list the initial linear regression model and the minimal adequate model. As we used all Q-PCR replicates (n = 6), we included replicate as a random effect. Where it was necessary to account for violation of homogeneity of variance, we included an appropriate weight. Hence, where appropriate, we provide a summary of the coefficient table. The coefficients indicate the relative performance of each level relative to the baseline, as indicated. Coefficients ± SE, t-values and significance values are presented.

Model I.1: $L_{surface}$, Bacterial *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(BamoaADNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 0.00, d.f. = 1, p = 0.993)

Model I.2: L_{mid}, Bacterial *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(BamoaADNA ~as.factor(Burrow Type))

(ii) Minimal adequate model:

lme(BamoADNA~as.factor(Burrow Type), random =~ 1 | Replicate, method="ML")

Table I.1 Coefficient table for Model I.2

Intercept ± SE (when baseline = closed): 8443 ± 670, t-value = 12.60, p<0.0001.

Coefficients ± SE, t-values and significance values (in parentheses) are presented.

	Blind-ended	**Open-ended**
Blind-ended	-	-6201 ± 947.9 -6.542 (0.0000654)
Open-ended	6201 ± 947.9 6.542 (0.0000654)	-

Model I.3: L_{max}, Bacterial *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(BamoaADNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 2.59, d.f. = 1, p = 0.107)

Model I.4: $L_{surface}$, Archaeal *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(AamoaADNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 0.01, d.f. = 1, p = 0.908)

Model I.5: L_{mid}, Archaeal *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(AamoaADNA ~as.factor(Burrow Type))

(ii) Minimal adequate model:

lme(AamoADNA~as.factor(Burrow Type), random =~ 1 | Replicate, method="ML")

Table I.2 Coefficient table for Model I.5

Intercept ± SE (when baseline = closed): 11377 ± 1899, t-value = 5.991, p = 0.0001.

Coefficients ± SE, t-values and significance values (in parentheses) are presented.

	Blind-ended	**Open-ended**
Blind-ended	-	-9042 ± 2686 -3.367 (0.00716)
Open-ended	9042 ± 2686 3.367 (0.00716)	-

Model I.6: L_{max}, Archaeal *amoA* DNA abundance (copies.mgwwsediment^{-1})

(i) Initial linear regression model:

lm(AamoaADNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 1.27, d.f. = 1, p = 0.260)

Model I.7: $L_{surface}$, Bacterial *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:
lm(BamoaARNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 1.71, d.f. = 1, p = 0.191)

Model I.8: L_{mid}, Bacterial *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:
lm(BamoaARNA ~as.factor(Burrow Type))

(ii) Minimal adequate model:

lme(BamoARNA~as.factor(Burrow Type), random =~ 1 | Replicate,
weights = varIdent(form = ~ 1|as.factor(Burrow Type)), method="ML")

Table I.3 Coefficient table for Model I.8

Intercept ± SE (when baseline = closed): 2.705 ± 9.903, t-value = 0.273, p = 0.790.
Coefficients ± SE, t-values and significance values (in parentheses) are presented.

	Blind-ended	**Open-ended**
Blind-ended	-	24.637 ±14.005 1.759 (0.109)
Open-ended	-24.637 ±14.005 -1.759 (0.109)	-

Model I.9: L_{max}, Bacterial *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:
lm(BamoaARNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 0.30, d.f. = 1, p = 0.581)

Model I.10: $L_{surface}$, Archaeal *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:
lm(AamoaARNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 1.32, d.f. = 1, p = 0.251)

Model I.11: L_{mid}, Archaeal *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:

lm(AamoaARNA ~as.factor(Burrow Type))

(ii) Minimal adequate model:

lme(AamoARNA~as.factor(Burrow Type), random =~ 1 | Replicate,
weights = varIdent(form = ~ 1|as.factor(Burrow Type)), method="ML")

Table I.4 Coefficient table for Model I.11

Intercept ± SE (when baseline = closed): 1.747 ± 14.759, t-value = 0.118, p = 0.908.

Coefficients ± SE, t-values and significance values (in parentheses) are presented.

	Blind-ended	**Open-ended**
Blind-ended	-	33.672 ± 20.873 1.613 (0.138)
Open-ended	-33.672 ± 20.873 -1.613 (0.138)	-

Model I.12: L_{max}, Archaeal *amoA* gene expression (DNA:cDNA abundance)

(i) Initial linear regression model:
lm(AamoaARNA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 2.09, d.f. = 1, p = 0.148)

Model I.13: $L_{surface}$, Bacterial:Archaeal *amoA* DNA abundance

(iii) Initial linear regression model:
lm(AOB:AOA ~as.factor(Burrow Type))

(iv) No minimal adequate model, intercept only (L-ratio = 0.58, d.f. = 1, p = 0.446)

Model I.14: L_{mid}, Bacterial:Archaeal *amoA* DNA abundance

(i) Initial linear regression model:
lm(AOB:AOA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 1.17, d.f. = 1, p = 0.280)

Model I.15: L_{max}, Bacterial:Archaeal *amoA* DNA abundance

(i) Initial linear regression model:
lm(AOB:AOA ~as.factor(Burrow Type))

(ii) No minimal adequate model, intercept only (L-ratio = 0.68, d.f. = 1, p = 0.410)

Appendix II: Chapter 3 Supplementary Information

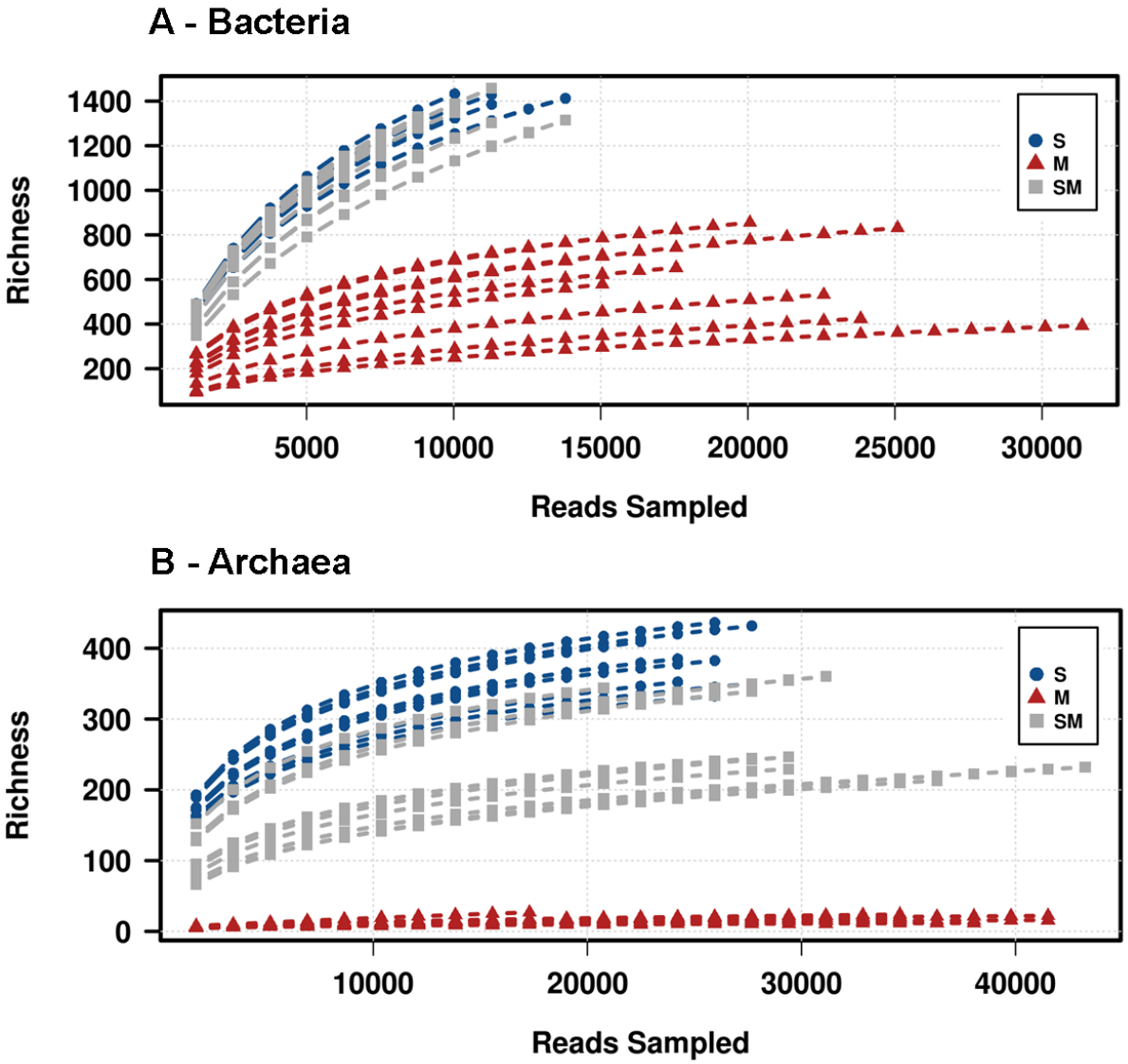

Figure II.1 Rarefaction curves for the bacterial (A) and archaeal (B) community.

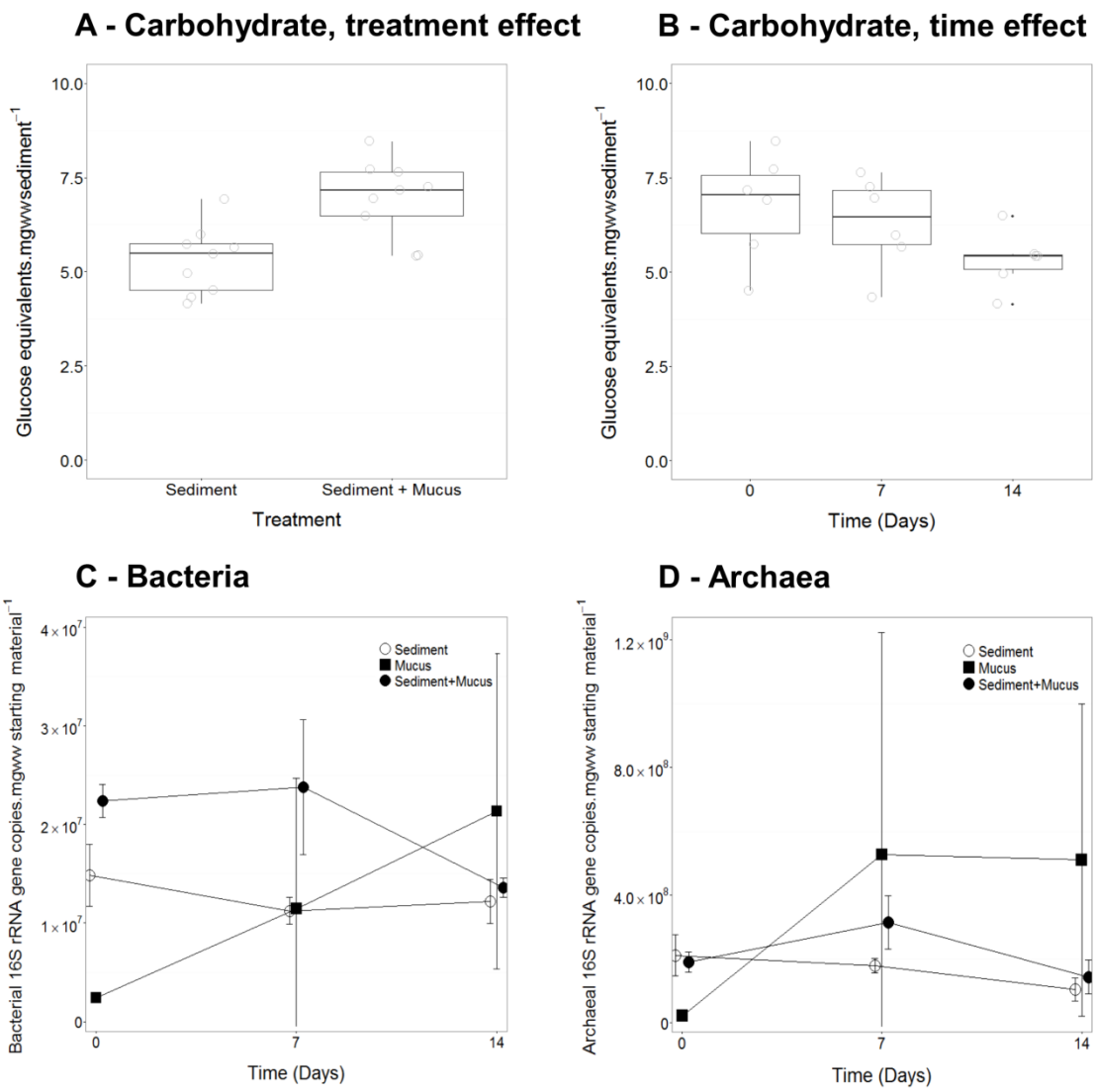

Figure II.2 Variations in sediment carbohydrate concentration and bacterial and archaeal abundance under varying mucus concentrations over time. Sediment carbohydrate concentration in mucus amended and non-mucus amended sediment treatments (A) (n = 9), and averaged over both treatments at each time point (B) (n = 6). Boxplot indicates median, 25% and 75% quartiles, and 95% confidence interval values. Impact of mucopolysaccharide on bacterial (C) and archaeal (D) 16S rRNA gene copy numbers. Data shown are means ± SD (n = 3 for mucus amended and non-mucus amended sediment slurries, except mucus treatment n = 2).

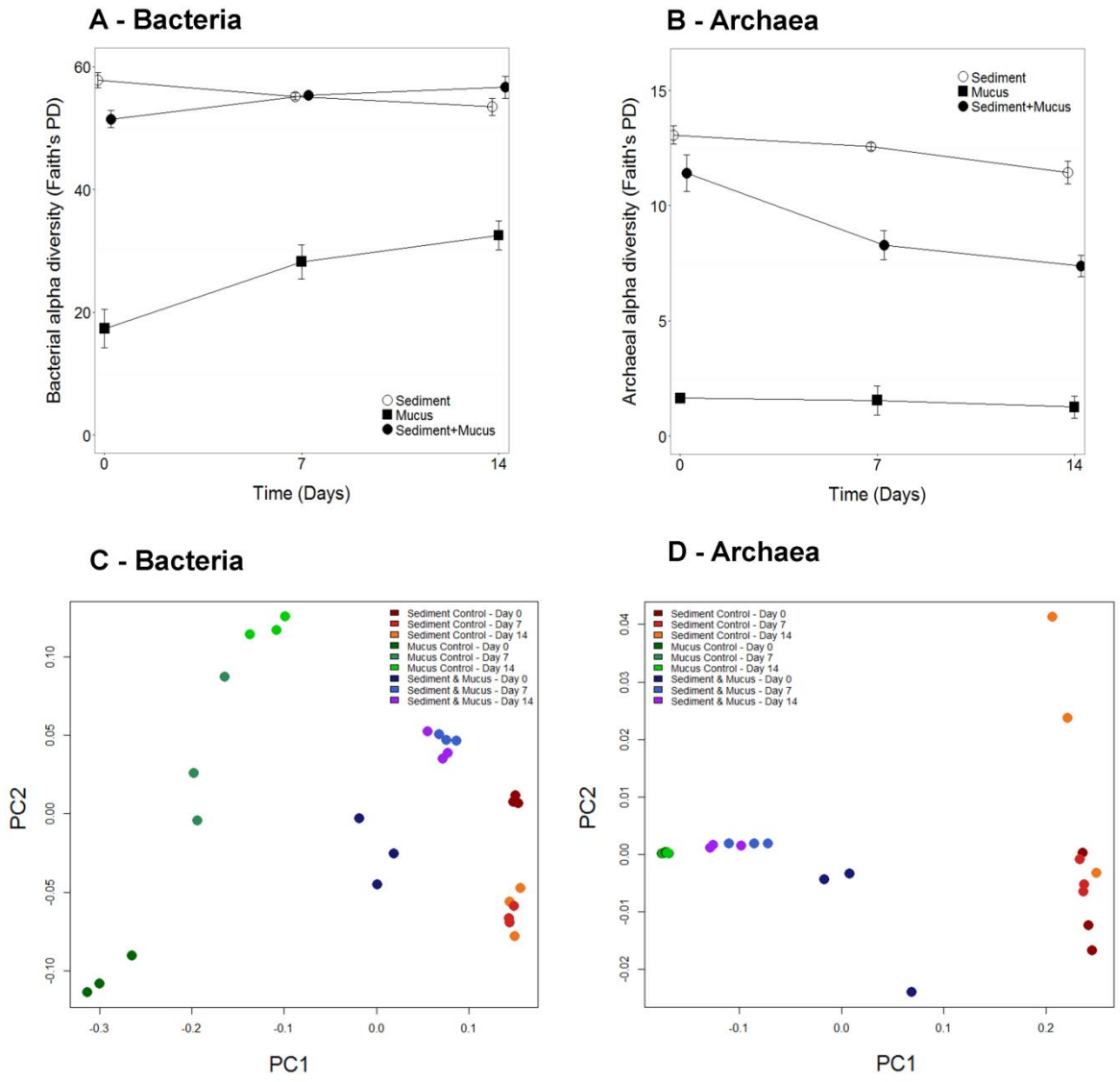

Figure II.3 Variations in bacterial and archaeal community structure in the presence of mucopolysaccharide

Impact 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.

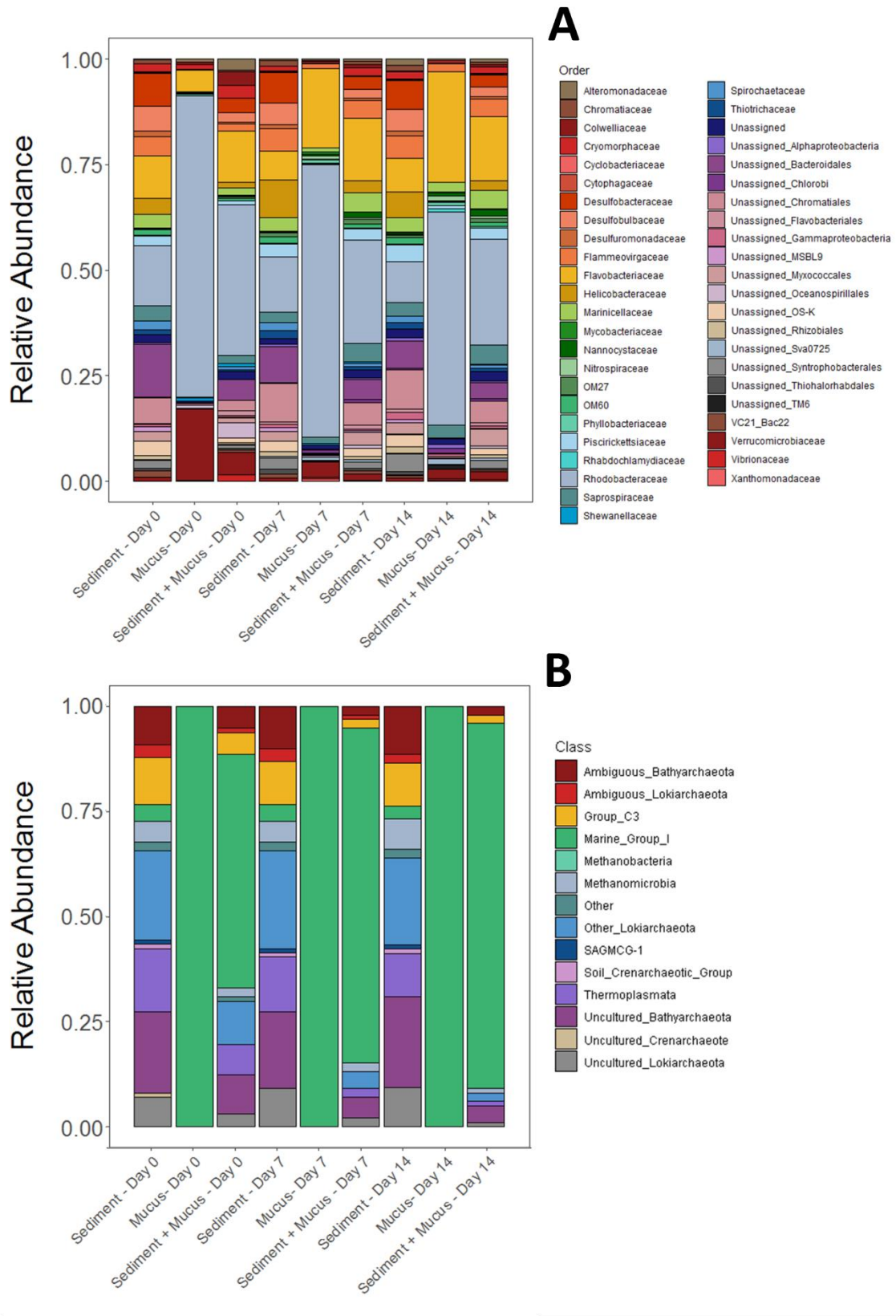

Figure II.4 Mean relative abundance of bacterial families (A) and archaeal classes (B). > 1% relative abundance in at least one sample for each of the three treatments on day 0, 7, and 14 of the incubation period (n = 3).

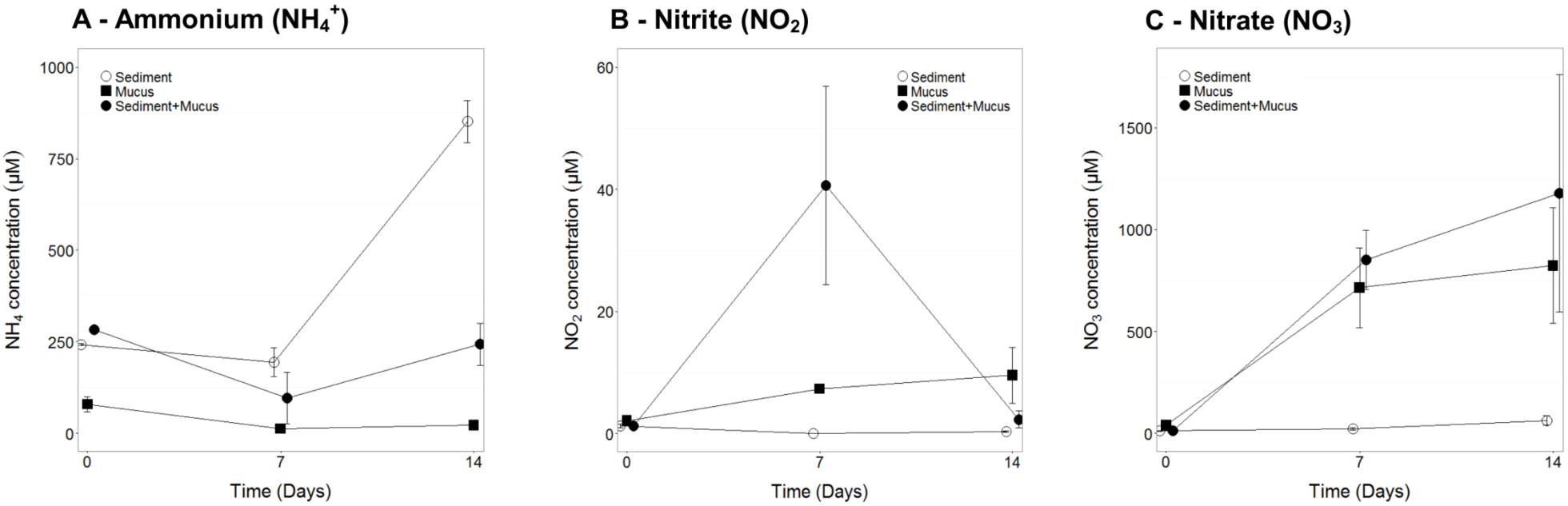

Figure II.5 Variations in ammonium (A), nitrite (B), and nitrate (C) concentrations in the presence of mucopolysaccharide

Data shown are means ± SD (n = 3, except sediment Day 0 and sediment + mucus Day 7 n = 2).

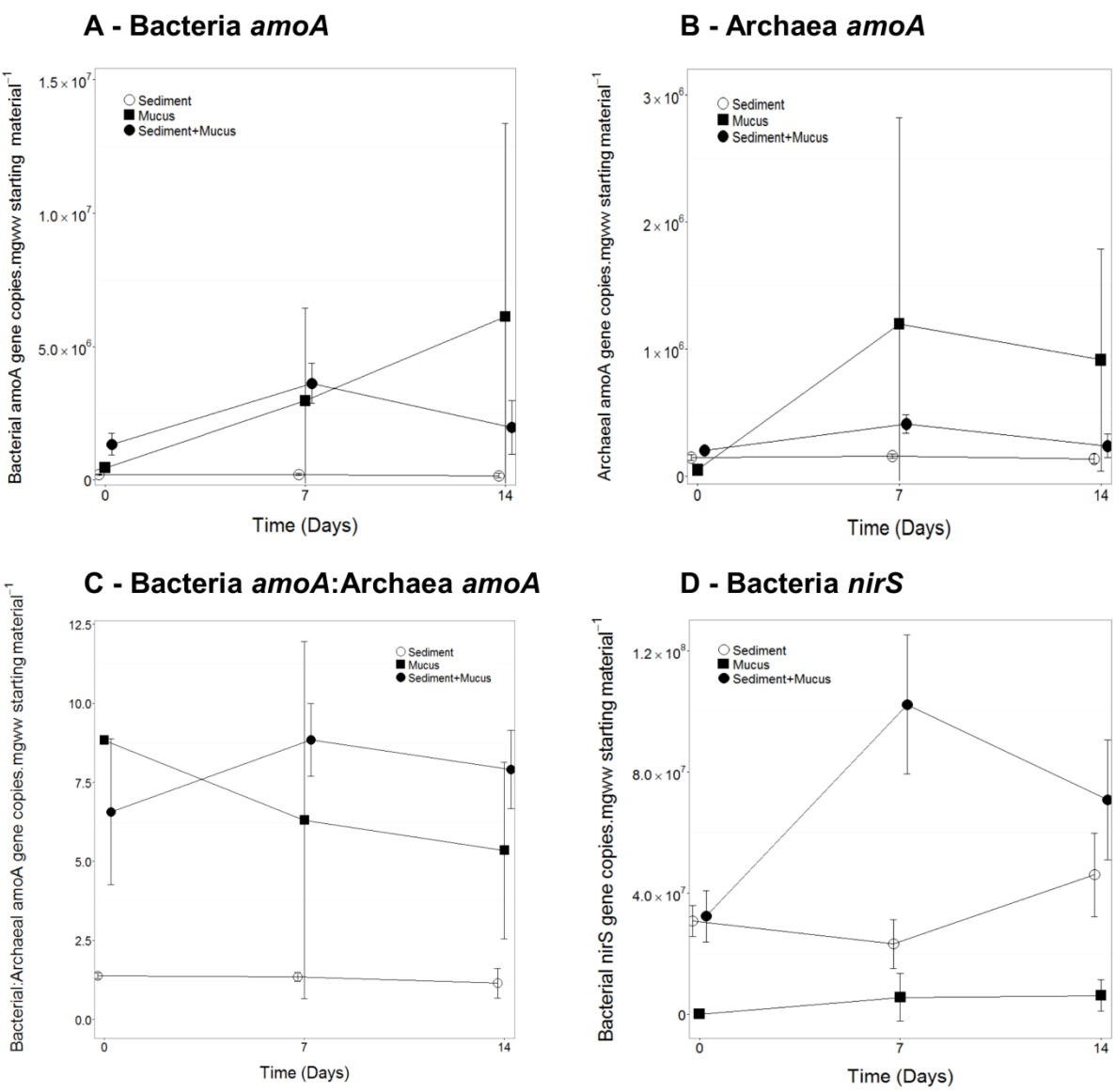

Figure II.6 Variations in nitrogen cycling functional gene abundance in the presence of mucopolysaccharide.

Bacterial *amoA* (A), archaeal *amoA* (B), bacterial:archaeal *amoA* copy ratio (C), and bacterial *nirS* (D). Data shown are means ± SD (n = 3).

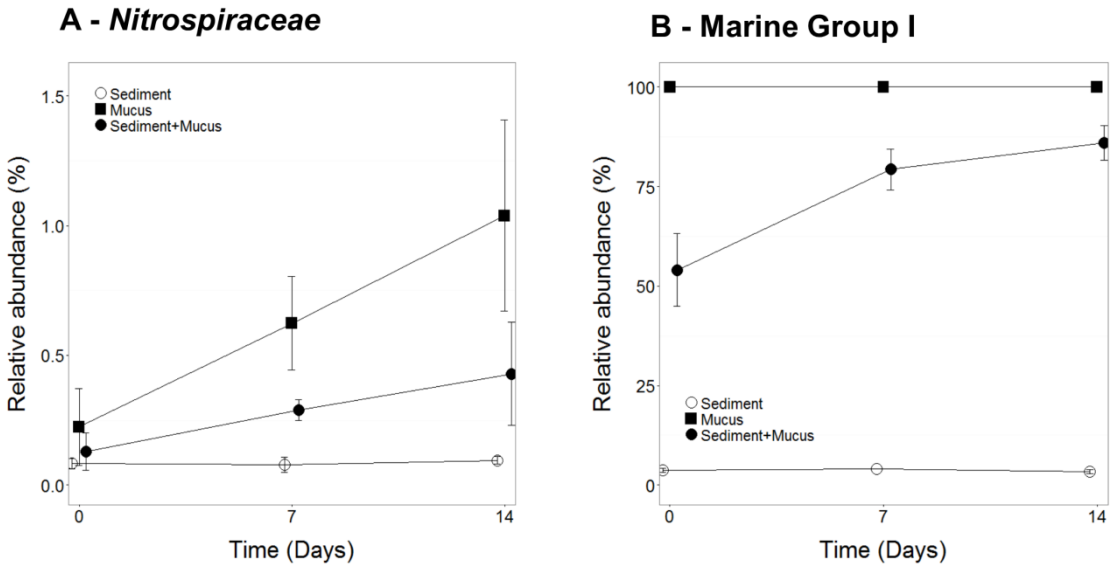

Figure II.7 Percentage of the bacterial community that belongs to the family of *Nitrospiraceae* (A), and the percentage of the archaeal community that belongs to the class Marine Group I (B).

Shown are the means ± SD (n = 3, except mucus treatment n=2).

Statistical Information

Summary of statistical models (Model II.1 to II.14). For each model, we list the initial linear regression model and the minimal adequate model. As bottles were sampled from repeatedly over time, we included botte ID as a random effect. Where it was necessary to account for violation of homogeneity of variance, we included an appropriate weight. Hence, where appropriate, we provide a summary of the coefficient table. The coefficients indicate the relative performance of each level relative to the baseline, as indicated. Coefficients ± SE, t-values and significance values are presented.

Model II.1: Carbohydrate concentration

(i) Initial linear regression model:

lm(Carbohydrate ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Carbohydrate~as.factor(Mucus)+as.factor(Time), random =~ 1 | BottleID, method="ML")

Model II.2: Bacterial 16S rRNA gene abundance

(i) Initial linear regression model:

lm(Bacterial 16S rRNA ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Bacterial 16S rRNA~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Time)), method="ML")

Model II.3: Archaeal 16S rRNA gene abundance

(i) Initial linear regression model:

lm(Archaeal 16S rRNA ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Archaeal 16S rRNA~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, method="ML")

Model II.4: Bacterial diversity

(i) Initial linear regression model:

lm(Bacterial diversity ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Bacterial diversity~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Time)), method="ML")

Model II.5: Archaeal diversity

(i) Initial linear regression model:

lm(Archaeal diversity ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Archaeal diversity~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, method="ML")

Model II.6: Ammonium (NH_4^+) (µM)

(i) Initial linear regression model:

lm(NH_4^+ ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(NH_4^+~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Time)), method="ML")

Model II.7: Nitrite (NO_2^-) (µM)

(i) Initial linear regression model:

lm(NO_2^- ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(NO_2^-~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus)), method="ML")

Model II.8: Nitrate (NO_3^-) (µM)

(i) Initial linear regression model:

lm(NO_3^- ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(NO_3^-~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus)*as.factor(Time)), method="ML")

Model II.9: Bacterial *amoA* abundance

(i) Initial linear regression model:

lm(AOB ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(AOB~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

Model II.10: Archaeal *amoA* abundance

(i) Initial linear regression model:

lm(AOA ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(AOA~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

Model II.11: Bacterial:Archaeal *amoA* abundance

(i) Initial linear regression model:

lm(AOB:AOA ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(AOB:AOA~as.factor(Mucus), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

Model II.12: Bacterial *nirS* abundance

(i) Initial linear regression model:

lm(nirS ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(nirS~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, method="ML")

Model II.13: *Nitrospiraceae* relative abundance

(i) Initial linear regression model:

lm(Nitro ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(Nitro~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

Model II.14: Marine Group I relative abundance

(i) Initial linear regression model:

lm(MGI ~as.factor(Mucus)*as.factor(Time))

(ii) Minimal adequate model:

lme(MGI~as.factor(Mucus)*as.factor(Time), random =~ 1 | BottleID, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

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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.

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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

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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

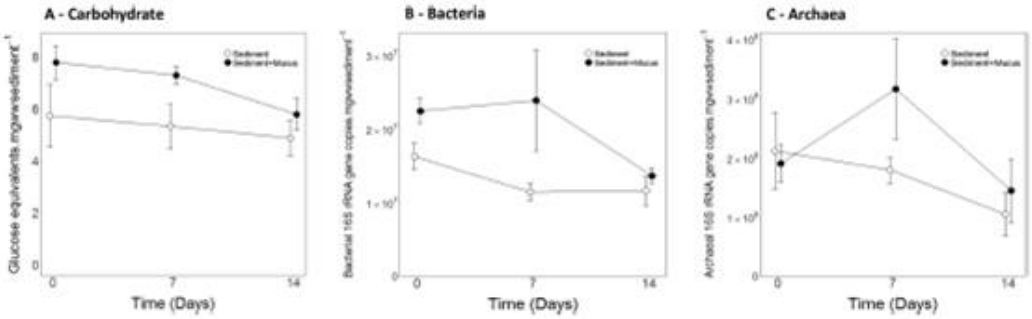

Figure 1. The effect of mucopolysaccharide on sediment carbohydrate concentration (A), and bacterial (B), and archaeal (C), 16S rRNA gene copy numbers. Data shown are means ± SD (n = 3).

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

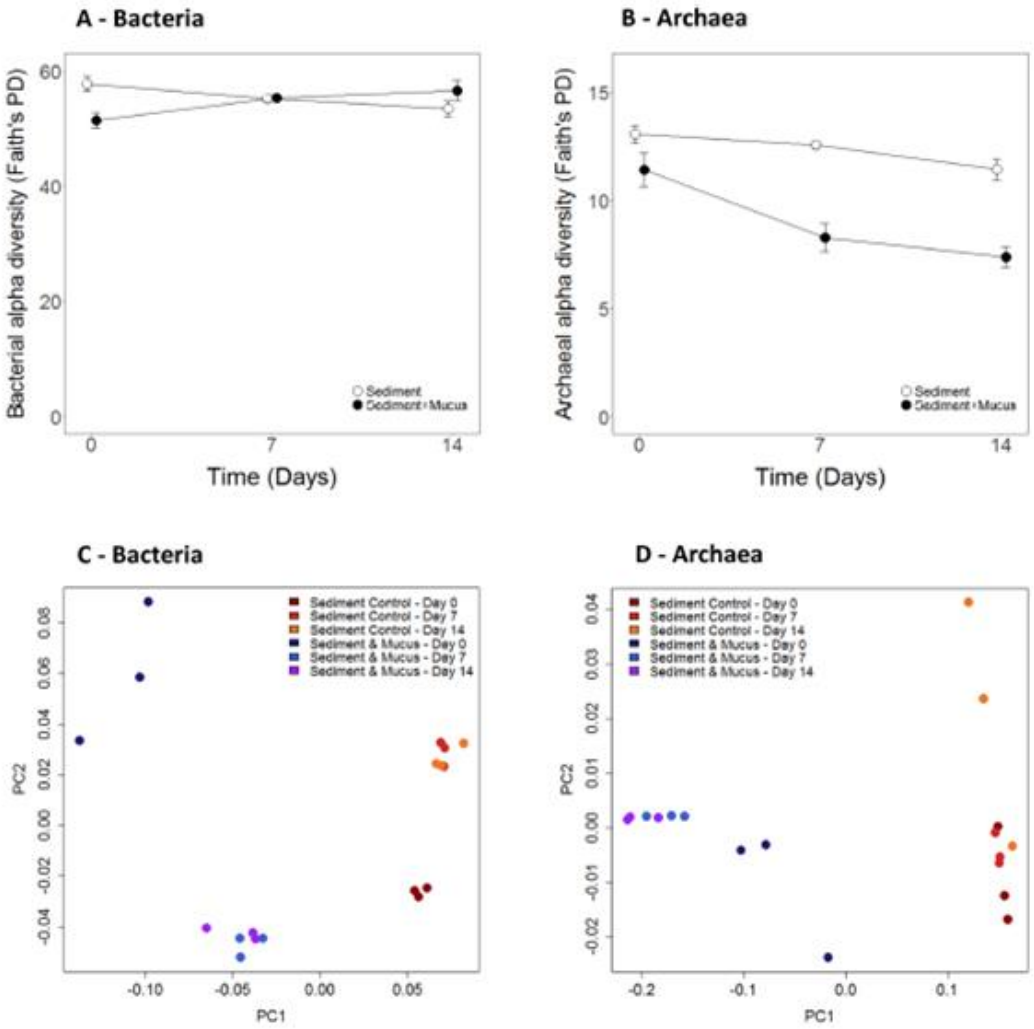

Figure 2. The effect of mucopolysaccharide on bacterial (A), and archaeal (B), alpha diversity. Data shown are means ± SD (n = 3). MDS (UNIFRAC) plots of bacterial (C), and archaeal (D), community structure.

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

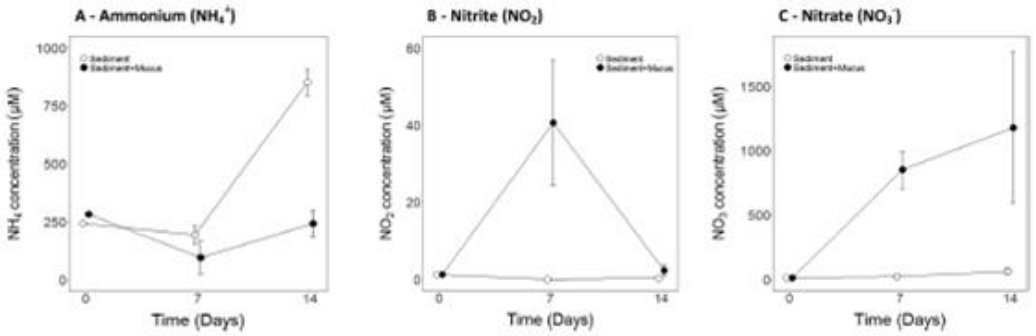

Figure 3. The effect of mucopolysaccharide on ammonium (A), nitrite (B), and nitrate concentrations (C). Data shown are means ± SD (n = 3, except sediment Day 0 and sediment + mucus Day 7 n = 2).

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

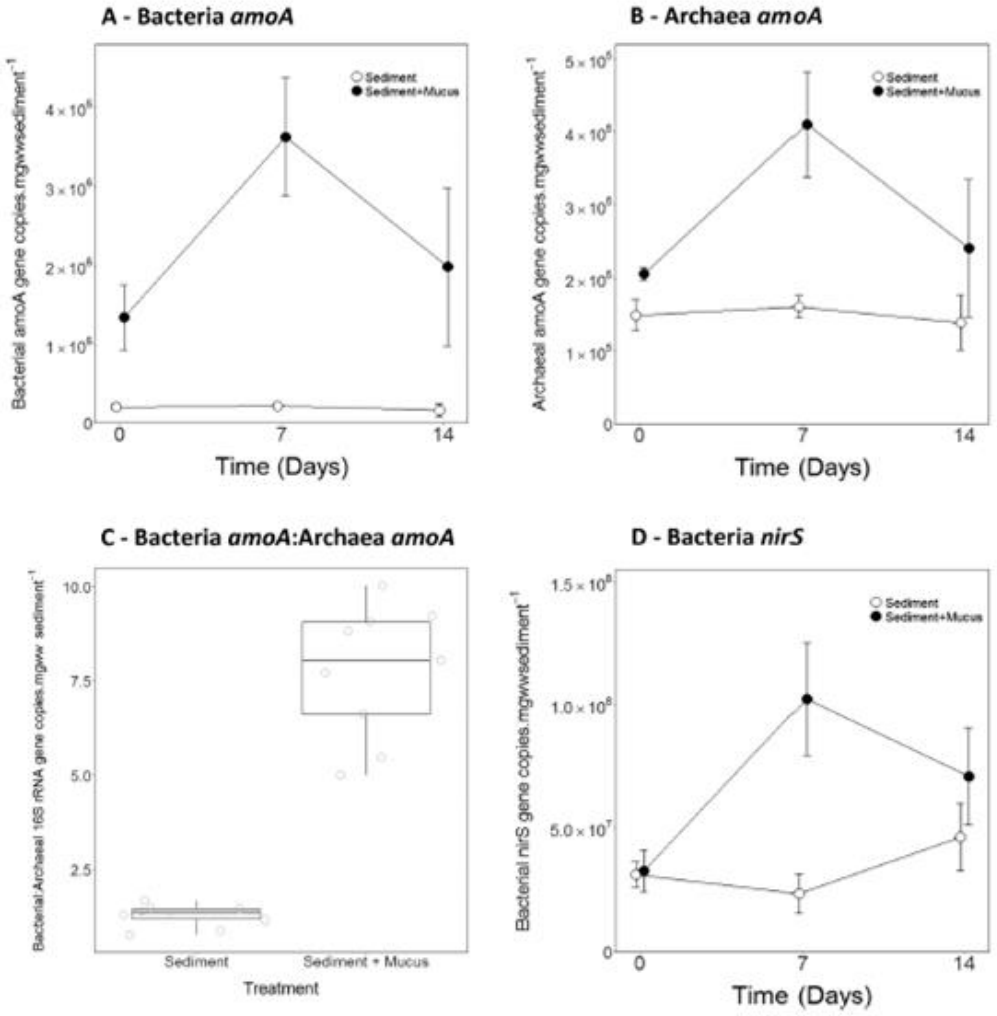

Figure 4. Variations in nitrogen cycling functional gene abundance in the presence of mucopolysaccharide. Bacterial *amoA* (A), archaeal *amoA* (B), bacterial:archaeal *amoA* copy ratio (C), and bacterial *nirS* (D). Data shown are means ± SD (n = 3).

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

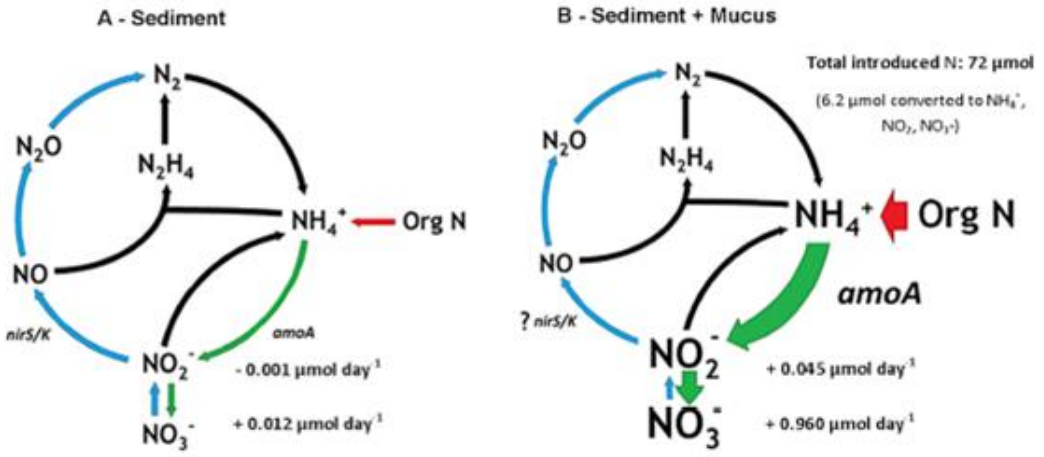

Figure 5. Summary of the proposed change in nitrogen cycling processes and microbial groups between sediments where invertebrate-derived mucopolysaccharide is (A), absent or (B), present, including rates of production for NO_2 and NO_3 between day 0 and day 7 of the incubation (Adapted from Jetten 2008).

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.

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Conflicts of interest. None declared.

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Appendix III: Chapter 4 Supplementary Information

Statistical Information

Summary of statistical models (Model III.1 to III.3), for NH_4^+, NO_2^-, and NO_3^- concentration. For each model, we list the initial linear regression model and the minimal adequate model. As bottles were sampled from repeatedly over time, we included bottle ID as a random effect. Where it was necessary to account for violation of homogeneity of variance, we included an appropriate weight. Hence, where appropriate, we provide a summary of the coefficient table. The coefficients indicate the relative performance of each level relative to the baseline, as indicated. Coefficients ± SE, t-values and significance values are presented.

Model III.1: NH_4^+ concentration (µM)

(i) Initial linear regression model:

lm(NH_4^+ ~as.factor(Mucus)* as.factor(O_2)* as.factor(Time))

(ii) Minimal adequate model:

lme(NH_4^+~ as.factor(Mucus)*as.factor(O_2), random =~ 1 | Bottle, weights = varIdent(form = ~ 1|as.factor(O_2)*as.factor(Time)), method="ML")

Model III.2: NO_2^- concentration (µM)

(i) Initial linear regression model:

lm(NO_2^- ~as.factor(Mucus)* as.factor(O_2)* as.factor(Time))

(ii) Minimal adequate model:

lme(NO_2^-~ as.factor(Mucus)*as.factor(O_2)*as.factor(Time), random =~ 1 | Bottle, weights = varIdent(form = ~ 1|as.factor(Mucus), method="ML")

Model III.3: NO_3^- concentration (µM)

(i) Initial linear regression model:

lm(NO_3^- ~as.factor(Mucus)* as.factor(O_2)* as.factor(Time))

(ii) Minimal adequate model:

lme(NO_3^-~ as.factor(O_2)*as.factor(Time), random =~ 1 | Bottle, weights = varIdent(form = ~ 1|as.factor(O_2)*as.factor(Time)), method="ML")

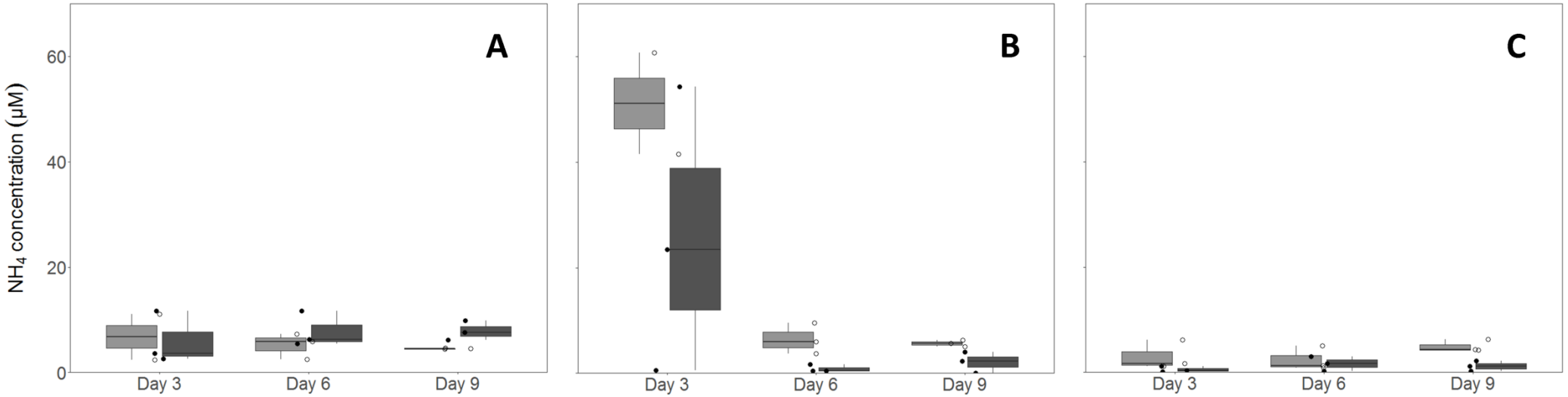

Figure III.1 - Variations in NH_4^+ concentration in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on NH_4^+ concentration under oxic (A; n = 3, except Sediment-only Day 3 n = 2), anoxic (B; n = 3 except Sediment-only Day 3 n = 2), and oscillating (C; n = 3 except Sediment-mucus Day 3 n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

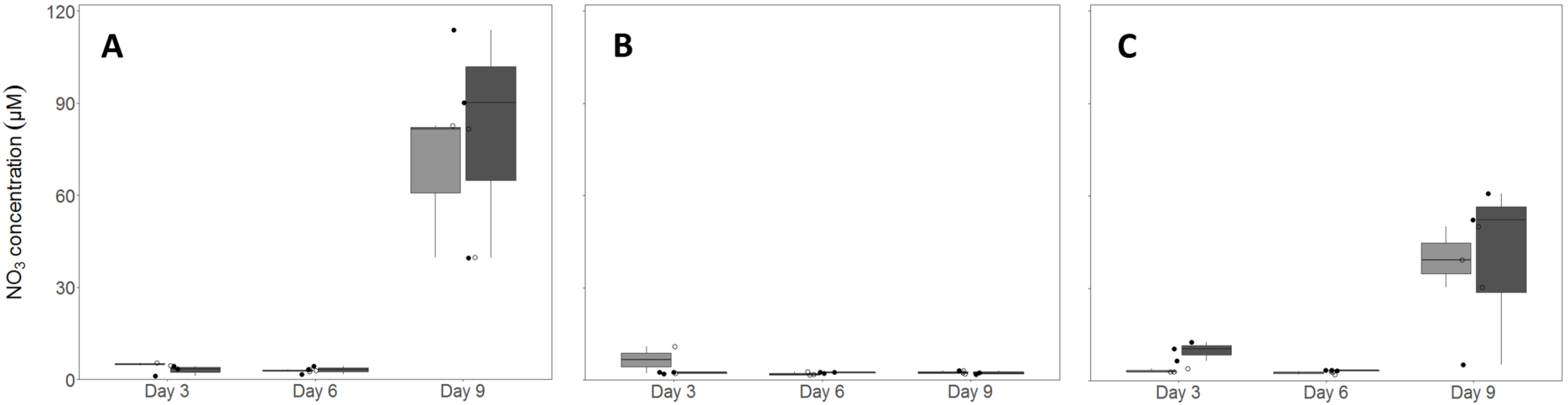

Figure III.2 - Variations in NO_3^- concentration in the presence of mucus under varying redox conditions over time.

The effect of mucopolysaccharide (Sediment-only: light grey, open circles; Sediment-mucus: dark grey, closed circles) and time on NO_3^- concentration under oxic (A; n = 3, except Sediment-only Day 3 n = 2), anoxic (B; n = 3 except Sediment-only Day 3 n = 2), and oscillating (C; n = 3 except Sediment-mucus Day 3 n = 2) O_2 conditions. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread.

Appendix IV: Chapter 5 Supplementary Information

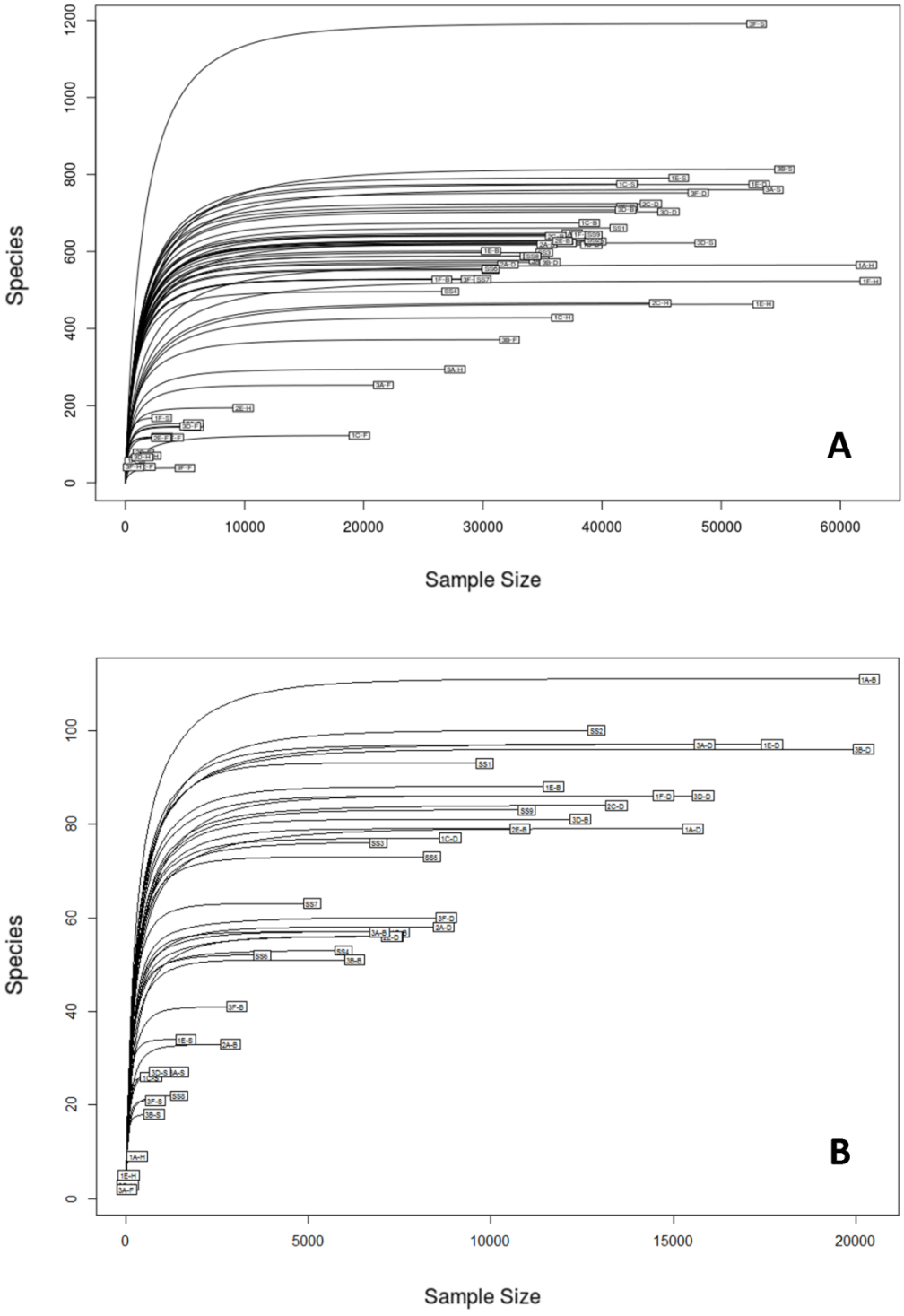

Figure IV.1 - Rarefaction curves for the bacterial (A) and archaeal (B) community.

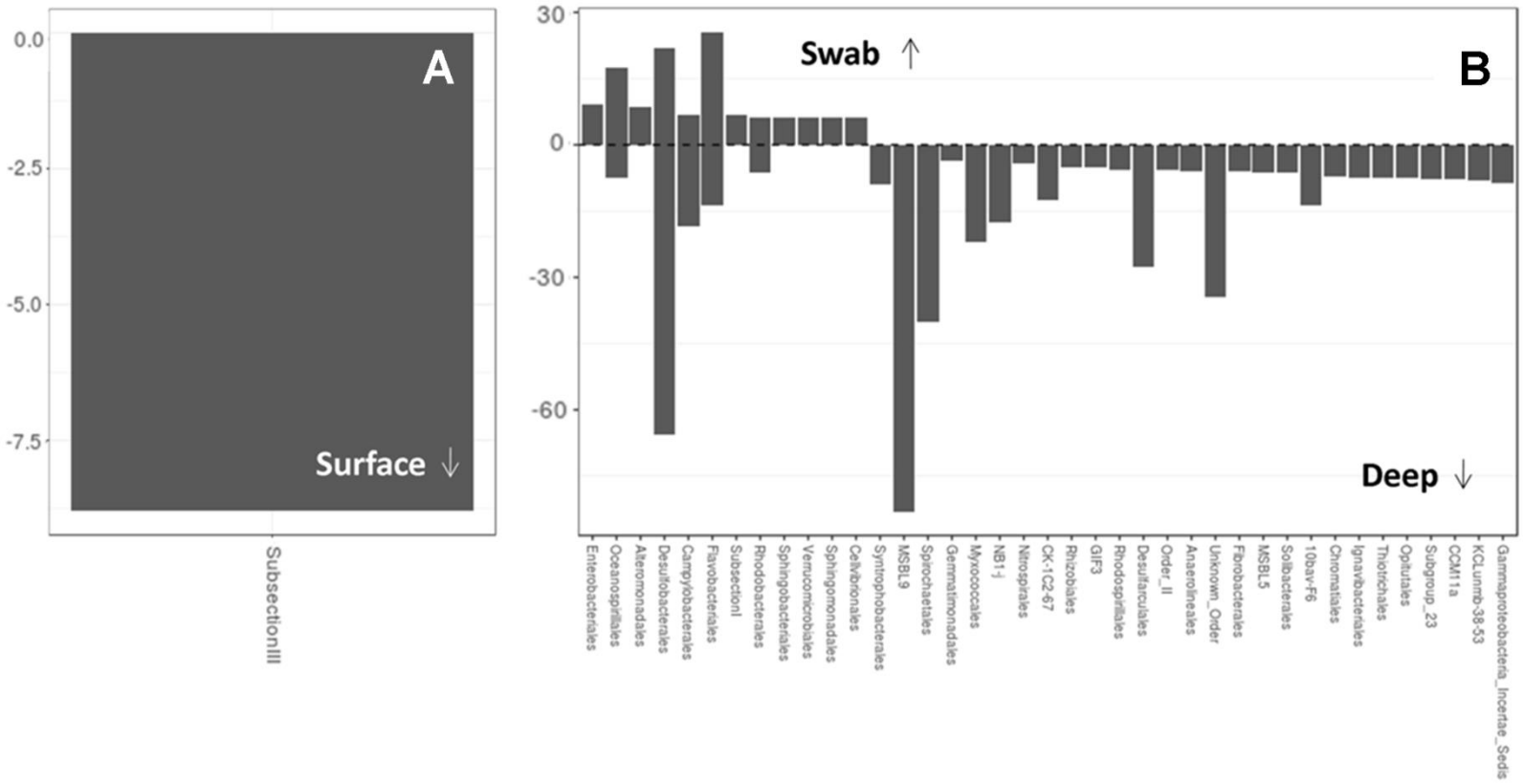

Figure IV.2 - Log2 fold change in abundance of taxa (p <0.05) between ecologically relevant combinations of samples. (A - Surface vs Swab; B – Deep vs Swab). For each combination, taxa below 0 are more abundant in first stated sample, taxa above 0 are more abundant in second stated sample.

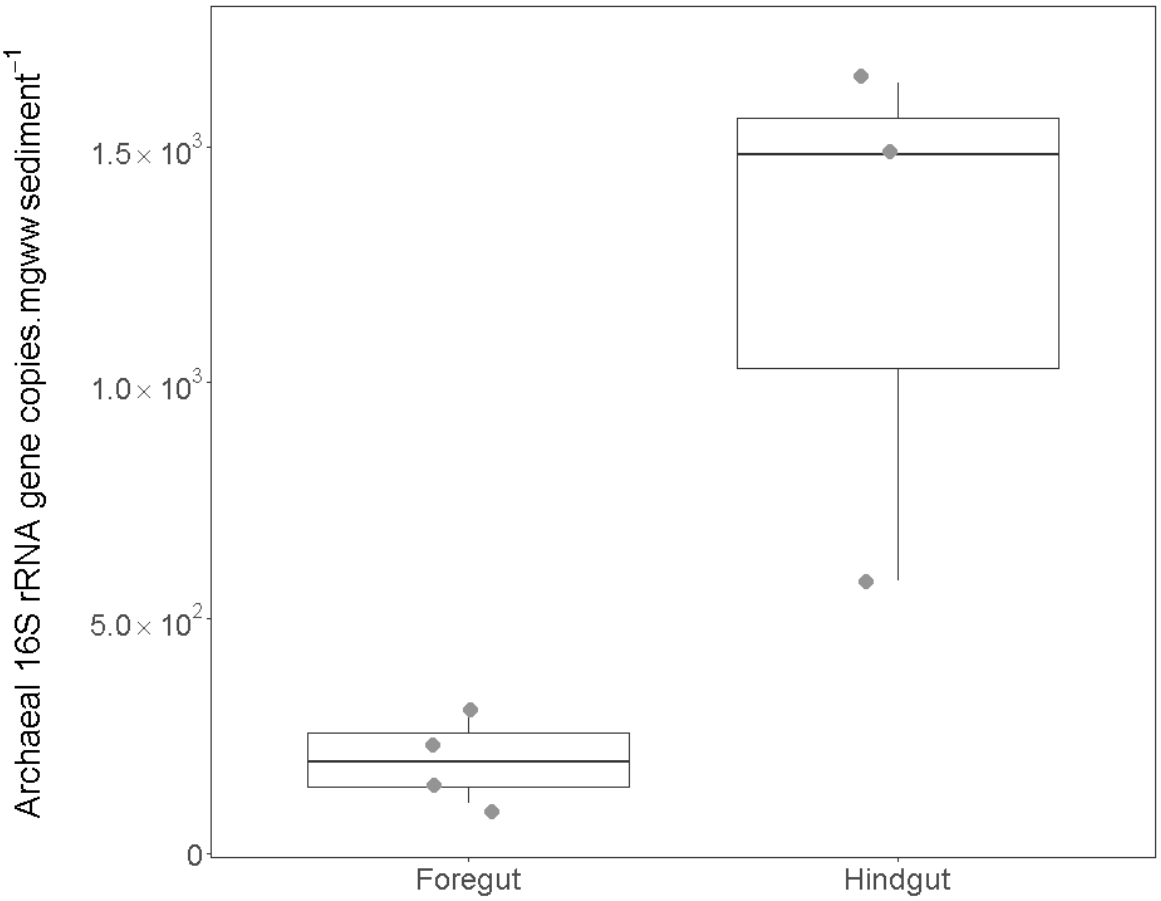

Figure IV.3 Variations in archaeal 16S rRNA gene abundance.

Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (n = 4, except hindgut n = 3). Untransformed data presented.

Table IV.1 - Summary of total read numbers for each sample from bacterial 16S rRNA gene sequencing analysis.

Burrow	Deep	Foregut	Hindgut	Swab	Surface
37008	37489	5713	62187	771	41369
38932	39108	19634	36654	42090	39242
30676	53189	1683	53516	46439	35148
26561	38306	4048	62500	3042	27260
25364	32099	5701	3070	54369	30630
36118	44100	1507	44876	55300	30646
36689	42092	3020	9883	48643	29958
34716	39475	21640	27658	52959	34155
33952	35620	32234	2120		39289
41976	45571	5446	1422		
28998	48074	4994	674		

Table IV.2 - Summary of total read numbers for each sample from archaeal 16S rRNA gene sequencing analysis.

Burrow	Deep	Foregut	Hindgut	Swab	Surface
20377	15541	24	297	678	9835
7483	8893		64	1639	12904
11717	17709		29	1421	6905
2857	14731		30	760	5947
7311	8708			923	8374
10803	12438			789	3723
6941	7271				5098
6266	15858				1447
12454	20215				10982
3034	15814				
	8783				

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Research Article

RESEARCH ARTICLE

Sediment microbial assemblage structure is modified by marine polychaete gut passage

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One sentence summary: Digestive tracts of the marine deposit feeder *Hediste diversicolor* contain distinct transitory sediment microbial assemblages.

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ABSTRACT

Invertebrate activities in sediments, predominantly the redistribution of particles and porewater, are well-known to regulate the structure of associated microbial assemblages; however, relatively little attention has been given to the effects of sediment ingestion, gut passage and excretion by deposit-feeding invertebrates. Here, we use high-throughput sequencing and quantitative PCR to examine how passage through the gut of the marine polychaete *Hediste diversicolor* affects the structure of bacterial and archaeal assemblages and the abundance of nitrogen cycling taxa. We show that the digestive tract of *H. diversicolor* contains unique transitory microbial assemblages that, during gut passage, become more like the surrounding sediment assemblages. Enrichment of similar microbial taxa in both the hindgut and the burrow wall suggest that these transitory gut assemblages may influence the composition of the local sediment community. The hindgut of *H. diversicolor* also forms a reservoir for unique ammonia-oxidising archaeal taxa. Furthermore, distinct microbial assemblages on external polychaete surfaces suggest that deposit-feeding invertebrates act as vectors that transport microbes between sediment patches. Collectively, these findings suggest that the passage of sediment and associated microbial assemblages through the gut of deposit feeding invertebrates is likely to play a significant role in regulating sediment microbial assemblages and biogeochemical functioning.

Keywords: invertebrate–microbe interactions; ecosystem functioning; functional traits; nitrogen cycling; microbiome

INTRODUCTION

Coastal sediment environments are globally important sites for organic matter decomposition and remineralisation (Middelburg, Soetaert and Herman 1997). The biological complexity associated with sediment ecosystems remains, however, insufficiently constrained in biogeochemical models to generate accurate projections (Snelgrove et al. 2018). In particular, the representation of microbial processes underpinning biogeochemical transformations requires more thorough consideration because sediment-dwelling invertebrates directly and indirectly alter the structure and activity of microbial communities (Mermillod-Blondin, Francois-Carcaillet and Rosenberg 2005; Gilbertson, Solan and Prosser 2012).

Invertebrate burrows are sites of steep chemical gradients, high levels of organic matter cycling and increased oxygen penetration (Kristensen 2000; Nielsen et al. 2004; Jovanovic et al. 2014)

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that vary in relation to the feeding, burying and ventilatory activity of the invertebrate inhabitant. Consequently, burrow walls support distinct microbial communities that display both spatial and inter-specific variation (Bertics and Ziebis 2009; Laverock *et al.* 2010; Pischedda *et al.* 2011; Taylor and Cunliffe 2015). Whilst it is known that changes in the structure and diversity of microbial assemblages related to invertebrate activities (e.g. mucopolysaccharide production) can have substantive effects on biogeochemical cycles (Satoh, Nakamura and Okabe 2007; Foshtomi *et al.* 2015; Dale *et al.* 2018; Foshtomi *et al.* 2018), studies to date have largely focused on the effects of particle and fluid displacement by infauna and do not consider the roles of other significant animal–environment–microbial interactions.

As marine sediment ecosystems are dominated by deposit-feeding invertebrates, transit of sediment through the digestive tract of these communities is likely to be particularly important in determining the benthic contribution to biogeochemical cycling (Thorsen 1998; Biron *et al.* 2014; Troussellier *et al.* 2017). In terrestrial systems, the specific organic matter, pH and redox conditions of the earthworm (*Lumbricus rubellus*) gut means that ingestion of soil significantly alters the abundance of certain microbial taxa in the transitory substrate assemblage (Furlong *et al.* 2002; Pass *et al.* 2015). In marine deposit-feeders, abundance-based techniques have demonstrated a general loss of bacteria in the foregut, followed by regrowth towards the hindgut (Plante, Jumars and Baross 1989; Hymel and Plante 2000). Bacteriolytic activity and digestion are centred in the stomach and decline towards the gut posterior (Plante and Mayer 1994; Mayer *et al.* 1997), where bacterial growth can be stimulated in the absence of competitors and the presence of elevated levels of organic matter (Andresen and Kristensen 2002). As the digestion and subsequent regrowth of bacterial assemblages can be species-specific and vary between individuals within an invertebrate population (Plante, Jumars and Baross 1989; Plante and Mayer 1994; Mayer *et al.* 1997), it follows that the nature of invertebrate–microbial gut interactions within a community will be of functional importance to ecosystem processes within the sediment profile. Clone library studies have also shown that gut passage can alter assemblage diversity and the ratio of aerobic to anaerobic taxa (Lau, Jumars and Ambrust 2002; Li *et al.* 2009), but the extent to which the surrounding sediment assemblages are influenced by these changes remains unclear (King 2018).

Invertebrate guts may introduce functionally important taxa into the surrounding sediment or they may act as a vector, which transports a subset of microbes between sediment patches (Troussellier *et al.* 2017). Earthworm guts have been shown to contain active nitrate-reducing populations (Furlong *et al.* 2002; Wust, Horn and Drake 2011) at abundances that are orders of magnitude higher than the surrounding substrate (Karsten and Drake 1997). The presence of these ingested denitrifying taxa means that earthworm guts are sources of nitrous oxide (N_2O) (Horn, Drake and Schramm 2006a; Horn *et al.* 2006b). In marine sediments, gut emissions from deposit feeding invertebrates also form a significant contribution to N_2O fluxes because of incomplete denitrification occurring in the gut (Heisterkamp *et al.* 2010). The significance of these emissions is constrained by a lack of mechanistic understanding of microbe–invertebrate interactions during gut passage, including consideration of whether gut conditions encourage the growth of microbial functional groups that contribute to sediment nitrogen cycling.

Here, we assess the variation between bacterial and archaeal assemblage structure in the surrounding sediment environment, the external body surface and the internal gut of the sediment-dwelling polychaete *Hediste diversicolor*, using both 16S rRNA gene amplicon sequencing and quantitative PCR (Q-PCR). Our aim was to determine whether the transitory microbial assemblages within the gut or on the external surfaces of the polychaete are distinct from the assemblages located in the local burrow wall or surrounding peripheral sediment, and to establish the extent to which these assemblages contribute to the wider sediment microbial community and potential nitrogen cycling.

METHODS

Sample collection and processing

Samples were collected from three mud flat sites (~15 m apart) at St Johns Lake, Cornwall, UK (50°21'51" N, 004°14'08" W), in September 2017 (Fig. 1a). A previous survey at this location showed that the sediment in the area is predominantly silt (16–63 μm) with an organic carbon content of 6.9% (Ecospan, 2010). For each site, three surficial sediment samples ('surface', upper 0.5 cm) were obtained using a sterile syringe and six burrows occupied by *H. diversicolor* were identified (Fig. 1b and c). Individual *H. diversicolor* were removed from each burrow, swabbed (Fisherbrand swabs, Fisher Scientific (Loughborough, UK)) and then anaesthetised in 40 ml of $MgCl_2$ in seawater (2.5% $MgCl_2$) to prevent gut evacuation (Rouse 2004). Sediment from the burrow wall ('burrow', 3 cm depth from the sediment-water interface, 0.5 cm of burrow wall thickness sampled) and from the surrounding, non-bioturbated area ('deep', 3 cm depth from the sediment–water interface, 4 cm from the burrow) was obtained with a sterile syringe. All sediment samples and swabs were snap frozen using a liquid N_2 dry shipper and stored at -80°C. Individual *H. diversicolor* were washed in distilled water, measured (10 ± 4 cm, n = 18) and dissected on the day of collection. An incision was made after the foregut apparatus (foregut, ~31 ± 6.9% body length, n = 13) and a separate incision towards the end of the hindgut (hindgut, ~79 ± 5.6% body length, n = 16) to facilitate the removal of two 1 cm sections of gut contents using sterile tweezers. Dissection tools were washed with ethanol between each incision. All samples were stored at -80°C.

DNA extraction and Q-PCR

DNA was extracted (sediment samples, 0.25 g wet weight; swab samples, whole swab; gut samples, available sediment content) from 11 burrow systems (Site 1, n = 4; Site 2, n = 3; Site 3, n = 4) using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA yield was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and the extracted DNA stored at −20°C.

Q-PCR was used to assess the abundance of 16S rRNA genes, *amoA* genes and the bacterial *nirS* gene in four of the burrow systems (full protocol, Text S1; reaction efficiencies, Table S1 (Supporting Information)). A total of 10 μL reactions contained 5 μL 2x SensiFast SYBR No-ROX 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, Australia), with duplicate technical replicates for each sample. Swab samples were excluded from Q-PCR analyses because of difficulties in obtaining accurate sample weights. Results were converted from ng μl^{-1} to copy number

Figure 1. Sample site at St John's Lake, Cornwall **(a)**, with a vertical cross-section of the sediment profile displaying visible *H. diversicolor* burrow systems with oxidised interfaces **(b)** and an individual worm within burrow **(c)**.

mgww.sediment^{-1}. Data are reported according to MIQE guidelines (Bustin *et al.* 2009).

16S rRNA gene sequencing and analysis

Bacterial and archaeal 16S rRNA gene PCR was used to establish sequencing viability (see Supplementary Methods for full protocol). Based on these results, the swab samples from site 2 ($n = 3$) were excluded from both bacterial and archaeal sequencing analyses, with additional samples excluded from the archaeal sequencing analysis (Table S2, Supporting Information). 16S rRNA gene sequencing was performed on the Illumina MiSeq platform using V6-V8 primer sets (Comeau, Douglas and Langille 2017). Poor quality sequencing runs excluded 4 archaeal samples from further analysis (Table S2, Supporting Information).

Sequences were analysed using the R package DADA2 (Callahan *et al.* 2016). Based on the forward and reverse read error profiles, reads were truncated at position 100 to account for the poor read quality of some of the gut and swab samples. The resulting read length was not enough for acceptable overlap between the forward and reverse reads; therefore, only the forward reads were used for the remainder of the analysis. Forward primer sequences were removed, and then reads were filtered to allow a maximum of four errors per read to obtain the best fit between the expected and estimated error rates. The sequence data were dereplicated to remove redundancy, sequence variance was inferred and a sequence table produced. Taxonomy was assigned to operational taxonomic units (OTUs) using the SILVA database (Quast *et al.* 2013) and loaded into the phyloseq package (McMurdie and Holmes 2013). Chloroplast plastid and other eukaryotic sequences were removed from the dataset before further analysis. Sequence data have been deposited in the European Nucleotide Archive (accession code PRJEB29031).

Statistical analysis

The single archaeal foregut sample was excluded from the statistical analyses because it was not replicated. Diversity was calculated with the phyloseq package using the Chao 1 diversity index and ANOVA to determine statistical significance. Variations in assemblage composition between environmental DNA sources (surface, burrow, deep, swab, foregut and hindgut) and sample sites (1, 2 and 3) were calculated from weighted Bray–Curtis dissimilarity matrices rarefied to the minimum number of reads using PERMANOVA (999 permutation), and visualised using Non-metric Multidimensional Scaling (NMDS) (vegan; v2.4–6; Oksanen *et al.* 2016) and Minimum Spanning Trees (phyloseqGraphTest). As sample site had no significant effect on assemblage structure, the three sites were pooled and site effects were disregarded. Pairwise PERMANOVA analyses (999 permutations) were also carried out between each sample type. Relative abundance plots were produced for the abundant bacterial orders and archaeal classes (>5% relative abundance). This high cut-off value was selected to minimise any potential bias introduced by the low read numbers obtained for some samples. Differential abundances of taxa (Log2-FoldChange) between pairs of sample types relevant to the aims of this study were calculated for the bacterial assemblages in the DESEQ2 package (Love, Huber and Anders 2014) using the Wald parametric test ($P < 0.05$). Changes in the abundance of nitrifying taxa were assessed using ANOVA once the key taxa were identified from the sequence table and sequence identity confirmed using online BLAST searches. Differences in bacterial and archaeal 16S rRNA genes, bacterial and archaeal *amoA* genes, the ratio of bacterial and archaeal *amoA* (AOB:AOA) genes and bacterial *nirS* genes between sample types were calculated using ANOVA,

and multiple comparisons to identify the source of any significant differences were performed using Tukey's post hoc tests. Bacterial and archaeal 16S rRNA, archaeal amoA and AOB:AOA were log transformed before analysis. For ecological clarity, we present untransformed data in the figures. All statistical analyses were performed with R (version, 3.2.2; R Core Team 2015)

RESULTS

For the bacterial community, sequencing analysis generated a total of 1 847 603 reads, which, after filtering, grouped into 4917 unique OTUs. Read depth was slightly higher and less variable in the sediment samples (burrow, 33 726 ± 5238; deep, 41 375 ± 6020; surface, 34 189 ± 4928) relative to the worm-associated samples (foregut, 9602 ± 10,144; hindgut, 27 687 ± 25 380; swab, 37 952 ± 22 679) (Table S3, Supporting Information). For the archaeal community, sequencing analysis generated a substantially lower total of 307 073 reads, which, after filtering, grouped into 268 unique OTUs. This low read depth, along with a more uneven distribution of reads between samples (burrow, 8924 ± 5187; deep, 13 269 ± 4303; surface, 7246 ± 3647; hindgut,105 ± 129; swab, 1035 ± 397) (Table S4, Supporting Information), means that the archaeal community cannot be assessed with the same degree of certainty but is included here to support the trends observed within the bacterial community.

Microbial abundance and alpha diversity

Bacterial abundance, determined by 16S rRNA gene QPCR, was dependent on the sample type (Log_{10}, $F_{4,14} = 3.7$, $P = 0.0295$), with a greater abundance of bacteria present in the hindgut (1 192 363 ± 293 596 copies mgww.sediment^{-1}) compared to the foregut (87 145 ± 61 045 copies mgww.sediment^{-1}) (Log_{10}, Tukey's, $P = 0.039$; Table S7, Supporting Information) (Fig. 2a). Archaeal abundance also differed between sample types (Log_{10}, $F_{4,14} = 59.82$, $P < 0.001$), with the greatest mean abundance in the burrow and deep sediment assemblages (burrow, 55 671 ± 29 315 copies mgww.sediment^{-1}; deep, 116 472 ± 51 386 copies mgww.sediment^{-1}; Log_{10}, Tukey's, $P < 0.001$ (Table S8, Supporting Information)), and the lowest mean abundance in the foregut assemblage (203 ± 94 copies mgww.sediment^{-1}; Log_{10}, Tukey's, $P < 0.05$ (Table S8, Supporting Information)) (Fig. 2b). Overall, archaeal abundance was lower than bacterial abundance, but followed a generally similar trend despite the low abundance in the hindgut.

Bacterial diversity, determined using the Chao1 index, was also dependent on sample type ($F_{5,55} = 17.79$, $P < 0.001$), with greater diversity in the sediment and swab assemblages (surface, 590 ± 55; burrow, 620 ± 50; deep, 674 ± 72; swab, 657 ± 372) than the gut assemblages (foregut, 144 ± 97; hindgut, 286± 206) (Fig. 3a). Archaeal diversity was generally lower, but was still dependent on sample type ($F_{5,35} = 15.59$, $P < 0.001$) and showed greater diversity in the sediment assemblages (surface, 68 ± 24; burrow, 65 ± 24; deep, 80 ± 15) than in the worm-associated (swab, 26 ± 24; hindgut, 6 ± 3) assemblages (Fig. 3b).

Microbial assemblage structure

Bacterial assemblage structure, determined by 16S rRNA sequencing, was dependent on sample type ($F_{5,55} = 10.27$, $P = 0.001$), with all sources being distinguishable from one another (Supplementary Table S5, Supporting Information). Overall, the sediment samples clustered separately from the gut samples. Within the sediment cluster, the burrow samples formed an intermediate cluster between the surface and deep sediment assemblage clusters (Fig. 4a and b), although the majority of the burrow samples were more affiliated to the surface assemblages (Fig. 4b). The gut assemblages clustered together, though in more variable and less distinct groups, with hindgut and sediment assemblages more closely affiliated with one another than the foregut and sediment assemblages (Fig. 4a and b). The external swab samples formed a distinct bacterial group between the sediment and gut clusters, being most closely affiliated to the surface and hindgut assemblages (Fig. 4a and b).

Sample type also affected archaeal assemblage structure ($F_{4,35} = 9.62$, $P = 0.001$), with each source forming an independent cluster (Supplementary Table S6, Supporting Information). As with the bacterial assemblages, sediment samples clustered together with the burrow samples forming an intermediate cluster between the deep and surface assemblages (Fig. 4c). Unlike the bacterial assemblages, however, the archaeal burrow assemblages appeared to be more closely affiliated with the deep sediment assemblages (Fig. 4d). Similarly, distinct hindgut assemblages showed greatest affiliation with the external swab assemblages, which formed an intermediate group between the gut and sediment assemblages (Fig. 4c and d).

Summary of abundant microbial taxa

The abundant bacterial assemblage (>5% relative abundance), determined by 16S rRNA gene sequencing, was made up of 18 orders that accounted for 54.6 ± 12.9% of the total OTU abundance (Fig. 5a). This abundant subset differed between the sediment (surface, burrow and deep) and gut environments (foregut and hindgut), with the sediment assemblages dominated by the orders Desulfobacterales, Flavobacterales and Xanthomonadales. Comparing the surface and deep sediment assemblages, the surface samples had greater abundances of Cytophagales and Rhodobacterales, whilst the deep samples had greater abundances of Desulfobacterales and Myxococcales. The burrow assemblages were a combination of both the surface and deep assemblages, with abundant populations of Desulfobacterales, Myxococcales and Rhodobacterales. In the gut, the majority of the samples were dominated by Flavobacterales and Rhodobacterales. Additionally, as observed previously in the community structure analyses (Fig. 4), there seems to be greater variation in the overall assemblage composition between the individual *H. diversicolor* gut tracts than between the individual burrow systems. The external swab assemblages were also more varied in composition relative to the burrow systems, with some individuals showing high abundances of Xanthomonadales, as observed in the sediment assemblages, whilst others showed high abundances of Rhodobacterales, similar to the gut assemblages.

The abundant archaeal assemblage (>5% relative abundance) was made up of five classes that accounted for 94.6 ± 3.5% of the total OTU abundance (Fig. 5b). This abundance assemblage also differed between the sediment and gut environments, with the external swab and sediment assemblages dominated by Group C3 and Thermoplasmata (Fig. 5b). Methanomicrobia was also abundant in the surface and swab assemblages, with some surface sediment assemblages also enriched in Marine Group I. As with bacteria, the burrow archaea assemblage was a combination of the surface and deep assemblages, which were dominated by Group C3 and Thermoplasmata, with high abundances of Marine Group I and Methanomicrobia in some individual burrows. In contrast, the gut assemblages were dominated by the Soil Crenarchaeotic Group, with Methanomicrobia only present in high abundances in the hindgut assemblages.

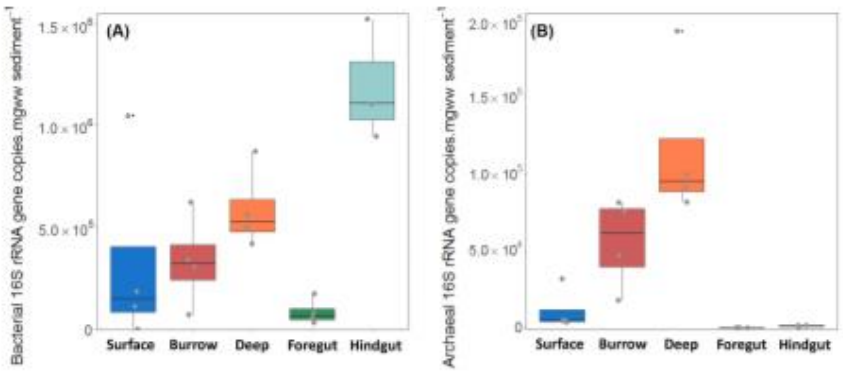

Figure 2. Variations in gene abundance between environmental sources. Bacterial (A) and archaeal (B) 16S rRNA genes. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (n = 4). Untransformed data presented.

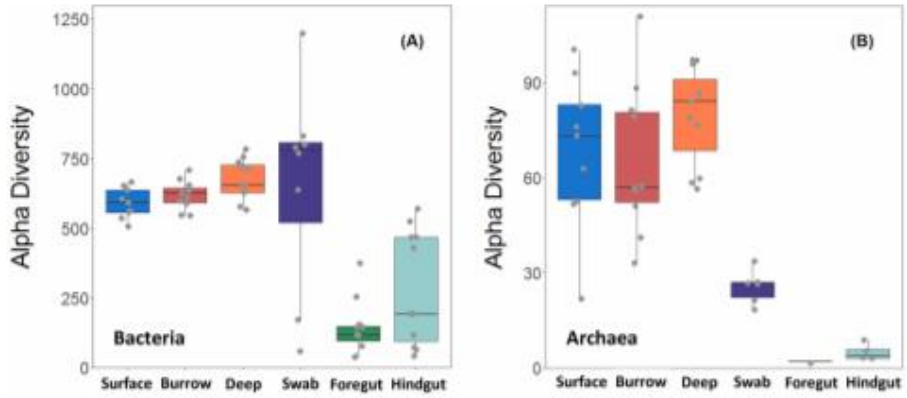

Figure 3. Variations in bacterial (A) and archaeal (B) alpha diversity between each of the environmental sources. Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (Bacteria: n = 11, except surface n = 9; Archaea: surface n = 9, burrow n = 10, deep n = 11, swab n = 6, foregut n = 1, hindgut n = 4).

Taxonomic variations between sample type assemblages

Pairwise comparisons of the abundance of taxonomic groups between sample types (plotted as log2 fold changes) revealed that bacterial burrow assemblages were significantly enriched in Desulfobacterales and Myxococcales relative to surface assemblages, which were enriched in Flavobacteriales and Rhodobacterales (Fig. 6a). In contrast, comparison between burrow and deep sediment assemblages revealed the reverse trend (Fig. 6b), which indicates that burrow assemblages were enriched in both surface and deep sediment taxa. Generally, fewer taxa varied between the foregut and hindgut, but greater abundances of Cytophagales, Flavobacteriales and Myxococcales were present in the hindgut relative to the foregut (Fig. 6c). Between the hindgut and the burrow assemblages, these three orders were generally enriched in the burrow environment, although some representatives were more abundant in the hindgut (e.g. *Ekhidna sp.*, *Actibacter sp.*) (Fig. 6d).

Between the swab and surface assemblages, only one Cyanobacteria representative differed between the two sample types (Supplementary Figure S1, Supporting Information). Swab assemblages were enriched in some Desulfobacterales taxa relative to burrow and hindgut assemblages and in Flavobacterales when compared to the hindgut assemblage alone (Fig. 6e and f). Bacterial orders enriched during gut passage (i.e. Cytophagales, Flavobacteriales and Myxococcales) were also more abundant in the external swab assemblage relative to the deep anoxic sediment (Supplementary figure S2, Supporting Information).

Abundance of nitrogen cycling taxa

Abundance of ammonia oxidising bacteria (AOB) was dependent on sample type ($F_{4,14} = 5.935$, $P = 0.005$), although the significance of this term was driven by a greater abundance in the burrow and deep sediment assemblages (burrow, 629 ± 210 copies mgww.sediment^{-1}; deep, 789 ± 365 copies mgww.sediment^{-1}) relative to the foregut assemblage (69 ± 32 copies mgww.sediment^{-1}; Tukey's, $P < 0.05$ (Table S9, Supporting Information)) (Fig. 7a). Concomitantly, the sequencing analysis showed that the majority of ammonia oxidising and nitrite oxidising (NOB) bacterial taxa (*Nitrosomonadaceae*, *Nitrosococcus*, *Nitrobacter* and *Nitrospina*) were either absent or did not differ with sample type. *Nitrospira* (NOB) was the exception ($F_{3,33} =$

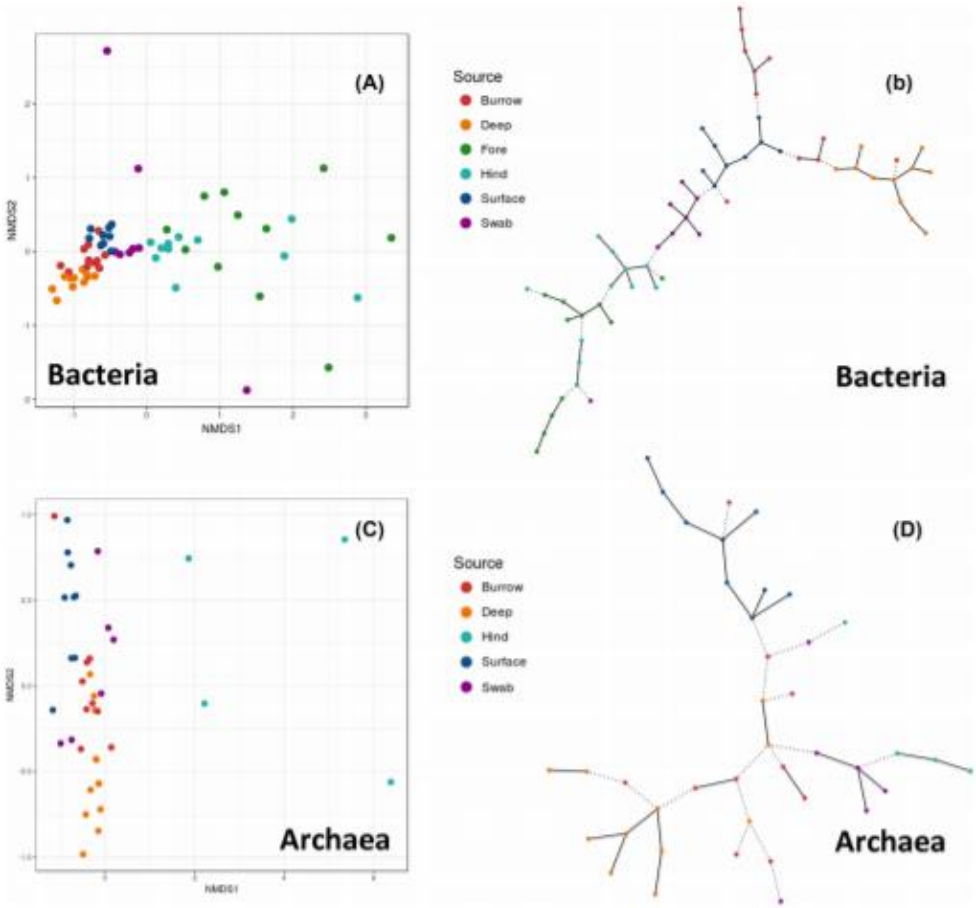

Figure 4. NMDS (Bray–Curtis) and network analysis (Minimum Spanning Tree, Bray–Curtis, P = 0.002) plots of bacteria **(A, B)** and archaeal **(C, D)** community structure.

2.97, P = 0.02) and had a lower relative abundance in the hindgut (0.02 ± 0.03% relative abundance) compared to the deep sediment assemblage (0.15 ± 0.10% relative abundance; Tukey's, $P < 0.05$).

AOA abundance was dependent on sample types (Log_{10}, $F_{4,14} = 10.65$, $P < 0.001$), with greater abundances in sediment and hindgut assemblages (burrow, 363 ± 66 copies mgww.sediment^{-1}; deep, 517 ± 121 copies mgww.sediment^{-1}; hindgut, 1951 ± 1129 copies mgww.sediment^{-1}) relative to the foregut assemblage (90 ± 148 copies mgww.sediment^{-1}; Log_{10}, Tukey's, $P < 0.05$ (Table S10, Supporting Information) (Fig. 7b). The ratio of AOB to AOA abundance (AOB:AOA) was also influenced by sample type (Log_{10}, $F_{4,14} = 4.08$, $P = 0.021$), with the lowest mean ratio present in the hindgut (0.267 ± 0.323), intermediate ratios in the sediment (surface, 1.11 ± 0.630; burrow, 1.70 ± 0.291; deep, 1.48 ± 0.406), and the highest mean ratio in the foregut (4.43 ± 2.86; Log_{10}, Tukey's, $P < 0.05$ (Table S11, Supporting Information) (Fig. 7c). A ratio less than one indicates an AOA dominated community, whilst a ratio greater than one indicates an AOB dominated community. Interestingly, the dominant AOA in most of the hindgut samples (*Nitrosocosmicus*) was from the Soil Crenarchaeotic Group, whilst the dominant group in the sediment samples (*Nitrosopumilus*) was from Marine Group I, which was only observed in some individual surface and burrow samples.

Bacterial *nirS* copy numbers, calculated as a proxy for the denitrifying bacterial assemblage, differed in abundance with sample type ($F_{4,14} = 10.26$, $P < 0.001$). Denitrifying bacterial abundance was generally lower in the gut assemblages (foregut, 671 ± 484 copies mgww.sediment^{-1}; hindgut, 27 442 ± 7546 copies mgww.sediment^{-1}) relative to the sediment assemblages (surface, 82 577 ± 40 786 copies mgww.sediment^{-1}, 112 541 ± 37 716 copies mgww.sediment^{-1}; deep, 106 859 ± 34 420 copies mgww.sediment^{-1}; Tukey's, $P < 0.05$ (Table S12, Supporting Information)), with the exception of the hindgut and surface sediment assemblage (Fig. 7d). All Q-PCR abundance data are provided in supplementary material (Table S13, Supporting Information).

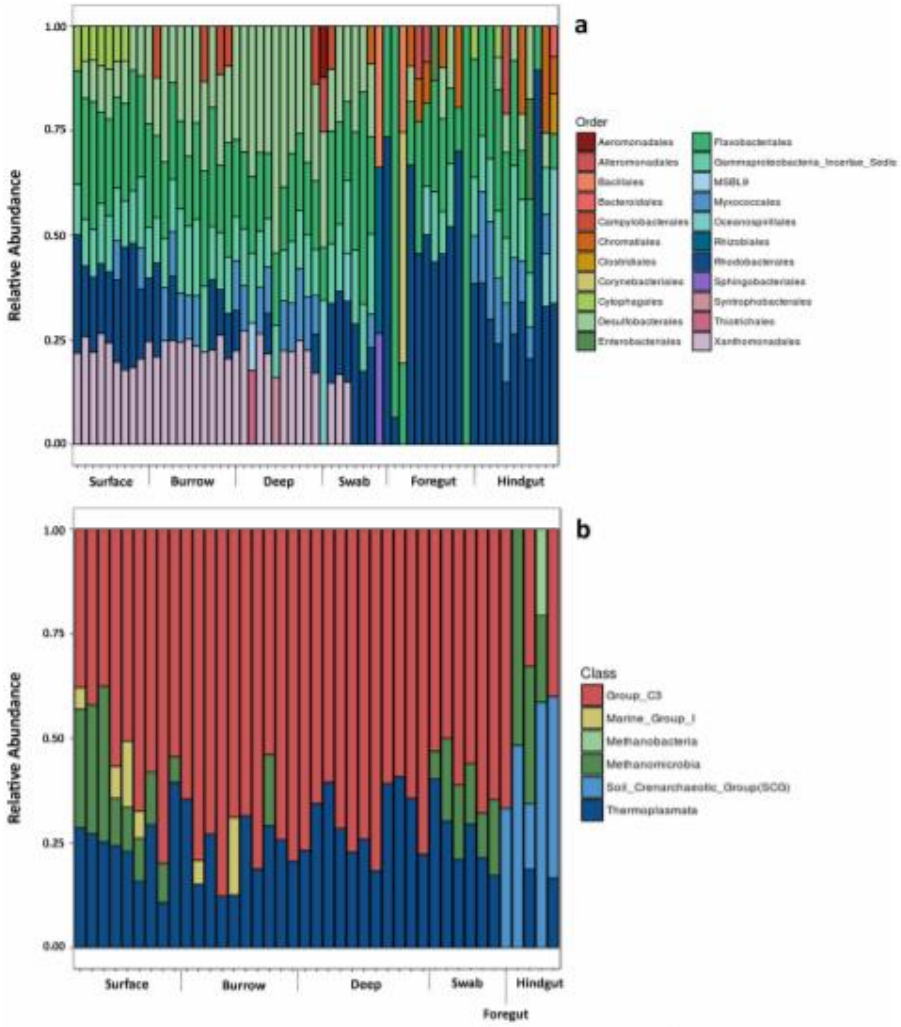

Figure 5. Relative abundance of bacterial orders **(a)** and archaeal classes **(b)** (> 5% relative abundance) between each of the samples for each site.

DISCUSSION

The passage of sediment through the gut of an invertebrate has the potential to be a key mechanistic ecological process that helps explain the influence of deposit feeding invertebrates on sediment microbial assemblages and subsequent biogeochemical cycling. Using high-throughput sequencing, we demonstrate that a common sediment-dwelling invertebrate (*H. diversicolor*) has a distinct transitory gut assemblage, with regrowth of both bacterial and archaeal taxa at the posterior end of the digestive tract. Hindguts of *H. diversicolor* also appear to be 'incubators' for distinct ammonia-oxidising archaeal assemblages. This specific transitory assemblage, and the distinct assemblage on the external surface of the polychaete, has the potential to introduce higher abundances of specific taxa to the surrounding sediment, and therefore modify the sediment assemblage structure and facilitate the transport of microbial taxa between sediment patches (Godbold, Bulling and Solan 2011).

Bacterial burrow assemblages have been shown to be similar to both surface (Laverock *et al.* 2010; Pischedda *et al.* 2011) and deep sediment assemblages (Papaspyrou *et al.* 2005; Papaspyrou *et al.* 2006). Here, we show that bacterial and archaeal abundances were generally similar between all surface, burrow and surrounding sediment, and abundances observed were consistent with those in other bioturbated sediments (Laverock *et al.* 2013). Assemblage composition analysis, however, indicated that the burrow assemblages were a combination of both

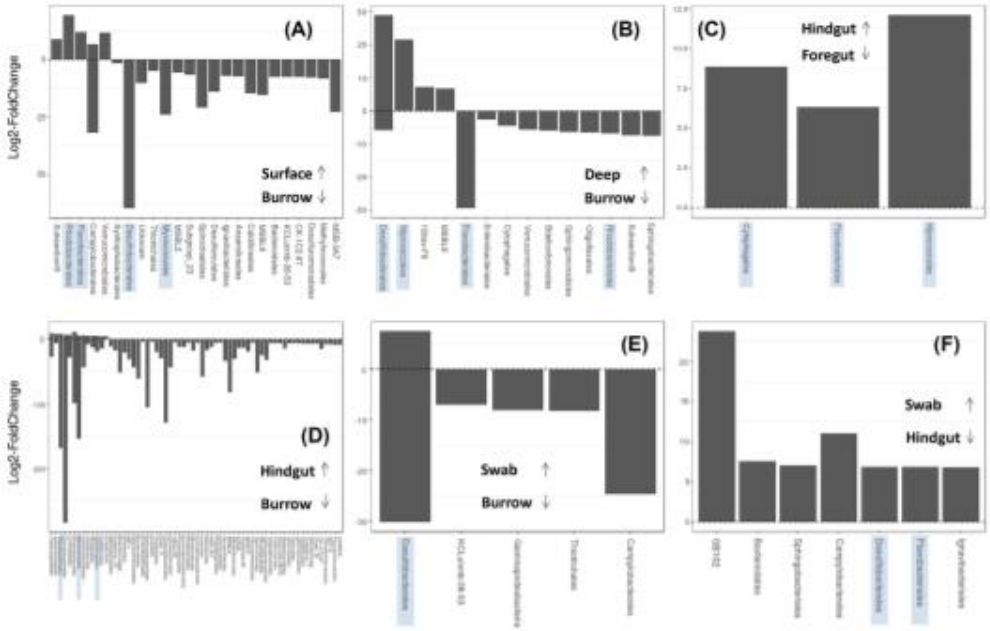

Figure 6. Change in abundance of taxa (Log2 fold; $P < 0.05$) between ecologically relevant combinations of environmental sources. (a—Burrow vs Surface; b—Burrow vs Deep; c—Foregut vs Hindgut; d—Burrow vs Hindgut; e—Burrow vs Swab; f—Hindgut vs Swab). For each combination, taxa below 0 are more abundant in first stated sample and taxa above 0 are more abundant in second stated sample. Key taxa are highlighted.

the surface and deep sediment assemblages, with the bacterial assemblage most similar to the surface sediment assemblage and the archaeal assemblage seemingly most similar to the deep sediment assemblage. Overall, this fits with the paradigm that intermittent irrigation of burrows by invertebrates creates an oscillating oxic-anoxic environment (Volkenborn *et al.* 2012), which switches the habitat between 'surface-like' and 'deep-like' available O_2 conditions and allows the coexistence of aerobic and anaerobic microbial taxa in the burrow. Other invertebrate activities occurring within burrows, such as mucopolysaccharide production (Dale *et al.* 2018) or the secretion of biocides (King 1988), are also likely to contribute to burrow assemblage structure.

The gut tracts of *H. diversicolor* contained distinct bacterial and archaeal assemblages that were less diverse than the surrounding sediment (King 2018) and included taxa, such as Thermoplasmatales and Methanomicrobiales, which have been previously observed in polychaete digestive tracts (Li *et al.* 2009). The transitory assemblage was most distinct from the surrounding sediment after passage through the foregut, where both bacterial and archaeal abundance and alpha diversity declined. Within the hindgut, the assemblages remained distinct but seemed to become more similar to the sediment and abundance increased, though this was most likely not due to the addition of taxa as there was no significant increase in bacterial or archaeal diversity. These observations follow the abundance patterns observed in other marine deposit feeders, where bacteriolytic activity is highest in the fore and midgut (Plante, Jumars and Baross 1989; Plante and Mayer 1994; Mayer *et al.* 1997), but are not consistent with previous studies of *H. diversicolor* where abundance-based techniques using epifluorescence microscopy have shown higher lytic activity and limited regrowth in the hindgut (Lucas and Bertru 1997; Lucas, Bertru and Hofle 2003). Here, the use of molecular tools with increased resolution has shown that Cytophagales, Flavobacteriales and Myxococcales increase in abundance during *H. diversicolor* gut transit. Both Cytophagales and Flavobacteriales have previously been observed in the guts of deposit-feeding shrimp (Lau, Jumars and Ambrust 2002) and are known to degrade complex macromolecules (Reichenbach and Dworkin 1981; McBride 2014) that are likely to be abundant in hindgut environments. As these taxa are also present in the surrounding sediment bacterial assemblages, gut conditions appear to affect assemblage composition by altering the abundance of existing transitory sediment taxa (Furlong *et al.* 2002).

As both bacterial and archaeal assemblages increased in abundance in the hindgut, it is possible that this microbial 'incubator' could contribute to the wider sediment assemblage once excreted. Because the distinct hindgut assemblage was more similar to the surrounding sediment than the foregut assemblage was, and greater abundances of the hindgut-enriched taxa were observed in the burrow environment relative to the surrounding deep anoxic sediment, it is possible that there was some introduction of hindgut enriched taxa to the sediment. Organic enrichment within the burrow environment, however, could have encouraged the proliferation of Cytophagales and Flavobacterales (Aller and Aller 1986; Papaspyrou *et al.* 2006), whilst Flavobacterales may have been translocated from surficial sediments. Overall, the majority of taxa were more abundant in the sediment than the hindgut, but the hindgut may act as a reservoir for at least some representatives of specific taxa

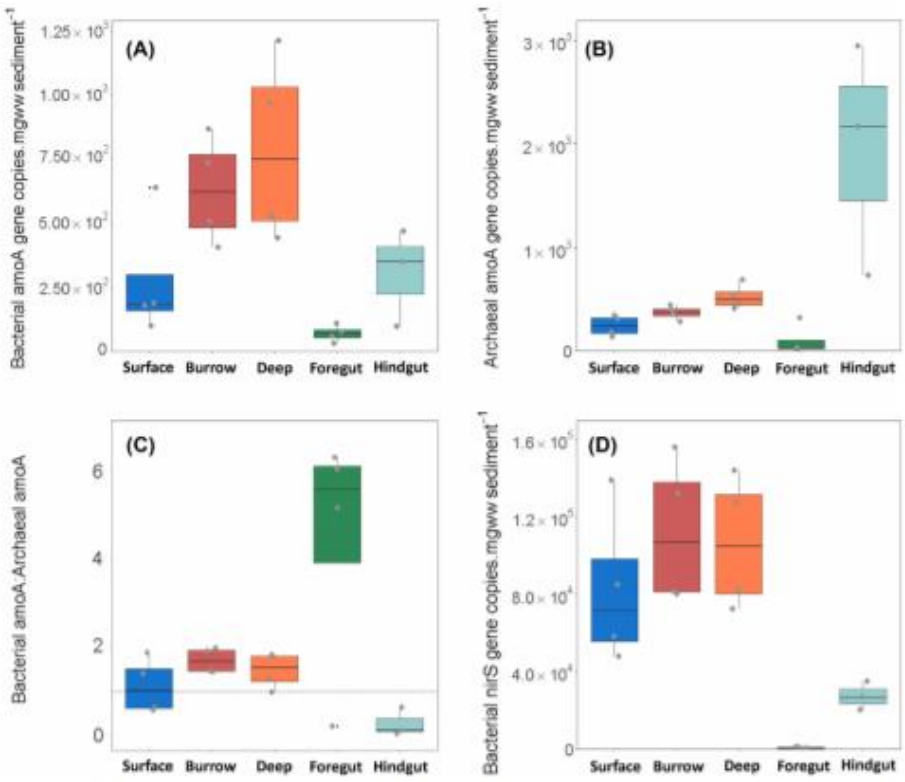

Figure 7. Variations in gene abundance between sample types. Bacterial (A) and archaeal (B) *amoA*, bacterial archaeal *amoA* copy ratio (C), and bacterial *nirS* (D). Boxplot indicates median, 25% and 75% quartiles, and 95% of the data spread (n = 4). Untransformed data presented.

(King 2018). Hence, to fully characterise how sediment functioning may be affected by gut passage and the introduction of specific taxa, it will be beneficial to consider the activity levels of microbial functional groups in both gut, faecal cast and sediment environments.

Invertebrates have been suggested as potential transporters of microbial groups within sediment (Troussellier *et al.* 2017), but the significance of such transport has not been assessed. In this study, the composition of the external microbial assemblages of *H. diversicolor* reflected a combination of both hindgut and sediment assemblages, particularly the surface sediment assemblage. This observation supports the view that a proportion of the hindgut assemblage may be excreted into the sediment. The close relationship with the surficial sediment also suggests that either *H. diversicolor* spends a portion of time at the surface (e.g. foraging, Vedel and Andersen 1994), or that the very inner burrow sediment immediately in contact with the invertebrate is more closely related to the surface sediment than the other burrow wall assemblages (Bertics and Ziebis 2009). As the *H. diversicolor* external assemblages had greater abundances of some Desulfobacterales taxa compared to both the burrow and hindgut assemblages, specific taxa may be concentrated on the external surfaces of individuals and therefore be redistributed within the burrow system. Additionally, the taxonomic groups that increased in abundance during gut passage were more abundant on the external surfaces of the polychaete than they were in the deep sediment, and so *H. diversicolor* individuals could transport taxa into anoxic sediment during burrow construction and extension (Davey 1994).

Nitrification is a significant process in benthic nitrogen cycling as it converts NH_4^+ released from organic matter back into NO_3^- (Herbert 1999) that can then support primary productivity in the overlying water column (Boynton and Kemp 1985). Although the abundances of AOB and archaea (AOA) in the sediment were consistent with other sediment environments (Laverock *et al.* 2013; Bowen *et al.* 2014), they were also consistent between the surface, burrow and surrounding sediment, which suggests that these burrow systems were not hotspots for nitrifying taxa. In the foregut of *H. diversicolor*, there seemed to be an overall reduction of both AOB and AOA in the transitory assemblage, though the high AOB:AOA ratio suggests that AOA taxa were generally more susceptible to potential digestive foregut loss processes.

Conversely, passage through the hindgut supported a substantial increase of AOA to abundances comparable with the surrounding sediment so that AOA dominated the nitrifying community within the hindgut environment. This suggests that the organic content differed between the surrounding sediment and the hindgut, most likely because of the digestion of organic matter in the foregut and the release of NH_4^+ in the hindgut,

and indicates that hindguts have the potential to contribute AOA to the surrounding sediment through excretion. Sequencing analysis, however, demonstrated that the dominant AOA taxon in the *H. diversicolor* hindguts was *Nitrosocosmicus* sp., which has a higher tolerance for NH_4^+ and NO_2^- than other AOA (Lehtovirta-Morley *et al.* 2016), whilst the dominant AOA in the sediment samples was instead *Nitrosopumilus* sp. (Reyes *et al.* 2017), which tends to dominate under low NH_4^+ conditions (Martens-Habbena *et al.* 2009). It is therefore possible that the high organic matter content in deposit feeder gut passages supports a unique AOA assemblage, which is then outcompeted when introduced to sediment. Whether the AOA are actively undertaking ammonia-oxidation in these low O_2 environments (Plante and Jumars 1992) to the extent that they significantly contribute to sediment nitrogen cycling remains to be determined.

Earthworm gut passages and faecal casts can contain larger and more active populations of denitrifying microbial groups than the surrounding soil (Karsten and Drake 1997; Furlong *et al.* 2002). Here, denitrifying bacteria were reduced during foregut passage and, although certain nitrite reducing taxa did form a significant portion of the transitory assemblage, there was no significant regrowth in the hindgut. *Hediste diversicolor* guts are, therefore, unlikely to contribute denitrifiers to sediment assemblages, but the presence of these taxa will still have functional value. Complete and incomplete denitrification by ingested soil taxa means that earthworms are sources of both N_2 and N_2O (Horn, Drake and Schramm 2006a; Horn *et al.* 2006b). Release of N_2O from deposit feeder guts have been shown to contribute to the overall flux from sediment systems (Stief *et al.* 2009; Heisterkamp *et al.* 2010), though this study also indicated that *H. diversicolor* has a slightly lower N_2O release rate than other deposit feeders (Heisterkamp *et al.* 2010). This may be due to a generally lower abundance of denitrifiers in *H. diversicolor* guts, though this would need to be confirmed by comparisons with other deposit-feeding invertebrate taxa. It also cannot be ruled out that complete denitrification (i.e. N_2 release instead of N_2O accumulation) may occur within *H. diversicolor* guts, but that remains to be determined via either direct activity measurements or identification of genes responsible for the final denitrification step (i.e. *nosZ*). Understanding how denitrifying assemblages differ between deposit feeding taxa, and whether this is related to subsequent variations in N_2O and N_2 release, will be beneficial to efforts seeking to improve current estimates of sediment N budgets.

Collectively, our findings indicate that the internal and external transport of microbial assemblages by deposit feeders has the potential to regulate sediment microbial assemblages. By accumulating both sediment and gut-associated taxa on external surfaces, burrowing invertebrates may alter local sediment microbial distributions. The transitory sediment assemblage in gut passages also has the potential to contribute to sediment nitrogen cycling, either by introducing key microbial functional groups or by supporting these taxa within the gut and excreting products. *Hediste diversicolor* often dominates the biomass of intertidal mudflats and is known to reach densities of 3700 ind. m^{-2} (Scaps 2002). As related polychaete populations have been estimated to ingest 5 kg (dry weight) sediment m^{-2} $year^{-1}$ (Cammen 1980), sediment microbial assemblages will be regularly and consistently exposed to gut conditions. Yet, the wider ecological consequences of this process are understudied, especially in the context of expanding polychaete fisheries (Watson *et al.* 2017; Cole, Chick and Hutchings 2018). Future effort should assess transitory taxa activity and establish whether the effects of gut passage vary between alternative invertebrate groups. Additionally, based on previous assessments of *H. diversicolor* emissions (Heisterkamp *et al.* 2010), these populations have the potential to release 8.8 μmol N_2O m^{-2} day^{-1} with significant implications for sediment denitrification and nitrogen fluxes. Further examination of gut passage and external transport as invertebrate functional traits will improve our understanding of invertebrate–microbe interactions, and the role this ecological process plays in regulating sediment ecosystem functioning.

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