Linking microbial communities and macrofauna functional diversity with benthic ecosystem functioning in shallow coastal sediments, with an emphasis on nitrifiers and denitrifiers

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Academic year 2016-2017

Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Science: Marine Sciences

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ACKNOWLEDGEMENTS

I am deeply indebted to all my family: my lovely spouse, Mehrshad; my dearest mother and father; my siblings especially my sister, Gilda; and my in-laws for their love and support at any conditions.

I would like to express my appreciation to my promotors, Prof. Magda Vincx, Prof. Jan Vanaverbeke and Prof. Anne Willems for their help and support during my PhD. It was a great honour to work under their supervision.

I would like to thank all members of reading and examination committee (Prof. Olivier De Clerck, Prof. Tom Moens, Prof. Steven Degraer, Dr. Melanie Sapp, Prof. Frederik Leliaert, Prof. Sofie Derycke, Prof. Nico Boon) for their useful comments.

I am very grateful to all staff of the research group Marine Biology and the Laboratory of Microbiology, Ghent University especially to Dirk Van Gansbeke and Bart Beuselinck for their technical assistance in the lab work.

I would like to express my gratitude to all my Iranian friends, to my friends from Belgium, to those friends from other nationalities who I met in Belgium for their kind help. I also want to thank all my diving friends in Belgium for their warm friendship.

در نهایت این پایان نامه را به پدر و مادر عزیزم، به همسرم، به اساتیدم در ایران و کشورم تقدیم می کنم.

Maryam
March 2017

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SUMMARY

Shallow coastal habitats represent some of the most valued ecosystems supporting complex communities of benthic organisms, delivering diverse ecosystem services. Cumulative activities of the benthic fauna affect the physical and chemical conditions of their environment and determine the rate of processes and benthic ecosystem functioning in coastal areas. However, excessive human use of the coastal zone puts extensive pressure on coastal ecosystems leading to biodiversity loss and habitat destruction. Therefore, it is essential to implement Ecosystem-Based Management (EBM) to ensure the protection of coastal resources whilst increasing the efficiency of their uses. Such EBM needs to be based on solid scientific evidence, including a detailed understanding of the link between biodiversity and ecosystem functioning.

Macrofaunal assemblages have been shown to impact microbial-mediated nitrogen cycling processes in marine sediments through their role in affecting the physical and chemical conditions as they affect the exchange processes across the sediment-water interface. However, how the functional traits of macro-organisms, through the interactions with micro-organisms, affect microbial-mediated ecosystem functioning is still not fully understood. This PhD thesis aims to investigate the effect of macrofauna (at the community and species level) on benthic microbial communities (total bacterial and archaeal communities and specific functional groups, nitrifiers and denitrifiers) and the N-cycle processes mediated by them (nitrification and denitrification). The nitrogen cycle is one of the most important biogeochemical cycles in ecosystems. It provides nitrogen as an important component for all living cells and for primary production. It also counteracts eutrophication in the coastal marine area; it also releases back the biologically useful nitrogen into the atmosphere by converting it into gaseous compounds in both terrestrial and marine ecosystems (Canfield *et al*., 2005).

Chapter 1 provides an overview of the present knowledge about the definition of the broad term “Ecosystem functioning” in relation with ecosystem services and relevance for management, and introduces the importance of shallow coastal habitats in marine environments. It further explains N-cycle processes, with focus on microorganisms mediating nitrification and denitrification and the environmental factors affecting these processes. Ultimately the impact of benthic fauna on nitrification and denitrification is

introduced. Objectives, study areas and outline of the thesis are also described in this chapter. In **Chapter 2**, the spatio-temporal variability of sedimentary biogeochemical cycling (nitrification and denitrification) was investigated over a yearly cycle (February to November 2011) at various stations in the Belgian Part of the North Sea (BPNS) representative of a wide range of coastal sediment types. We explored this with respect to variability in environmental parameters and the impact of macrofaunal communities in terms of structural and functional characteristics. The Bioturbation Potential of a macrofaunal community (BPc) was used as an integrated proxy for macrofaunal activity. Our results showed that statistical models based on BPc perform better than models based on structural characteristics, confirming the current knowledge that it is rather the functional biodiversity than taxonomic biodiversity that matters for benthic ecosystem functioning (Emmerson and Raffaelli, 2000; Ieno *et al.,* 2006; Cardinale *et al*., 2012) and further explaining this link on a relatively wide spatial scale in the BPNS over a yearly cycle.

To further detail our knowledge on the link between the macrofauna and benthic processes, we selected certain moments in time considering the annual phytoplankton bloom sedimentation (April, June and September) from the extensive sampling campaign of 2011 for a detailed investigation of the microbial communities of the upper cm of sediment (**Chapter 3**). This yielded a unique dataset allowing us to investigate the link between aspects of microbial diversity and biogeochemical processes on the one hand, and how this link is modulated by macrofaunal structure and/or activity at the community level in space and time, on the other hand. Microbial communities were assessed as total 16S rDNA bacterial and archaeal communities (excluding extracellular DNA) and RNA-based nitrifying communities (bacteria and archaea). Our results in the BPNS, support the link between microbial biodiversity and N-cycle processes in marine sediments as aspects of diversity of the total bacterial community and the ammonia-oxidizing bacteria and archaea (β-AOB and AOA) were correlated, with respectively the rate of N-mineralization and nitrification in the sediment. This link is modulated by the macrofaunal density and/or activity (BPc) and is further affected by the annual spring phytoplankton bloom (water and sediment chl-*a* concentration), and changes in the water temperature, C:N ratio, and grain size in space and time. While

nitrification and denitrification rates were low in permeable and muddy sediments, fine sandy sediments, characterized by highest macrofaunal densities, biomass and bioturbation potential (BPc), showed highest nitrification rates in the summer and highest denitrification rates throughout the year. In addition, our results indicated that bacteria (total and β-AOB) showed more spatio-temporal variation than archaea (total and AOA) as sedimentation of organic matter and the subsequent changes in the environment had a stronger impact on their community composition and diversity indices. The establishment of such links strengthens the call to include benthic microbial indicators as well as macrofaunal indicators and to construct methods integrating these indicators for assessment of sea-floor integrity in policy affairs.

There is a lot of evidence that ecosystem engineers have important effects on biogeochemical processes. In **Chapter 4**, we performed a detailed experiment on the effect of the tube building polychaete, *Lanice conchilega*, on the expression of the functional gene *nosZ*, involved in the final step of the denitrification process. We also aimed to investigate nitrifying organisms but the bacterial *amoA* gene failed to amplify due to the low primer melting temperature for this library. *Lanice conchilega* is known to manifest both autogenic and allogenic ecosystem engineering properties by, respectively its own physical structure and bio-irrigating the tube. This polychaete is present throughout the North Sea and can be found in dense aggregations, referred to as biogenic reefs and is therefore considered as one of the important ecosystem engineers that can be used in the implementation of marine Ecosystem-Based Management in the North Sea. However, our knowledge of how *L. conchilega* affects sedimentary processes through its effect on microorganisms is very limited. We therefore investigated horizontal and vertical patterns in denitrifying microbial communities in *L. conchilega* biogenic reefs in the intertidal zone of Boulogne-sur-Mer (France) at different densities of *L. conchilega* in its patches. Our results showed that high density of *L. conchilega* (3,185-3,440 ind. m^{-2}) has significant effects on the vertical and horizontal distribution of *nosZ* transcripts as well as on variations in composition of *nosZ* transcripts compared with sediments where *Lanice* was absent. The patterns observed in composition and diversity indices of *nosZ* transcripts in *Lanice* reefs are associated with the heterogeneity in geochemical environments (oxygen penetration

depth, oxygen concentration in space and time, % silt+clay and chl *a* concentrations) in the sediment and resulted mainly from the allogenic effects of *Lanice* in our study site.

In **Chapter 5**, the gained knowledge on the structural and functional effects of macrofauna on the microbiota-ecosystem functioning link in subtidal and intertidal bioturbated sediments is summarized and discussed with regard to the need for such integrated link in implementing Ecosystem-Based Management. We further suggest to develop better descriptions of bioturbation potential or extend the BPc index with a proxy for bio-irrigation in future investigations. We also briefly discuss methodological challenges when studying the link between microorganisms and their activity and how our research strategy avoided some of these issues. We showed that the use of active functional genes (*amoA* bacteria, *amoA* archaea and *nosZ* genes) reveals better insights in the macrofauna – microbe – processes interactions compared to DNA-based or the total microbial community investigation.

SAMENVATTING

Ondiepe kust habitats behoren tot de meest waardevolle ecosystemen die complexe gemeenschappen van benthische organismen herbergen die instaan voor diverse ecosysteemdiensten. Cumulatieve activiteiten van de benthische fauna dragen bij tot fysische en chemische omgevingsparameters en beïnvloeden zo de snelheid van biogeochemische processen en het functioneren van benthische systemen in kustgebieden. Het intensief gebruik van de kustzone door de mens resulteert echter in grote druk op kustecosystemen wat leidt tot het verdwijnen van habitats en verlies aan biodiversiteit. Het is daarom noodzakelijk om Ecosysteem-gebaseerd beheer (Ecosystem-Based Management, EBM) in te voeren, om natuurlijke kustrijkdommen te beschermen en ook efficiënter te gebruiken. Dergelijk EBM moet steunen op een sterke wetenschappelijke basis, inclusief een grondig begrip van het verband tussen biodiversiteit en het functioneren van het ecosysteem.

Er werd reeds aangetoond dat macrofauna gemeenschappen microbiële processen in de stikstofcyclus in mariene sedimenten beïnvloeden door hun effect op de fysische en chemische eigenschappen van de omgeving, beïnvloeden ze ook uitwisselingsprocessen over het raakvlak van water en sedimenten. Hoe de functionele kenmerken van macro-organismen, in interactie met micro-organismen, precies bijdragen tot het microbiële ecosysteem functioneren, blijft echter grotendeels onbekend. Deze doctoraatstudie had tot doel het effect van macrofauna (op gemeenschaps- en soortniveau) na te gaan op benthische microbiële gemeenschappen (totale gemeenschap en bepaalde functionele subgroepen) en hun activiteiten in de stikstofcyclus (nitrificatie en denitrificatie). De stikstofcyclus is een van de meest belangrijke biogeochemische cycli in ecosystemen. Hij voorziet alle levende cellen van de noodzakelijke stikstof ook voor primaire productie en remedieert eutrofiëring in mariene kustzones door biologisch actieve stikstof om te zetten tot gassen die vrijgesteld worden in de atmosfeer in terrestrische en mariene systemen (Canfield *et al*. 2005).

Hoofdstuk 1 geeft een overzicht van de huidige kennis rond de definitie van het brede begrip “Ecosysteem functioneren” in relatie tot ecosysteemdiensten en belang voor beheer, en introduceert het belang van ondiepe kust habitats in mariene systemen. Het

geeft een overzicht van de stappen in de stiktofcyclus, met nadruk op de micro-organismen die instaan voor nitrificatie en denitrificatie en de bepalende omgevingsfactoren. Tot slot wordt het effect van benthische fauna op nitrificatie en denitrificatie besproken. In **Hoofdstuk 2** werd de ruimtelijke en temporele variabiliteit van sedimentaire biogeochemische processen (nitrificatie en denitrificatie) onderzocht over een jaarcyclus (feb.- tot nov. 2011) in verschillende stations van het Belgische deel van de Noordzee (BPNS) die een brede waaier aan kustsedimenttypes vertegenwoordigen. We onderzochten de variatie in omgevingsparameters en de impact van structurele en functionele kenmerken van de macrofaunagemeenschap. Het Bioturbatie Potentieel van een macrofaunagemeenschap (BPc) werd gebruikt als een geïntegreerde proxy voor macrofauna activiteit. Onze resultaten toonden aan dat statistische modellen gebaseerd op BPc beter waren dan modellen op basis van structurele kenmerken en bevestigden dat de functionele eerder dan de taxonomische biodiversiteit van belang is voor het functioneren van benthische ecosystemen (Emmerson and Raffaelli, 2000; Ieno *et al.,* 2006; Cardinale *et al.,* 2012) en verklaren deze link over een relatief brede ruimtelijke schaal in het BPNS over een jaarcyclus.

Om onze kennis over het verband tussen macrofauna en benthische processen verder uit te bouwen, selecteerden we tijdspunten van fytoplanktonbloei (april, juni, september 2011) voor een uitgebreide staalname voor een grondige studie van de microbiële gemeenschappen in de bovenste cm van het sediment (**Hoofdstuk 3**). Dit leverde een unieke dataset die ons toeliet het verband te onderzoeken tussen specifieke functionele aspecten van microbiële diversiteit en biogeochemische processen enerzijds, maar anderzijds ook hoe deze interacties beïnvloed worden door structurele eigenschappen en/of fysiologische activiteiten van de marcofauna gemeenschap over tijd en plaats. Microbiële gemeenschappen werden gekarakteriseerd door onderzoek van van de totale 16S rDNA bacteriële en archaea gemeenschap (exclusief extracellulair DNA) en de RNA-gebaseerde nitrifiërende gemeenschap(bacteria en archaea). Onze resultaten voor het BPNS ondersteunen het verband tussen microbiële biodiversiteit en processen in de stikstofcyclus in mariene sedimenten, aangezien er een correlatie was tussen diversiteitsaspecten van de totale bacteriële gemeenschap en de ammonia-oxiderende bacteria en archaea (β-AOB en AOA) en respectievelijk stikstofmineralisatie en

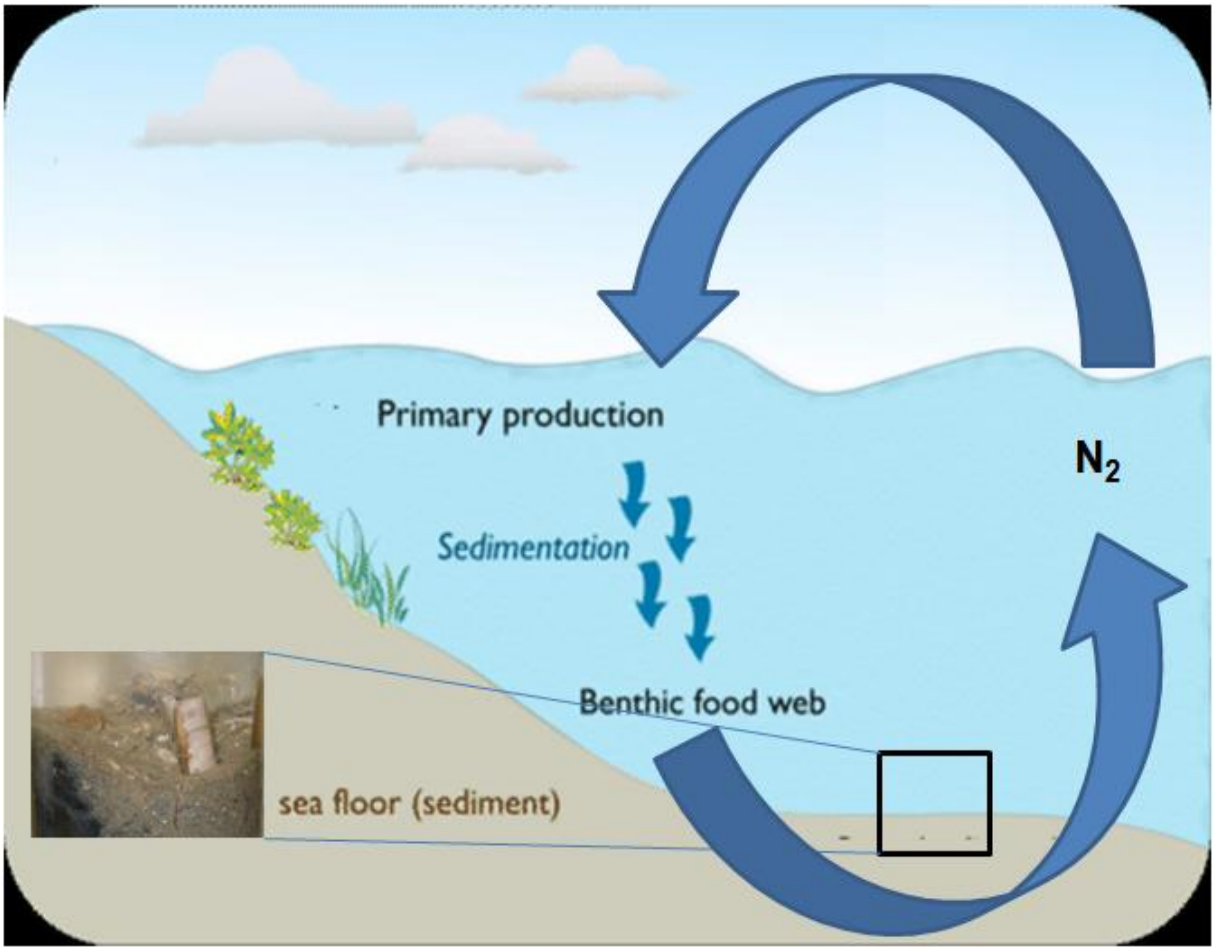
nitrificatie snelheid in het sediment. Deze relatie wordt beïnvloed door de macrofauna densiteit en/of activiteit (BPc) en ook door de jaarlijkse fytoplankton lentebloei (chl-*a* concentratie in water en sediment), en temporele en spatiale veranderingen in watertemperatuur, C:N verhouding, korrelgrootte. Terwijl nitrificatie- en denitrificatiesnelheden laag zijn in doorlaatbare modderige sedimenten, vertoonden fijne zandige sedimenten met hoge densiteiten aan macrofauna, biomassa en bioturbatie potentieel (BPc), de hoogste nitrificatiesnelheden in de zomer en de hoogste denitrificatiesnelheden gedurende heel het jaar. Ook toonden onze resultaten dat bacteria (totale gemeenschap en β-AOB) meer varieerden over ruimte en tijd dan Archaea (totale gemeenschap en AOA) aangezien sedimentatie van organisch materiaal en daaropvolgende veranderingen in de omgeving een groter effect hadden op hun gemeenschapssamenstelling en diversiteitsparameters. De sterke relatie tussen BPc en biogeochemsiche processen in fijne zandige sedimenten weerspiegelt het belang van macrofauna activiteit en toont het belang aan van een beheer dat rekening houdt met functionele kenmerken van benthische gemeenschappen bij het beslissingsproces. De vaststelling van deze verbanden versterkt de nood om benthische microbiële indicators en ook macrofauna indicators mee te nemen en methoden te ontwikkelen om deze te integreren bij het beheer van zeebodem integriteit.
Er zijn veel aanwijzingen dat “ecosysteem ingenieers” belangrijke effecten hebben op biogeochemische processen. In **Hoofdstuk 4** voerden we een grondig experimenteel onderzoek uit naar het effect van de buisvormende polychete worm *Lanice conchilega* op de expressie van het functionele gene *nosZ*, betrokken bij de laatste stap van het denitrificatieproces. We planden ook om nitirifiërende organismen te onderzoeken, maar konden het bacteriële *amoA* gen niet amplificeren wegens de lage primer smelttemperatuur voor dit gen. *L. conchilega* vertoont zowel autogene als allogene ecosysteem wijzigende eigenschappen door respectievelijk zijn eigen fysische structuur en het bio-irrigeren van zijn buis. Deze polycheet komt voor in heel de Noordzee en kan dichtbevolkte aggregaten vormen, biogene riffen genaamd, en worst daarom beschouwd als een van de belangrijke “ecosysteem engineers” die kunnen gebruikt worden bij de implementering van EBM in de Noordzee. Onze kennis over hoe *L. conchilega* de sedimentaire processen beïnvloedt door zijn effect op micro-organismen

is echter zeer beperkt. We onderzochten daarom horizontale en verticale patronen in de denitrifiërende microbiële gemeenschappen in *L. conchilega* biogene riffen in de intertidale zone van Boulogne-sur-Mer (Frankrijk) bij verschillende densiteiten van *L. conchilega*. Onze resultaten toonden aan dat vergeleken met sedimenten zonder *Lanice*, hoge dichtheden (3,185 - 3,440 ind.m^{-2}) significante effecten hebben op zowel de verticale en horizontale verspreiding van *nosZ* transcripten als op variaties in de samenstelling van deze transcripten. De waargenomen patronen in samenstelling en diversiteitsindices van *nosZ* transcripts in *Lanice* riffen zijn gerelateerd aan de heterogeniteit van de geochemische parameters van het sediment (zuurstofdiepte en zuurstofconcentratie over tijd en ruimte, %slib+klei en chl *a* concentraties) en werden vooral veroorzaakt door allogene effecten van *Lanice* in ons studiegebied.

In **Hoofdstuk 5** wordt de bekomen kennis over de structurerende en functionele effecten van macrofauna op de relatie tussen microbiota en ecosysteem functioneren in subtidale sedimenten met bioturbators, samengevat. Het belang van integratie van deze interacties bij de implementering van “Ecosystem-Based Management” wordt besproken. We stellen verder voor om betere beschrijvingen van het bioturbatie potentieel te ontwikkelen of om de BPc index uit te breiden met een proxy voor bio-irrigatie bij toekomstig onderzoek. Ook bespreken we kort de methodologische uitdagingen bij het onderzoek naar het verband tussen micro-organismen en hun activiteiten en hoe we in onze onderzoeksstrategie hiermee omgingen. We toonden aan dat het gebruik van actieve functionele genen (*amoA* bacteria, *amoA* archaea en *nosZ* genen) grondiger inzichten geeft in de macrofauna – micro-organisme interacties en processen in vergelijking met DNA-gebaseerde of totale microbiële gemeenschap studies.

Chapter 1

General Introduction, Aims and Thesis Outline



1. Biodiversity, Ecosystem Functioning, Ecosystem Services and Ecosystem Management

"Ecosystem functioning" means the overall processes that sustain an ecological system (Jax, 2005; Pinto *et al*., 2014) in terms of efficient circulation of matter and energy (Boero and Bonsdorff, 2007) such as primary productivity, decomposition and nutrient cycling (Naeem *et al*., 1999; Duncan *et al*., 2015). Ecosystem functioning ultimately provides ''ecosystem services'', such as food, medicines, cleansing air and water (Daily *et al*., 1997; Hooper *et al.,* 2005; Duncan *et al*., 2015) which are of utilitarian value to society. On the other hand, society is managing ecosystems, by regulating land or sea use and the intensity of the human activities. Both using and managing natural resources affects the abiotic environment, biodiversity and ecosystem functioning and hence the provision of ecosystem services (Figure 1).

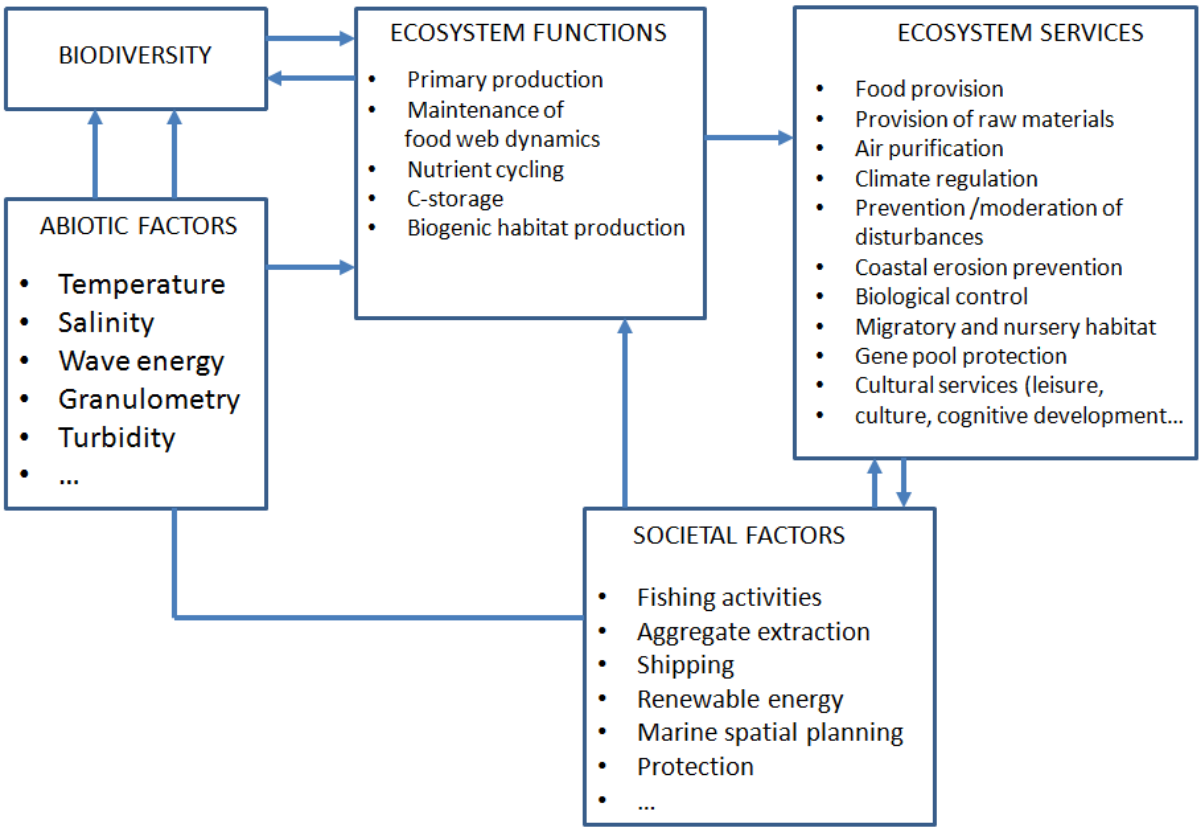


Figure 1: Conceptual framework showing the links between biodiversity, ecosystem functioning, ecosystem services, abiotic factors and society (modified from Duncan *et al*., 2015).

In the marine environment, human activities are extensive and only few areas remain untouched by these activities. Especially in coastal marine areas, the development of

a wide variety of activities results in competition for space and resources which leads to calls for a more effective management. Since the 1990s, marine management has shifted from a sectoral approach towards a management based focusing on interactions between ecosystem components and the position of humans within these systems (Atkins *et al*., 2011). The understanding of ecosystem services is central to this approach, as human activities indirectly affect the provision of direct and indirect contributions to human well-being (de Groot *et al*., 2010). It is now acknowledged that the relation between biodiversity and the provisioning of *ecosystem services* should be tackled by investigating the relations between biodiversity and *ecosystem functioning* while at the same time moving away from investigating biodiversity from a species-identity perspective to an approach taking into account functional traits (Duncan *et al*., 2015).

Ecosystem processes reflect the collective activities of plants, animals, and microbes, interactions of different biota and the effects of these activities - feeding, growing, moving, excreting waste, competition on resources etc - on the physical and chemical conditions of their environment (Naeem *et al*., 1999; Naeem, 2002; Hooper *et al.,* 2005; Rennenberg, 2015). These processes are therefore controlled by structural biodiversity (i.e. community complexity; Naeem *et al*., 1999; Duncan *et al*., 2015; Brose and Hillebrand, 2016) and functional biodiversity (i.e. the value and the range of those species and organismal traits that influence ecosystem functioning; Tilman, 2001) as well as by abiotic factors (e.g. inorganic nutrient inputs, resource availability, temperature, disturbance) (Hooper *et al*., 2005; Duncan *et al*., 2015). In this thesis, we will focus on aspects of ecosystem functioning in shallow coastal sea-floor habitats, hence on “benthic ecosystem functioning”. In Europe, the importance of benthic ecosystem functioning has been recognized trough the Marine Strategy Framework Directive (MSFD, EC Directive 2008/56/EC), a legislative framework aiming to achieve the Good Environmental Status (GES) of EU’s marine waters by 2020 and to protect the resource base upon which marine-related economics and social activities depend. Descriptor 6 of the MSFD describes GES as “*Sea-floor integrity is at a level that ensures that the structure and functions of the seafoor are safeguarded and benthic ecosystems, in particular, are not adversely affected*” (http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32008L0056&from=EN).

An important function of coastal sea floors is the mineralization of organic matter. Wollast (1998) estimated that over 80% of global benthic mineralization occurs at

shelf sediments as up to 50% of the primary production is deposited on the bottom, while cycling of primary production in the open ocean is merely a water-column process. Mineralization of this organic matter in the sediment replenishes the pelagic nutrient pool available for primary production (Blackburn, 1988) which is another important ecosystem function at the basis of the provision of food. Primary production rates and phytoplankton dynamics depend largely on the timing and magnitude of nutrient release during benthic organic matter mineralization (Soetaert and Middelburg, 2009). As such, benthic mineralization processes should be considered important in the framework of MSFD Descriptor 6, however developing indicators for these processes is not straightforward due to a lack of knowledge on the mechanisms structuring these processes. Mineralization is essentially a microbial process affected by a suite of environmental variables (Langenheder *et al*., 2010) and modulated by the activity of larger, macrofaunal organisms (see Stief, 2013). Most of the available information stems from single-species incubations in limited sedimentary environments, while the development of indicators for assessing the environmental status requires information on a wide range of field situations. While the ultimate goal of this thesis is not a direct development of relevant indicators, it does aim at providing sound scientific information on the interaction between environmental variables, macrofaunal activity, and functional aspects of microbial communities that can be applied in an evidence-based development of such indicators.

2. Importance of Shallow Coastal Habitats

Shallow coastal habitats and continental shelf sediments represent some of the most valued ecosystems (Snelgrove, 1999); with high productivity and rich species diversity, supporting complex communities of benthic organisms, delivering diverse ecosystem services and providing considerable social, economic, and aesthetic value to human populations (Wilson and Farber, 2008). These areas are bordered by the highest human population densities and thus experience multiple stressors such as habitat destruction and modifications (Clarke Murray *et al*., 2014), increasing nutrient supply (derived from agricultural activities) (Beach, 2002; Howarth *et al.,* 2002; Seitzinger *et al.,* 2002) and eutrophication-induced oxygen stress (hypoxia) (Diaz and Rosenberg, 2008; Van Colen *et al*., 2012). Shallow coastal habitats are also vulnerable to ocean acidification and increasing temperature (Anthony *et al.,* 2009).

Although shallow coastal ecosystems represent less than 2% of the oceanic surface, they produce about 20% of the global marine primary production (Pedersen *et al.,* 2004). Sediment and water column interactions are of significant ecological importance to these shallow waters (Reay *et al.,* 1995). The significance of these interactions has been demonstrated in terms of both nutrient and oxygen dynamics (Zeitzschel, 1979; Garber, 1987). Nutrient regeneration in sediments and subsequent release to the overlying water column, commonly referred to as benthic-pelagic coupling, fuels a significant portion of water column production in shallow marine systems (Nixon *et al.,* 1976). As such, the exchange of particulate and dissolved nutrients between sediment and water is particularly important for the functioning of shallow coastal and estuarine ecosystems (Brigolin *et al.,* 2011; Brady *et al.,* 2013). Factors such as water depth, turbidity, sedimentation rates, organic matter mineralization rates, bioturbation (particle mixing), bio-irrigation (solute transfer) and diffusive or advective transport (Jahnke *et al.,* 2000; Huettel *et al.,* 2003; Woodin *et al*., 2016) influence the magnitude and efficiency of the benthic-pelagic coupling (Porubsky, 2008).

Due to the high structural and functional diversity among species, high productivity, benthic-pelagic coupling and contrasting sediment types from muddy to permeable, coastal areas are characterized as an ecosystem with significant ecological importance to biogeochemical exchanges and therefore nutrient cycles (e.g., carbon and nitrogen cycles). These regions are also under the influence of terrestrial (via rivers and groundwater) and atmospheric nutrient loadings (e.g., nitrate, ammonium, and phosphate) (Jickells, 1998; Siefert, 2004), which vary regionally and seasonally (Siefert, 2004). The dynamic recycling also operates on a much shorter time scale especially in the shallow coastal areas (Jørgensen, 1983). Therefore, coastal areas are valuable to investigate biogeochemical cycling and the factors deriving these processes in marine ecosystems.

3. Nitrogen Cycling Processes

The nitrogen cycle is one of the most important biogeochemical cycles in ecosystems. It provides nitrogen as an important component for all living cells and for primary production; while counteracting eutrophication in the coastal marine area, it also releases back the biologically useful nitrogen into the atmosphere by converting it into gaseous compounds in both terrestrial and marine ecosystems (Canfield *et al*.,

2005). Therefore, it is of importance to investigate the nitrogen cycle, to understand the mechanisms and microorganisms involved as well as the biotic and abiotic factors affecting these processes. Below, the nitrogen cycle is explained in more detail.

Nitrogen is a key constituent of many important biomolecules, such as amino acids, nucleic acids, chlorophylls, and thus it is essential to all living organisms. Sediments and sedimentary rocks are among the largest reservoirs of nitrogen. However, they are not actively cycled by organisms. Of comparable size is the reservoir of atmospheric N_2, which accounts for 78% of the gas in the atmosphere (Canfield *et al.,* 2005; Osman, 2013). Living biomass, dead organic (detrital) pool and inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) comprise comparably the small nitrogen reservoirs in aquatic and terrestrial ecosystems. However, the biologically available forms of nitrogen, which can be used for primary production, consist of dissolved inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) which accounts for < 1% of other nitrogen reservoirs (Mackenzie, 1998; Canfield *et al.,* 2005). The biological N-fixation process (changing N_2 into a biologically useful form) and the processes of nitrogen loss to the atmosphere (denitrification and anammox) interact with the large atmospheric N_2 pool. However, the turnover time of the atmospheric N_2 pool is slow (Canfield *et al.,* 2005; Bernhard, 2010) as most biota are unable to fix molecular nitrogen (Möller, 2014). In terrestrial environments, next to biological nitrogen fixation by a specialized group of bacteria and archaea (Canfield *et al.,* 2005; Dos Santos *et al*., 2012), industrial nitrogen fixation into synthetic fertilizer by the Haber-Bosch process increases the amount of available inorganic nitrogen (Canfield *et al.,* 2005). In marine environments, nitrogen availability is the limiting factor for primary production over much of the ocean (Ryther and Dunstan, 1971; Herbert, 1999; Howarth and Marino, 2006; DeVries *et al*., 2012). Knowledge on the nitrogen cycle is therefore of high importance for the understanding of the functioning of these environments.

Recent research suggests that the state of balance of the present-day marine nitrogen budget is in doubt (Ganeshram *et al.,* 1995; Middelburg *et al.,* 1996; Alkhatib *et al.,* 2012). Some studies suggest that global estimates of denitrification are rising, leading to a significant loss of nitrogen from marine areas (Ryther and Dunstan, 1971; Herbert, 1999; Canfield *et al.,* 2005); others argue that an almost balanced budget exists (e.g. DeVries *et al.,* 2012). On the other hand, many coastal environments today are subjected to increasingly high levels of anthropogenic

nitrogen enrichment and eutrophication problems arisen due to agricultural activities, aquaculture and industrial development (Kennish, 2002; Boyer and Howarth, 2008; Mosier, 2011).

Organic matter arrives at the sea floor in coastal sediments by phytoplankton bloom sedimentation (Lancelot *et al.,* 2005; Provoost *et al.,* 2013), river runoff (Fagervold *et al.,* 2014) and microphytobenthos and macrophyte (macro algae) production depending on depth and light (Hardison *et al.,* 2013). A critical process in the nitrogen cycle is the N-mineralization or conversion of organic nitrogen in particulate organic materials and dead biomass (detritus) into simpler compounds that can be taken up by other microbes together with a concurrent release of inorganic nitrogen (NH_4^+) (e.g. Robertson and Groffman, 2007). N-mineralization is therefore also known as ammonification (Rydin and Jeglum, 2013) and carried out by a wide range of microorganisms (bacteria and archaea) from aerobes to anaerobes and also fungi (e.g. Robertson and Groffman, 2007). The NH_4^+ produced (Figure 2) can be incorporated back into biomolecules (assimilation) by microphytobenthos when there is light, transported to the water column by molecular diffusion as a source of recycled nitrogen for primary production and/or oxidized by nitrifying organisms under oxic conditions (Seitzinger, 1990; Canfiled *et al*., 2005; Fenchel *et al*., 2012).

Nitrification is a two-step aerobic oxidation process carried out by nitrifying organisms (bacteria and archaea) in which ammonia is converted to nitrite with hydroxylamine as an intermediate ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^-$; ammonia oxidation) and nitrite is converted to nitrate in the second step ($NO_2^- \rightarrow NO_3^-$; nitrite oxidation), performed by different microorganisms. However, recently it was found that complete oxidation of ammonia to nitrate can also be carried out in a single organism (complete ammonia oxidation; comammox) (Daims *et al*., 2015; van Kessel *et al*., 2015). Nitrifying organisms are generally chemoautotrophs (see heterotrophic nitrification in Box 1), and use CO_2 as their carbon source for growth. Nitrification is an important process in the nitrogen cycle especially in marine coastal area as it links degradation of organic matter (N-mineralization) with removal of bioavailable nitrogen to the atmosphere (denitrification) (Blackburn and Blackburn, 1992; Canfield *et al.,* 2005).

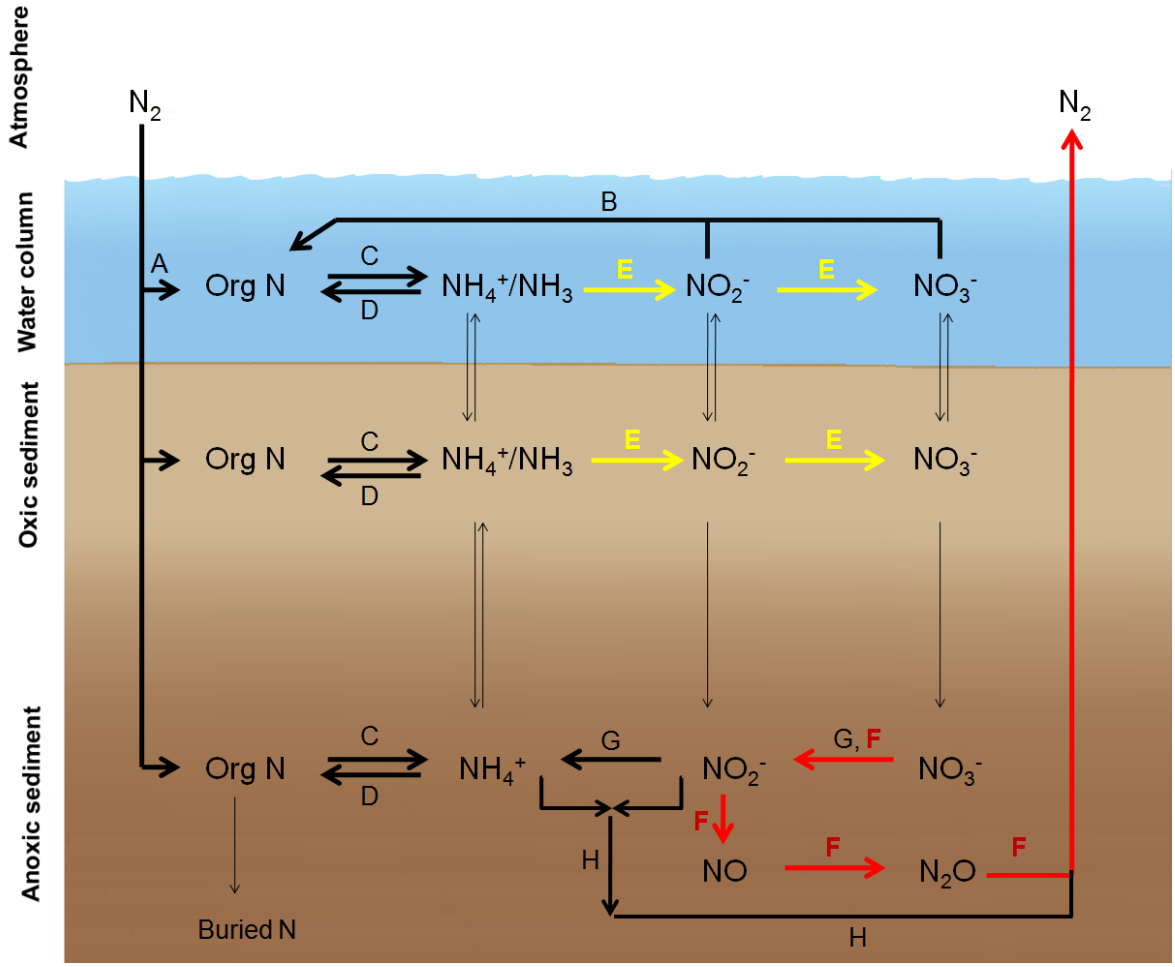


Figure 2: Microbial nitrogen cycling in aquatic environments. A: nitrogen fixation; B: NO_x assimilation; C: ammonification (mineralization); D: NH_4^+ assimilation; E: nitrification (NH_3 and NO_2^- oxidation); F: denitrification; G: NO_3^- ammonification (DNRA); H: anammox (modified after Canfield *et al.,* 2005). Note: Ammonia is used as a substrate for the first step of nitrification (ammonia oxidation). Nitrification and denitrification processes are shown in different colours (yellow and red, respectively).

BOX 1:

Anoxic nitrification

The chemolithoautotrophic oxidation of NH_4^+ might also occur under anoxic conditions. Conventional understanding of the nitrogen cycle in marine sediments has changed in recent years with the discovery of this alternative pathway for ammonia oxidation via the reduction of manganese oxides (MnO_2) as an electron acceptor in anoxic sediments (Canfield *et al*., 2005; Bartlett *et al*., 2008).

$$NH_4^+ + 4MnO_2 + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O$$

However, there are still few reports of the occurrence of anaerobic nitrification. Due to complex recycling of redox species, these processes are difficult to discern and therefore poorly understood (Fernandes *et al*., 2015).

Heterotrophic nitrification

Heterotrophic nitrification is the oxidation of inorganic (NH_4^+) or organic reduced forms of N, to nitrate by a wide range of fungi and *heterotrophic* bacteria (Canfield *et al*., 2005; Prosser, 2005; Ward, 2008).
It has been argued that heterotrophic nitrification involves enzyme systems that are quite different from those of the autotrophs (Canfield *et al*., 2005; Ward, 2008). Heterotrophic nitrification cannot serve as an energy generating mechanism, as the autotrophic process does. Therefore, the nitrification rate of heterotrophs is slower (generally 1000 to 10,000 times; Canfield *et al*., 2005) than for chemolithoautotrophs (Prosser, 2005; Ward, 2008). However, it may be important in acid soils or where C:N ratio and heterotrophic biomass are high (Prosser, 2005). Nevertheless, our knowledge on the role of heterotrophic nitrification in aquatic ecosystems is still limited (Canfield *et al*., 2005; Ward, 2008).
Recent research has highlighted the existence of bacteria with the marine origin (*Pseudomonas* sp. ADN-42) that are capable of performing simultaneously heterotrophic nitrification and aerobic denitrification (Jin *et al*., 2015).

Denitrification, a heterotrophic microbial process (Bernhard, 2010), consists of four respiratory reduction steps, in which nitrate (NO_3^-) is reduced sequentially to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas (N_2) (Philippot, 2002; Francis *et al.,* 2007). When the oxygen supply is limited, facultative denitrifiers substitute nitrate for oxygen as an electron acceptor for respiration (e.g. Skiba, 2008). Therefore, denitrification has been considered as an anaerobic microbial process (Vymazal, 2006; Bernhard, 2010). This is a cellular adaptation process during oxygen

depletion (Laass *et al*., 2014). However, recent studies revealed that in the presence of oxygen, some denitrifying organisms are also able to co-consume O_2 and NO_3^- (termed aerobic denitrification) (Gao *et al*., 2010). Therefore, the oxygen level for denitrification can differ between denitrifiers. While inhibition of oxygen on the denitrification enzymes of *Agrobacterium tumefaciens* was reported (Kampschreur *et al*., 2012), *Pseudomonas stutzeri* is able to denitrify under aerobic conditions (Su *et al.*, 2001).

Denitrification is a key process in the N-cycle as a primary loss mechanism for N in the N budget (Jenkins and Zehr, 2008) of coastal waters, counteracting eutrophication on the one hand, but contributing to global warming and ozone depletion (Knowles, 1982; Sanford *et al*., 2012) on the other hand (see Box 2) .

BOX 2:

N_2O is an important greenhouse gas as one molecule of N_2O has a global warming potential that is 296 times that of a molecule of CO_2 (although the N_2O emission is much less than CO_2; https://www.epa.gov/ghgemissions/overview-greenhouse-gases). In addition, the production of NO is detrimental for the ozone layer (Kohn, 2015). Some denitrifying organisms may have truncated pathways, where the end product of nitrate reduction is N_2O rather than N_2 (Zumft, 1997; Wang *et al*., 2008) although N_2O reduction is energy conserving (Zumft, 1997). It is likely they lack the gene for N_2O reduction (the *nosZ* gene) (Zumft, 1997). Some strains do not encode the gene for NO reductase (Casella *et al*., 2006). However, denitrification is not the only possible route of production and consumption of these intermediates (van Groenigen *et al*., 2015). For example, non-denitrifiers possessing the “atypical” N_2O reductase gene play also a significant role in N_2O consumption especially in soil and/or there is evidence of N_2O fixation via nitrogenase to NH_3 (van Groenigen *et al*., 2015). Published estimates of current natural emission of N_2O from terrestrial, marine and atmosphere sources range from 10 to 12 Tg N_2O-N yr^{-1}, of which 3.8 Tg N_2O-N yr^{-1} is emitted from marine environments. Anthropogenic N_2O emissions for example from agriculture and industry are about 5.3 Tg N_2O-N yr^{-1} (Davidson and Kanter, 2014).

For a long time denitrification was considered the most important pathway for reducing nitrate and nitrite (NO_x^-) in the environment. However, the dissimilatory nitrate reduction to ammonium (DNRA or NO_3^- ammonification) and anammox ($NH_4^+ + NO_2^- \rightarrow N_2$; anaerobic ammonium oxidation) have been recognized as important pathways, alternative to denitrification, that can reduce NO_x^- in the absence or near

absence of O_2 (Figure 2; Van De Graaf *et al*., 1995 and 1996; An and Gardner, 2002; Canfield *et al.,* 2005; Burgin and Hamilton, 2007). However, DNRA yields NH_4^+ instead of N_2 and thus increases nitrogen retention in the ecosystem (Canfield *et al.,* 2005). Anammox, which produces N_2 gas, is low in shallow sediments where denitrification is orders of magnitude higher because of the availability of labile sedimentary organic matter as electron donors (Seitzinger, 1988; Canfield *et al.,* 2005; Op den Camp *et al.,* 2006; Thamdrup, 2012).

As denitrification depends mainly on the supply of nitrate by nitrification, the two processes are coupled functionally in sediment systems (Klingensmith and Alexander, 1983), which is referred to as the benthic coupled nitrification-denitrification.

Coupled nitrification-denitrification constitutes an important aspect of marine benthic ecosystem processes, often interacting in close proximity in the environment across oxic/anoxic interfaces involving the diffusion of substrates and products along concentration gradients over scales ranging from micrometers to centimeters (Joye and Hollibaugh, 1995). Many studies have investigated these biogeochemical processes (so-called because of an interaction between physical, chemical and biological processes in an ecosystem; Inglett *et al.,* 2008) in various ecosystems including terrestrial (Stange and Neue, 2009; Chen *et al.,* 2013), estuarine (Bernhard *et al*, 2010; Wankel *et al.,* 2011) and coastal systems - both in the water column (De Corte *et al.,* 2009; Christman *et al*, 2011; Veuger *et al.,* 2013) and sediments (Nold *et al.,* 2000; Liu *et al.,* 2003; Beman *et al.,* 2012) - as well as in wastewater in order to remove potentially harmful levels of ammonium (Bernhard, 2010).

4. Microorganisms Mediating Nitrification and Denitrification

The two steps of nitrification (ammonia and nitrite oxidation) are mainly accomplished by different groups of organisms together referred to as nitrifiers: ammonia-oxidizing bacteria and ammonia-oxidizing archaea (AOB and AOA; oxidizing ammonia to nitrite) and nitrite-oxidizing bacteria (NOB; oxidizing nitrite to nitrate) (Koch *et al*., 2015).

Ammonia oxidation is the first and rate-limiting step in nitrification. Previously, scientists believed that the most important contributors to ammonia oxidation were restricted to two groups of Proteobacteria: *(i)* the ammonia-oxidizing Betaproteobacteria (β-AOB): the genera *Nitrosomonas* and *Nitrosospira* comprising

the main genera of β-AOB in marine ecosystems that have been the subject of many studies (Head *et al*., 1993; Purkhold *et al.,* 2003; Freitag and Prosser, 2004; Canfield *et al.,* 2005; Ward, 2008) and *(ii)* ammonia-oxidizing Gammaproteobacteria (γ-AOB): the genus *Nitrosococcus* (Ward and O'Mullan, 2002) with a single species (*Nitrosococcus oceanus*) in marine sediments (Canfield *et al.,* 2005). γ-AOB are generally less common and widespread in the environment compared to β-AOB (Ward, 2011).

Recently, ammonia-oxidizing archaea (AOA) have been found to be also potentially important in nitrogen cycling in a variety of environments such as marine waters and sediments (Francis, *et al.,* 2007). The cultivation and the discovery of AOA showed that these organisms outnumber their bacterial counterparts in many habitats (Ward, 2011; Hatzenpichler, 2012) such as the water column of the North Sea and Atlantic Ocean (Wuchter *et al.,* 2006). However, there are also studies indicating the opposite view (Wankel *et al.,* 2011; Gilbertson *et al.,* 2012). For example, in Elkhorn Slough estuary in California, impacted by agricultural activities, AOB *amoA* gene copies outnumbered AOA *amoA* gene copies in the sediment (Wankel *et al.,* 2011).

Nitrite-oxidizing bacteria (NOB) are chemolithoautotrophic or mixotrophic organisms (augmenting their autotrophic life with heterotrophic metabolisms) (Canfield *et al.,* 2005; Abeliovich, 2006; Spieck and Lipski, 2011) and are known as fastidious organisms, which are difficult to grow and work with (Abeliovich, 2006; Spieck and Lipski, 2011). Their growth is slow due to the poor energy balance (Spieck and Lipski, 2011) as nitrite-oxidation yields less biochemically useful energy in comparison with ammonia-oxidation (Canfield *et al.,* 2005; Ward, 2013; Figure 3).

Recently, the discovery and cultivation of special nitrifying bacteria from the genus *Nitrospira*, a globally distributed group of nitrite oxidizers, has been reported. It is capable of complete nitrification (complete ammonia oxidation; comammox) and grow by the oxidation of ammonia to nitrate. This process is also energetically feasible and advantageous since it yields more energy ($\Delta G^{\circ\prime}$ = -349 kJ mol^{-1} NH_3) than either single step (Daims *et al*., 2015; van Kessel *et al*., 2015).

New methods targeting functional genes encoding enzymes involved in specific N transformations allow the direct (cultivation-independent molecular surveys) quantification and identification of microorganisms involved in N-cycling. These approaches are rapidly increasing the knowledge on the diversity, distribution and activities of microbial communities involved in N-cycling (Bernhard *et al.,* 2010; Reed

et al., 2010; Singh *et al.,* 2010; Christman *et al.,* 2011; Herrmann *et al.,* 2011; Wankel *et al.,* 2011; Beman *et al.,* 2012). Nitrification steps are catalyzed by various enzymes encoded by different genes. The "***amo****"* genes, encoding the active subunits (A, B, C) of the ammonia monooxygenase enzyme, are responsible for the first reaction of ammonia oxidation converting NH_3 to NH_2OH ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^-$) (e.g. Canfield *et al.,* 2005; Figure 3). *amoA* is a widely used marker for molecular studies of ammonia-oxidizing microorganisms in environmental systems (e.g. Abell *et al*., 2011). This gene is conserved across known ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Archaeal and Bacterial *amoA* only share 25% AA sequence identity, which allows for definitive phylogenetic distinction between AOA and AOB populations (Nicol and Schleper, 2006).

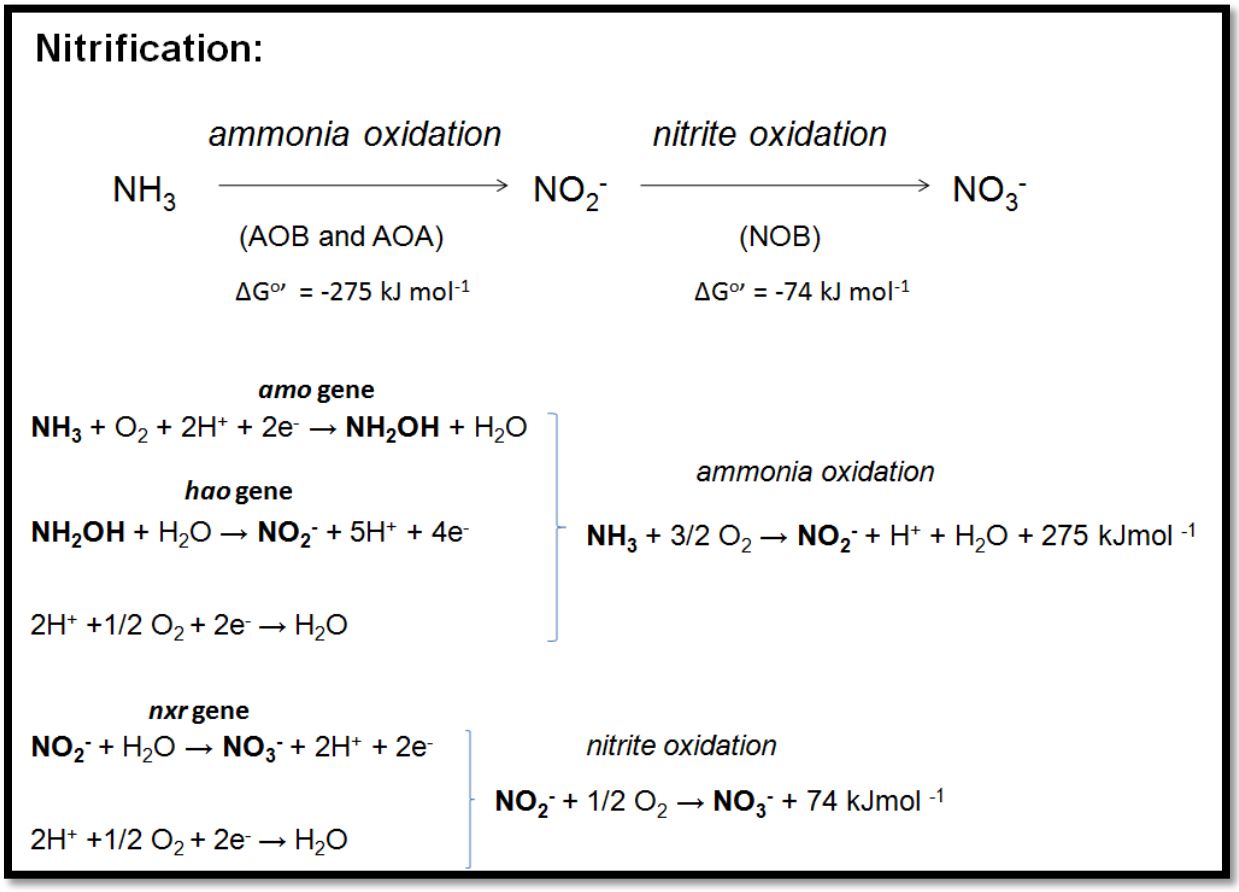


Figure 3: The two steps of nitrification (ammonia and nitrite oxidation): stoichiometry of reactions; genes encoding each step (*amo* and *hao* genes encoding ammonia oxidation and *nxr* encoding nitrite oxidation); microorganisms involved in each step (AOB and AOA: ammonia-oxidizing bacteria and archaea carry out ammonia oxidation; NOB: nitrite oxidizing bacteria involve in nitrite oxidation); and the amount of energy gained from each step (Prosser, 2005; Ward, 2008; Pester *et al*., 2014; Daims *et al*., 2015).

Identified *Nitrospira* species capable of complete nitrification (comammox) encode all enzymes necessary for ammonia oxidation via nitrite to nitrate in their genomes. Their ammonia monooxygenase (AMO) enzymes are also phylogenetically distinct from currently identified AMOs (Daims *et al*., 2015; van Kessel *et al*., 2015).

Denitrification is mediated by a taxonomically diverse group of microorganisms, mainly bacteria (Canfield *et al*., 2005). Nearly 130 denitrifying bacterial species have been reported within more than 50 genera and most of these were found within the Alpha, Beta and Gamma (α, β, γ) classes of the Proteobacteria (Zumft, 1997; Canfield *et al*., 2005). These classes of denitrifying bacteria have been also reported in marine ecosystems (Hunter *et al*., 2006; Mills *et al*., 2008; Wittorf *et al*., 2016). Among archaea, denitrification is described in halophiles and hyperthermophiles (Canfield *et al*., 2005; Sanford *et al*., 2012).

There is recent evidence that some eukaryotes are also capable of denitrification (Risgaard-Petersen *et al.,* 2006; Bernhard, 2010). Several species of fungi, for example, have been shown to display denitrifying activity with the genes encoding homologs of *nirK* and *nor* located in the mitochondria. They reduce NO_3^- or NO_2^- to NO and N_2O (Nakanishi *et al*., 2010; Rőszer, 2012). There is also evidence of anaerobic denitrification in fungi from coastal marine sediments (Cathrine and Raghukumar, 2009).

The four reductive steps of denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) are catalysed by different enzymes (Figure 4), in which nitrate (NO_3^-) reductases are encoded by *narG* and/or *napA* genes; nitrite (NO_2^-) reductases by *nirK* or *nirS* genes; nitric oxide (NO) reductases by two variants of *norB* gene and finally nitrous oxide (N_2O) reductase by the *nosZ* gene (Zumft, 1997).

The genes encoding the enzymes for NO_2^- (*nirS* and *nirK*) and N_2O (*nosZ*) reduction have been the focus of phylogenetic studies in the denitrification process as NO_2^- reduction is the defining step of denitrification and N_2O (*nosZ*) reduction leads to the loss of biologically available nitrogen from the environment as N_2 (Canfield *et al*., 2005; Mills *et al.,* 2008; Bai *et al.,* 2012).

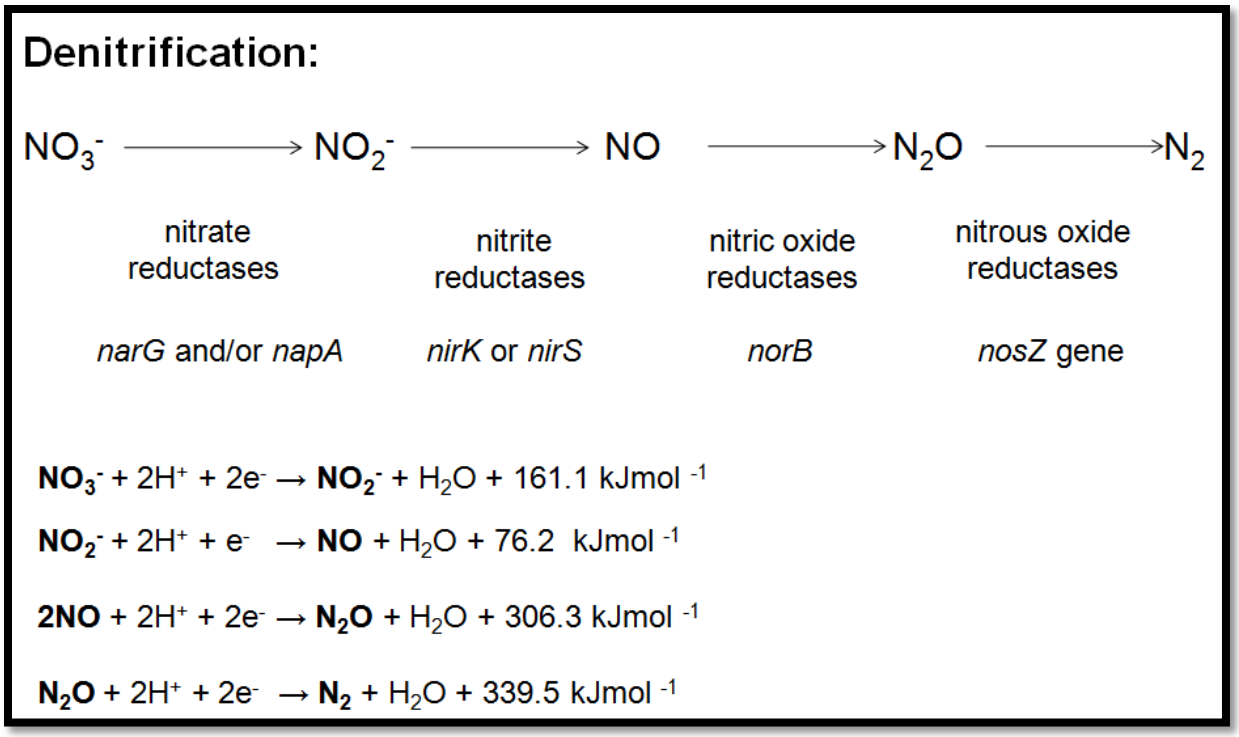


Figure 4: The four reductive steps of denitrification (stoichiometry of reactions; Zumft, 1997) and the genes (*narG* and/or *napA, nirK* or *nirS, norB* and *nosZ*) encoding the enzymes catalysing each step

5. Environmental Factors Affecting Nitrification and Denitrification

Nitrification can take place both in the water column and the sediment. However, nitrification rates in the water column of shallow marine and estuarine ecosystems appear to be at least an order of magnitude lower than the nitrification rates per unit of volume in the sediment (Canfield *et al.,* 2005; Gürel *et al.,* 2005). Physico-chemical factors regulating nitrification in coastal marine sediments (Figure 5) include salinity, pH, temperature (Cao *et al.,* 2011), ammonia concentration (Herrmann *et al*, 2011), dissolved oxygen concentration (Park *et al.,* 2010), the presence of any inhibitory compounds and the dissolved CO_2 concentration (Gürel *et al.,* 2005).

The ammonia concentration plays an important role in nitrification rates. Nitrifying organisms have distinct substrate affinities for ammonia. For example, *Nitrosomonas* is characterized by low substrate affinity and is able to live and even be dominant at high ammonia concentrations (Hunik *et al*., 1992; Webster *et al*., 2005; Wankel *et al*., 2011). Within the genus *Nitrosospira* different affinities for ammonia were noted (Webster *et al*., 2005). When comparing different nitrifying organisms (AOA and AOB), AOA display generally higher substrate affinities for ammonia and are able to

live in low ammonia concentration (Erguder *et al*., 2009; Martens-Habbena *et al*., 2009; Abell *et al*., 2010; Park *et al*., 2010; Hatzenpichler, 2012).

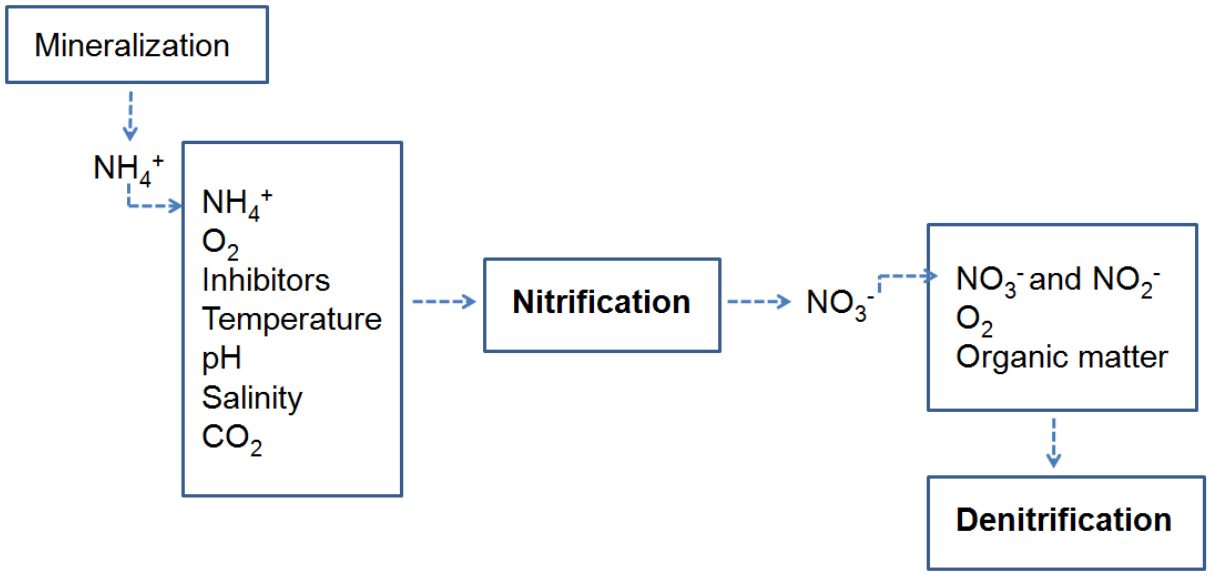


Figure 5: Physico-chemical factors regulating the rate of nitrification and denitrification in coastal marine sediments

Nitrifying organisms are aerobes and thus their activities depend on the availability of O_2 (see also anoxic nitrification in Box1). The minimum O_2 concentration required for their activities in the sediment ranges from 1.1 to 6.2 µM (Gürel *et al.,* 2005). Surprisingly, many nitrifiers prefer relatively low O_2 conditions (Canfield *et al.,* 2005). However, their O_2 optimum further appears to be location-specific and depends on adaptations to the prevailing environmental conditions (Canfield *et al.,* 2005). Further evidence suggests that AOB and AOA demonstrate differential responses to changes in sediment dissolved oxygen concentrations (Abell *et al.,* 2011). While abundance and diversity of bacterial *amoA* transcriptions is reduced at sediment hypoxia, no such changes were observed in archaeal *amoA* transcriptions (Abell *et al.,* 2011). AOA in fact display higher affinities for oxygen (Martens-Habbena *et al*., 2009; Abell *et al*., 2010; Park *et al*., 2010; Hatzenpichler, 2012). Nitrifiers in sediments may survive periods of inactivity when they are periodically exposed to anoxia, and recover their activity following O_2 exposure (Canfield *et al.,* 2005). Under low O_2 conditions, sulphide production would be stimulated while sulphide oxidation is limited. The resulting increase in sulphide concentration could promote inhibition of nitrification (Joye and Hollibaugh, 1995). The ability of nitrifiers to recover from

environmental O_2 fluctuations may be impeded if sulphide exposure also occurs (Ward, 2008). AOA appear to tolerate higher sulphide exposure (up to 0.5 mM) while AOB are inhibited at very low sulphide concentrations (Erguder *et al*., 2009).

Temperature influences the metabolic activities of nitrifying organisms. Nitrifiers would exhibit optimal growth and/or activity during the summer months when the temperature is highest (Gürel *et al.,* 2005). However, different temperature optima are reported in different kinds of environments. Nitrifiers adapted to low temperature can nitrify under low temperature conditions at rates comparable to the rates attained by nitrifiers adapted to higher temperatures living under high temperature conditions (Ward, 2008). While temperature is an important variable for many biological processes, nitrification is if anything less sensitive to regulation by temperature and is usually regulated in the environment by some other variable (Ward, 2008). For example, nitrification rates in some shallow Danish fjords are lowest during the summer and highest during the winter, which is attributed primarily to reduced solubility and availability of O_2 to the nitrifying populations and also to increased concentration of toxic sulphide in summer time (Henriksen and Kemp, 1988).

The growth and activity of most nitrifiers are optimal in the neutral to slightly alkaline pH range (pH 7 to 8.5) which coincides with the pH levels in most natural aqueous environments (Cao *et al.,* 2011).

Salinity has both physiological (Rysgaard *et al*., 1999; Gürel *et al.,* 2005) and non-physiological effects (Gürel *et al.,* 2005) on nitrification processes, lowering the rate of activities when salinity increases. As a consequence of non-physiological effect, the concentration of cations increases at high salinities. These compete with NH_4^+ for adsorption on the sediment increasing the NH_4^+efflux from the sediment (Gürel *et al.,* 2005). However, nitrifiers are generally able to acclimate to a broad salinity range and their tolerance differs from species to species (Canfield *et al.,* 2005).

Although AOA and AOB are chemolithoautotrophic using carbon dioxide as a carbon source, some AOA can also use organic carbon as an electron donor independent of ammonia oxidation (Erguder *et al*., 2009; Mußmann *et al*., 2011; Xu *et al*., 2012). This is consistent with both autotrophic and heterotrophic lifestyles and these microorganisms may function either as a strict autotroph or as a mixotroph utilizing both carbon dioxide and organic material as carbon sources (Erguder *et al*., 2009; Xu *et al*., 2012).

In general, AOA, being ubiquitous, seem to have a wide range of growth conditions, and some ecotypes might be unique to specific environments as well. It is proposed that the AOA might be important actors within the nitrogen cycle in low-nutrient, low-pH, and sulphide containing environments (Erguder *et al*., 2009).

Denitrification can also take place both in the water column and sediment but the rate of this process in the water column is lower than in the sediment (DeVries *et al*., 2012).

Rates of benthic denitrification are affected by organic matter content arriving at the sediment surface, concentration gradients of dissolved inorganic nitrogen (DIN: NO_3^-, NO_2^- and NH_4^+) and the availability of oxygen (Middelburg *et al*., 1996; Liu *et al*., 2003; Deutsch *et al*., 2010; Huang *et al*., 2011). The availability of organic matter in marine systems is one of the important factors favouring denitrification (Gilbert *et al*., 1997; Bertics *et al*., 2012). Organic matters serve as the electron donor in this process (van Haandel and van der Lubbe, 2007). As coastal marine environments act as centres of deposition of organic materials, most denitrification in marine sediment occurs in coastal regions rather than deep-sea environments (Gürel *et al.,* 2005). It is not only the quantity but also the quality of organic matter that is an important control on denitrification (Eyre *et al*., 2014). However, it is far less known how organic matter quality affects denitrification in ecosystems (Eyre *et al*., 2014; Stelzer *et al*., 2014). It seems that the effect of the quality of organic matter on denitrification rates is through its effect on the quantity and quality of DOC (dissolved organic carbon) and redox conditions in the sediment (Stelzer *et al*., 2014).

Despite co-consuming O_2 and NO_3^- during aerobic denitrification (Gao *et al*., 2010), denitrification is known mainly as an anaerobic process (Canfield *et al*., 2005; Vymazal, 2006; Bernhard, 2010) occurring when O_2 is absent or nearly absent and when NO_3^- is present. With increasing sediment depth in a vertical sediment profile, oxygen concentrations decrease and denitrifiers shift from facultative anaerobes to strict anaerobes (Tiquia *et al*., 2006). However, maximum denitrification rates do not necessarily meet the lowest O_2 concentration in deep layers (Tiquia *et al*., 2006; Gao *et al*., 2010; Li *et al*., 2010) due to the low/absent NO_3^- and NO_2^- concentrations (Tiquia *et al*., 2006; Gao *et al*., 2010).

Denitrification is often coupled with nitrification across oxic/anoxic interfaces in the sediment (Zehr and Ward, 2002). Therefore, some of the factors that affect nitrification also impact on denitrification (Figure 5). For example, seasonal hypoxia

resulting from anthropogenic activities in Himmerfjärden estuary (Baltic Sea) causes partial decoupling of nitrification from the production of ammonium by mineralization. Decreasing nitrification (considering also ammonia oxidation is the rate-limiting step in nitrification), reduces nitrogen loss via denitrification. The seasonal recovery increases the oxygenation of the bottom water, causing a re-establishing of nitrification, and this resulted in increasing denitrification rates (Bonaglia *et al.,* 2014a).

In organically enriched environments, oxygen depletion causes a shift in the source of nitrate for denitrification from benthic coupled nitrification-denitrification to the water column (Banks, 2011). In addition, in such environments, DNRA (converting NO_x^- to NH_4^+) can be a significant N-cycling process competing with denitrification for NO_x^- and thus potentially limiting N losses from the marine environment (Nizzoli *et al.,* 2006). Irreversible shifts of nitrogen recycling to the pelagic zone due to DNRA stimulation also in turn exacerbate eutrophication in an area that is already sensitive to oxygen stress (Banks, 2011; Bonaglia, 2015).

6. The Impact of Benthic Fauna on Nitrification and Denitrification

Macrofaunal assemblages have been shown to impact nitrogen cycling in marine sediments (Snelgrove, 1998) through their role in affecting the physical and chemical conditions and the exchange processes across the sediment-water interface (Ziebis *et al.,* 1996). The resulting changes are known to influence microbial activities (Chung and King, 1999; Dollhopf *et al.,* 2005; Papaspyrou *et al.,* 2006; Braeckman *et al.,* 2010; Laverock *et al.,* 2011; Gilbertson *et al.,* 2012), abundance (Papaspyrou *et al.,* 2005; Bertics and Ziebis, 2009) and diversity (Laverock *et al.,* 2010). However, this influence on benthic microbial populations and their activities through the creation of microniches within sediments and the burrow wall (Bertics and Ziebis, 2009) is based on research on individual macrofaunal functional species (Howe *et al.,* 2004; Braeckman *et al.,* 2010). It depends on the body size (Howe *et al.,* 2004), density (Nizzoli *et al.,* 2007; Braeckman *et al.,* 2010), mobility and reworking mode (bioturbation) such as burrowing and feeding activities of individual species (Howe *et al.,* 2004; Solan *et al.,* 2004; Bertics and Ziebis, 2009; Gilbertson *et al.,* 2012). At community level, the reworking characteristics of all species, and most likely those of the dominant ones (Biles *et al.,* 2002; Kristensen *et al.,* 2014) determine the impact of macrofauna on microbiota.

Different functional groups are distinguished within macrofaunal communities (Figure 6): *(i)* Epifauna (such as the hermit crab, *Pagurus bernhardus*) whose activities occur predominantly above the sediment-water interface (Solan *et al*., 2004; Solan and Wigham, 2005; Buatois and Mángano, 2011) and therefore have negligible contribution to vertical particle transport (Buatois and Mángano, 2011); *(ii)* surficial modifiers (such as sea urchin, *Brissopsis lyrifera*), whose sediment reworking activities are restricted to < 1-2 cm of the sediment profile (Solan *et al*., 2004; Solan and Wigham, 2005), are therefore shallow vertical bioturbators (Buatois and Mángano, 2011). These organisms rarely venture above the sediment-water interface (Solan and Wigham, 2005); *(iii)* biodiffusers (such as bivalves, i.e. *Abra alba*) whose activities result in a constant and random diffusive transport of particles over short distances. Particles are progressively transported through every level of the sediment profile by the activities of the organisms (Solan *et al*., 2004; Solan and Wigham, 2005); *(iv)* burrow dwellers and tube builders: burrow dwellers such as the amphipod, *Corophium volutator* (Emmerson *et al.,* 2001), the polychaete, *Nereis (Hediste) diversicolor* (Christensen *et al.,* 2000)*,* the ghost shrimp, *Neotrypaea californiensis*, and the fiddler crab, *Uca crenulata*, (Bertics and Ziebis, 2009) suspend large amounts of sediment into the water column*,* through excavating burrow constructions. Burrow construction stimulates fluxes between the sediment and water column through extending the surface area available for diffusive solute exchange (sediment-water interface) (Howe *et al.,* 2004, Nizzoli *et al.,* 2007). Depending on burrow morphologies, macrofaunal species creating burrows can have different impacts on microbial processes. For example, the two crustaceans *N. californiensis* and *U. crenulata* exhibit different burrowing behaviour and ventilating activities, which has contrasting effects on bacterial communities associated with the burrows (Bertics and Ziebis, 2009). Various metabolic properties of benthic microorganisms determining their ecological niches in the sediment, also determine their distribution and rate of activities in burrow constructions of macrofauna. Burrow walls are not geochemically uniform structures and often exhibit patterns of zonation and heterogeneity (Chung and King, 1999; Kristensen, 2000). There are numbers of studies which have focused on such individual functional effects of burrowing organisms (Biles *et al.,* 2002; Howe *et al.,* 2004; Bertics and Ziebis, 2009; Gilbertson *et al.,* 2012). Tube builders, such as the polychaetes *Sabellaria spinulosa* and *Lanice conchilega*, building tubes into the sediment that are also elevated to the water, have

autogenic effects (by their own physical structures) on associated communities (Braeckman *et al*., 2014a). The autogenic engineering effect of *L. conchilega*, for example, provides new habitat for associated species by increasing bed stability (Rabaut *et al*., 2009) and trapping organic matter from the water column. It substantially affects the structure and abundance of associated communities and food-web properties (Callaway, 2006; De Smet *et al*., 2015 and 2016).

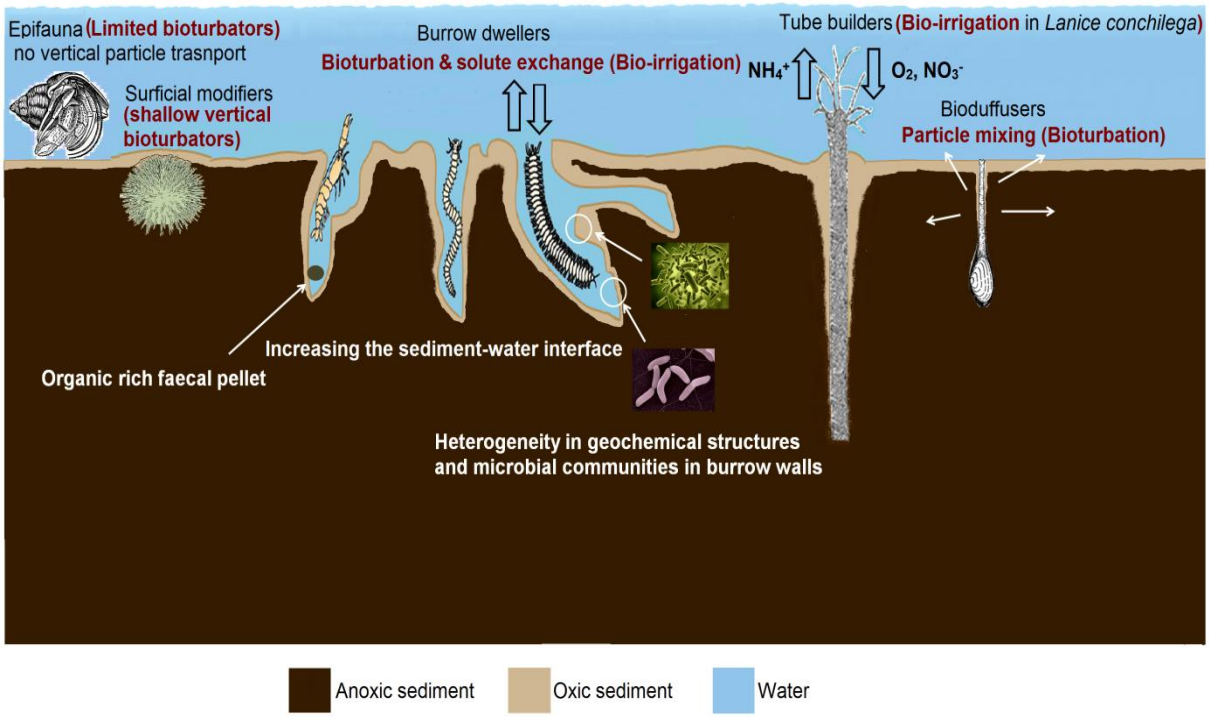


Figure 6: Different functional groups in macrofaunal communities (Solan *et al*., 2004; Solan and Wigham, 2005; Buatois and Mángano, 2011) and their effects (through bioturbation [particle mixing] and bio-irrigation [solute transfer]) on the physical and chemical conditions across the sediment-water interface and microbial communities in the sediment. The big arrows show solute exchange (bio-irrigation) across the sediment-water interface. Random diffusive transport of particles (bioturbation) by biodiffusers was also illustrated by white arrows.

In general, species that actively burrow or move through the sediment have a larger impact on the sediment structure and subsequently on microbial communities than sedentary species. However, tube-building species such as *L. conchilega* can stimulate microbial-mediated processes through bio-irrigation activity (Braeckman *et al*., 2010). *Lanice conchilega* is therefore known to manifest both autogenic and allogenic (transforming living/non-living materials from one physical state to another) ecosystem engineering properties (Godet *et al*., 2008). The allogenic engineering capacity of *L. conchilega* stems from its piston-pumping activity (Forster and Graf,

1995) causing water and solute exchanges between tubes and the overlying water (bio-irrigation). *Lanice conchilega* was shown to have more pronounced influences on benthic respiration, nutrient release and denitrification than the biodiffusing bivalve *A. alba* in coastal sediments (Braeckman *et al*., 2010).

In fact, macrofauna, by their bioturbation and bio-irrigation activities and by transferring water and solutes from the overlying water influence the biogenic mixing depth of sediments (BMD: an indicator of bioturbation depth. The BMD is delimited at the lower boundary as the interface between the oxidized and reduced sediment; Figure 7) (Solan *et al.,* 2004) and supply different microbial populations with electron-donors and electron-acceptors (Kristensen, 1988; Kristensen and Kostka, 2004). The bioturbation potential of macrofauna can be estimated using an appropriate trait-based index (BPc) (see Box 3).

BOX 3:

The macrofaunal bioturbation potential can be estimated by calculation of the contribution of each species using an appropriate trait-based index e.g. BP_i (bioturbation potential of each species) and elaborated to population ($BP_p = A_i$ x BP_i) and community levels ($BP_c = \sum BP_p$) (Solan *et al*., 2004; Queirós *et al*., 2013).

$$BPi = \sqrt{(Bi/Ai)} \times Mi \times Ri$$

$$BPc = \sum_{i=1}^{n} \sqrt{(Bi/Ai)} \times Mi \times Ri \times Ai$$

Bi and Ai are the biomass and abundance of species

Mi (mobility) ranging from 1 (living in a fixed tube) to 4 (free three dimensional movement via burrow system)

Ri (sediment reworking) ranging from 1 (epifauna that bioturbate at the sediment–water interface) to 5 (regenerators that excavate holes, transferring sediment at depth to the surface)

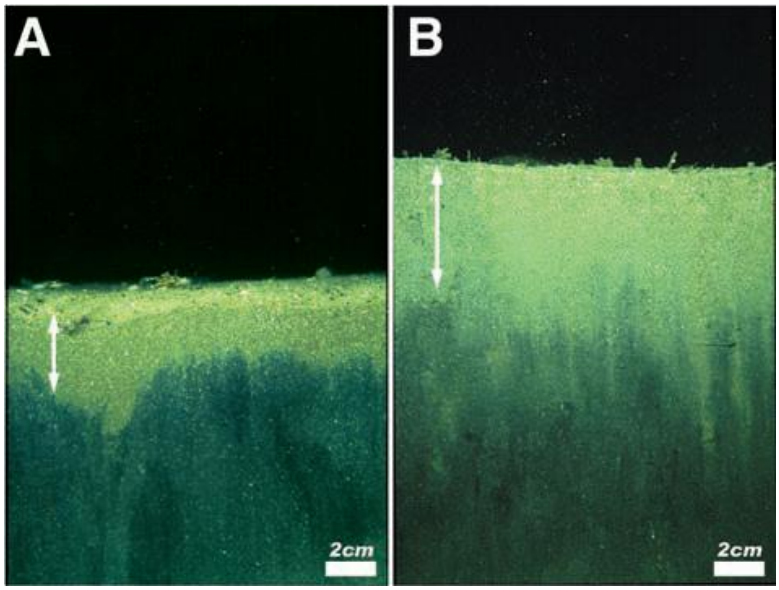


Figure 7: The biogenic mixing depth (BMD, white arrows) of sediments. A and B shows BMD in two different sites. Site A is perturbed by fresh water discharge and untreated domestic sewage. Site B is identified as a pristine area. Site A harbours a lower diversity of taxa compared to the site B (Solan *et al*., 2004).

The depth distribution of nitrifying organisms in the sediment is constrained by the downward dissolved oxygen diffusion, which is mostly less than one centimeter (Ziebis *et al.,* 1996; Gürel *et al.,* 2005). The presence of these organisms in anaerobic sediments at depths well below the zone into which oxygen can penetrate is attributed to macrofaunal irrigation of sediment by physical resuspension and bioturbation (Gürel *et al.,* 2005). High potential nitrification activity is found in the lining of permanent infauna burrows, and this rate is reported to be even higher in the burrow wall than at the sediment surface (Henriksen and Kemp, 1988).

Nitrification rates in the sediment are stimulated by macrofaunal activities through increasing O_2 and/or NH_4^+ concentrations in the sediment. NH_4^+/ NH_3 concentrations, fueling nitrification, increase through the direct release by macrofauna as observed in Antarctic sediments in the presence of amphipods (Nedwell and Walker., 1995), by excretion by the worms (Blackburn and Henriksen, 1983; Kristensen, 1985) or by increasing mineralization rates. The mineralization rate intensifies in the presence of macrofauna as the efficiency of solute exchange with the overlying water increases (Aller and Aller, 1998). The nitrate produced by the nitrification process can then serve as a substrate for denitrification (coupled nitrification-denitrification stimulation). Howe *et al.* (2004) noted that the increase in denitrifcation in the presence of the

burrowing shrimp *Upogebia deltaura* is largely fuelled by NO_3^- generated by nitrification from the burrow wall. In nitrate rich water, however, macrofauna stimulate denitrification both by increasing nitrification in the sediment and by providing the nitrate from the overlying water (Nizzoli *et al.,* 2007) (see also Box 4 for microorganisms living in the gut of aquatic animals).

BOX 4:

A large variety of aquatic animals was found to harbor denitrification by ingested bacteria in their gut when nitrate is present in the environment. High N_2O to N_2 production ratio indicates delayed induction of the last step of denitrification in the anoxic environment in the gut of these animals (Stief *et al*, 2009; Heisterkamp *et al*., 2010). Soil-living earthworms are also known as important emitters of nitrous oxide (Drake *et al*., 2006). The amount of emission of nitrous oxide in benthic invertebrates differs and depends on factors such as body weight, habitat and animal diet (Stief *et al*, 2009; Heisterkamp *et al*., 2010). It seems as if nitrous oxide emission by aquatic animals is quantitatively important in aquatic environments (Stief *et al*, 2009; Heisterkamp *et al*., 2010). However, studying this group of organisms is out of scope of this PhD thesis as the aim is to investigate the effect of macrofauna on microorganism-mediated N-cycle processes in the sediment and further use this knowledge on Ecosystem-Based Management approach.

Recently, bioturbation effects of benthic organisms of less than 1mm in size (meiofauna) have been reported to increase the removal of fixed nitrogen from aquatic ecosystems by stimulating nitrification and denitrification in the oxic–anoxic transition zone of the marine sediment in the absence of macrofauna (Bonaglia *et al.,* 2014b). This study involved mainly nematoda as the dominant meiofaunal group (92% of total abundance) in the sediment (Bonaglia *et al.,* 2014b). High meiofaunal bioturbation might not deepen oxygen penetration significantly as these organisms are mainly active in the uppermost millimetres (Bonaglia *et al.,* 2014b). In addition, the presence of macrofauna (> 1mm) in the interaction with meiofauna might hide their effect on microbial activities in sediments (Bonaglia *et al.,* 2014b). In some meiofaunal groups, such as some species of Foraminifera, the enhanced production of nitrogen gas is not through the stimulation of microorganisms but rather through the direct respiration of nitrate by the organism (Risgaard-Petersen *et al.,* 2006; Høgslund *et al.,* 2008).

7. Aims and Objectives of the PhD Thesis

The overall aim of this PhD thesis is to gain insight in the effect of biological factors on the coastal benthic biogeochemical cycling.

It is already established that irrigated burrows of macrofauna enhance total microbial metabolism by stimulating oxic and suboxic reactions in the burrow wall and adjacent surrounding sediments (Kristensen and Kostka, 2004). However, we are still at the beginning of our understanding of how macrofaunal activities interact with the sediment and its microbial communities, and how this affects important biogeochemical cycles in the marine realm (Laverock *et al.,* 2011). Furthermore, actual observations are very often limited to individual macrofaunal species and also to the total microbial communities in the sediment with less attention to the various microbial functional groups.

In this thesis, the interactions between macrofauna and microorganisms mediating N-cycle processes (nitrification, denitrification) are investigated both from an environmental as well as from a functional point of view. Our shallow coastal study area is situated in the Belgian Part of the North Sea and in an intertidal zone in the North of France (see further for a short site description).

The three main chapters (**Chapters 2, 3** and **4**; Figure 8) are focusing on the understanding of benthic ecosystem functioning in relation to the N-cycle and the microorganisms that drive these reactions in coastal sediments.

A combination of multiple techniques to investigate microbial communities will be applied in this thesis. These techniques include DGGE (Denaturant Gradient Gel Electrophoresis), a commonly used technique for studies of microbial composition and diversity (Frossard *et al*., 2012; Graue *et al*., 2012; Tang *et al*., 2013; Tiodjio *et al*., 2014; von Scheibner *et al*., 2014); and HTS (High Throughput Sequencing) using Illumina sequencing which allows fast and deep amplicon sequencing (e.g. Kircher, 2011; Chao *et al*., 2015). We applied these techniques to investigate 16S rDNA gene in total microbial communities (DNA-based methods) as well as the expression of the functional genes (RNA-based methods) involved in N-cycling processes.

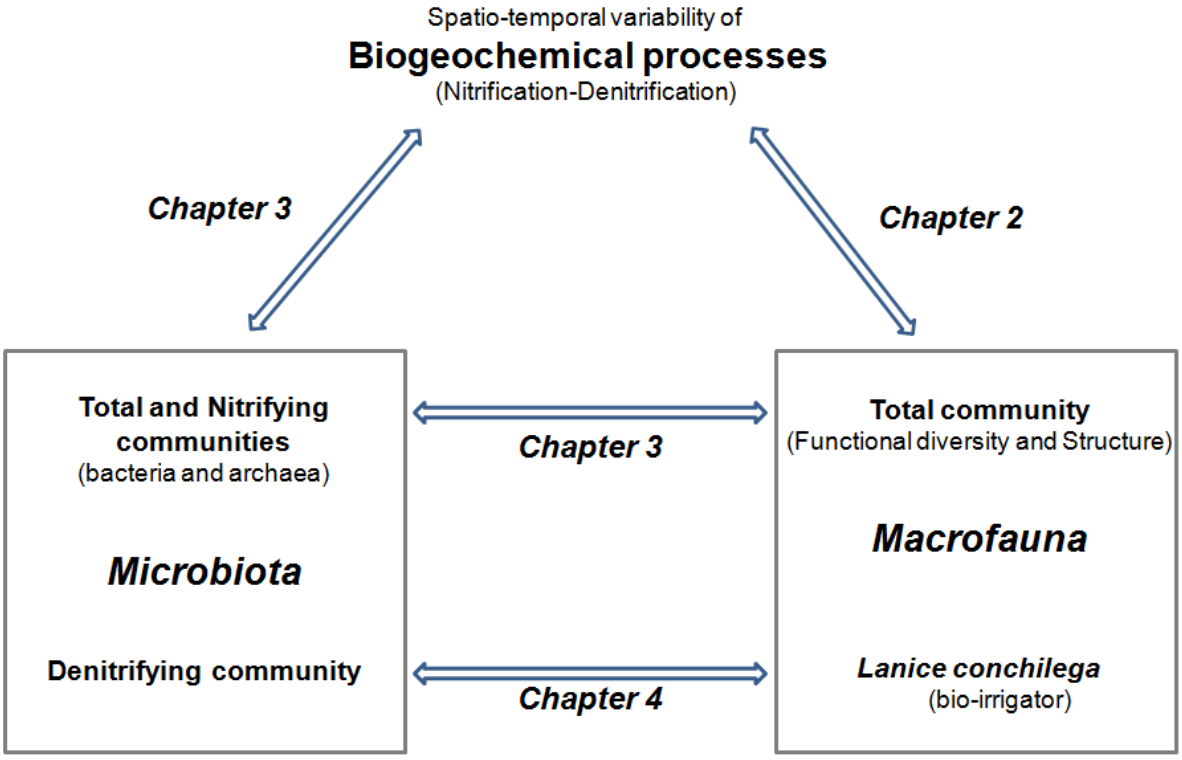


Figure 8: Schematic overview of the macrofauna – microbiota – biogeochemical processes interactions, which were studied in the present PhD thesis

These chapters aim at answering following questions:

Does macrofaunal density and/or functional diversity influence benthic biogeochemical cycling over a large horizontal (km) and temporal scale? (Chapter 2)

- How does the coastal benthic biogeochemical cycling change in space and time at the scale of the Belgian Part of the North Sea?
- How are these changes influenced by total macrofaunal density and functional diversity at the community level in the study area?

Does macrofaunal density and/or functional diversity affect the link between microbial communities and benthic biogeochemical cycling at large (km) horizontal and temporal scales? (Chapter 3)

- How are the N-cycle processes (nitrification, denitrification and N-mineralization) linked with diversity of the total microbial community (bacteria

and archaea) and functional genes (bacterial and archaeal *amoA*) involved in nitrification in space and time in shallow subtidal marine sediments?

- Is this link modulated by total macrofaunal density and functional diversity at the community level?

How does bio-irrigation by macrofauna (with the tube-building polychaete, *Lanice conchilega* as a case study) affect an important biochemical process such as denitrification at horizontal (m) and vertical (cm) scales? (Chapter 4)

- How is the composition and diversity of expressed *nosZ* genes affected by heterogeneity in the biogeochemical environment (oscillation in O_2 concentration, sediment grain size and chlorophyll *a*) in the *L. conchilega* reef vertically at small (cm) scales along the sediment depth profiles and also at larger horizontal scales (m) at different natural *Lanice* densities within the reef?

8. Study Area

8.1. Belgian Part of the North Sea (Belgium)

The Belgian Part of the North Sea (BPNS) is a shallow coastal area (average 20 m water depth) which extends to ≈ 40 km offshore and is situated between 51 and 52° N in the Southern Bight of the North Sea (M'harzi *et al.,* 1998). This area with the salinity usually < 33 psu is influenced by the residual current entering from the English Channel on the one hand, and largely by freshwater sources from e.g. Schelde estuary on the other hand. Temperature ranges between 5 and 20 °C (Provoost *et al*., 2013). Within this area, turbulence of the water column is so high that even during summer temperature stratification does not take place (M'harzi *et al.,* 1998; Rousseau *et al.,* 2006), also because of strong tidal currents (Degraer *et al.,* 2006). The combination of the complex bathymetry and hydrodynamics is responsible for the high diversity of sediment types on the BPNS varying from very fine mud to coarse sand (Degraer *et al.,* 2006). This area is also characterized by high concentrations of anthropogenic nutrients from terrestrial input in the near coastal zone (Rousseau *et al.,* 2002) resulting in high primary production and algal biomass (Rousseau *et al.,* 2006). Spring phytoplankton blooms begin in March and peak in April and May spreading in almost the entire Belgian Coastal Zone and adjacent waters (Rousseau *et al.,* 2006). Nearly 24% of the biomass produced by

primary production is deposited on the sediment while most of the settled biomass (70–75%) is under the form of *Phaeocystis* colonies, and is mainly mineralised by bacterial activity (Rousseau *et al.,* 2006).

The highly variable sediments in the BPNS harbor different macrofaunal communities (Van Hoey *et al*., 2009). Four subtidal macrofaunal communities can be distinguished based on the habitat preferences: (1) the *Macoma balthica* (2) the *Abra alba* (3) the *Nephtys cirrosa* and (4) the *Ophelia limacina* community. The offshore sandy sediments with low mud content are suited for the *O. limacina* and *N. cirrosa* communities. The *Abra alba* community, located in fine sandy sediments with relative high mud content displays high density and species richness. Muddy sediments are inhabited by the *Macoma balthica* community. This community is characterized by average density and number of species (e.g. Degraer *et al*., 2008; Van Hoey *et al*., 2009).

The BPNS was selected as a case study area because of the high diversity of sediment (e.g. Degraer *et al*., 2006), strong ecological knowledge (e.g. Lancelot *et al*., 2005; Van Hoey *et al*., 2009; Vanaverbeke *et al*., 2011), high amount of available data on macrofauna (e.g. Degraer *et al*., 2008; Van Hoey *et al*., 2009) and increasing knowledge on the biogeochemical cycling (Braeckman *et al*., 2010; Provoost *et al*., 2013).

8.2. Boulogne-sur-mer (France)

The experimental area was located in soft-bottom intertidal zone of Boulogne-sur-mer along the northern part of the English Channel (50° 44.01’ N, 01° 35.15’ E; Northern France). The tidal regime is semi-diurnal ranging up to 7m (De Smet*,* 2015). This area is sheltered by two harbor walls where well-established *L. conchilega* aggregations occur, covering an area of about 51,500 m^2*.* The largest zone (about 45,000 m^2) is subtidal and only exposed at extreme low water spring tide conditions (LWST) while the other two reef zones located higher on the beach are exposed at every low water: a western zone (about 4,000 m^2) and an eastern zone (2,500 m^2) can be identified (Rabaut *et al.,* 2008).

The bio-irrigating polychaetes, *L. conchilega*, as described earlier, manifest both allogenic and autogenic ecosystem engineering properties (Godet *et al*., 2008). They are present throughout the North Sea (Van Hoey *et al*., 2008) and can be found in

dense aggregations (Van Hoey *et al*., 2008), referred to as biogenic reefs (Rabaut *et al*., 2009).

Figure 9: *Lanice conchilega* reef in the intertidal and individual polychaete (modified after http://www.arkive.org/sand-mason/lanice-conchilega/ and http://borea.mnhn.fr/fr/biogenic-reefs-affect-multiple-components-intertidal-soft-bottom-benthic-assemblages-lanice-0)

9. Outline of the Thesis

Apart from the general introduction (**Chapter 1**) and discussion (**Chapter 5**), this thesis is a compilation of three research articles; published (**Chapters 2 and 3**) or in preparation for submission (**Chapter 4**). Each chapter is therefore intended to be an autonomous part, which can be read on its own. Inevitably, there may be some overlap between the introduction sections of the different chapters. Cited literature is compiled in a single list at the end of the thesis. **Chapter 2** has joint authorship (Maryam Yazdani Foshtomi as the second author). The other chapters have MYF as a first author.

In **Chapter 1**, the general introduction, the scientific setting of this PhD thesis was outlined by introducing the definition of the broad term “Ecosystem functioning” in relation with ecosystem services and relevance for management, the importance of shallow coastal habitats focusing on N-cycle processes and the role of micro-and macro-organisms in these processes. **Chapter 2** guides the reader through spatial and temporal differences in biogeochemical cycling (nitrification, denitrifcation) at various stations in the BPNS representative of a wide range of coastal sediment types and macrobenthic communities over a yearly cycle (February to November 2011). Models were developed to predict nitrification and denitrification as well as oxygen, ammonium, and alkalinity fluxes from abiotic environmental variables and functional biodiversity (that is bioturbation potential) of macrobenthos. The chapter

has been published as “Braeckman U, Yazdani Foshtomi M, Van Gansbeke D, Meysman F, Soetaert K, Vincx M and J. Vanaverbeke. 2014. Variable importance of macrofaunal functional biodiversity for biogeochemical cycling in temperate coastal sediments. Ecosystems. 17, 720–737”. I was involved in this project from April to October 2011 contributing in the field sampling (water and sediment sampling), lab experiments and incubations, data measurements (O_2 winkler, O_2 and pH profiles, SCOC, DIN, ...). **Chapter 3** focuses on the impact of macrofauna at community level on microbiota and how this affects nitrogen cycling. In this chapter, we simultaneously investigated spatio-temporal patterns in microbial community composition and diversity, macrofaunal abundance and their sediment reworking activity, and N-cycling processes (N-mineralization, nitrification, and denitrification) in seven subtidal stations in the BPNS, with different timing with regard to the phytoplankton bloom deposition. Microbial samplings in **Chapter 3** were carried out during the same sampling campaigns as in **Chapter 2**, thus allowing maximal linkage between the results of these chapters. In this chapter, four communities of microbiota were investigated including total communities (bacteria and archaea) and active nitrifying communities (ammonia-oxidizing bacteria (AOB) and archaea (AOA)) by targeting the functional gene, *amoA*. **Chapter 3** has been published as “Yazdani Foshtomi M, Braeckman U, Derycke S, Sapp M, Van Gansbeke D, Sabbe K, Willems A, Vincx M, Vanaverbeke J. 2015. The link between microbial diversity and nitrogen cycling in marine sediments is modulated by macrofaunal bioturbation. PLoS ONE. 10(6), e0130116”. **Chapter 4** represents the impact of bio-irrigation by the tube-dwelling polychaete, *L. conchilega* on denitrifying organisms. Our objective was to understand how horizontal and vertical variations in denitrifying gene (*nosZ*) expression are affected by bio-irrigation activity and heterogeneity in the biogeochemical environment (oscillation in O_2 concentration, sediment grain size and chlorophyll *a*) in the *L. conchilega* reef. Sampling was carried out in the *Lanice* reef in the intertidal zone of Boulogne-sur-mer, France. Sediment was collected from three different areas within the reefs: *(i)* an area with an average density of *Lanice* individuals of 25-27 tubes per core surface (3,185-3,440 ind. m^{-2}) (referred to as “high *Lanice* treatment”) *(ii)* an area with lower *Lanice* densities (5 tubes per core surface; 637 ind. m^{-2}) located on the edge of the patches (“low *Lanice* treatment”) *(iii)* and sediments without *Lanice* between the reef patches (“control treatment”). Sediment oxygen concentrations were measured as vertical profiles in the ambient

sediment (1 to 2 cm distance from tubes) and also by logging changes of sedimentary O_2 concentrations every second for 30-35 min at 1.5 cm sediment depth and at 1 cm distance from the tube. The results of this chapter are in preparation as "Yazdani Foshtomi M, Leliaert F, Derycke S, Willems A, Vincx M, Vanaverbeke J. The effect of bio-irrigation by the tube-building polychaete *Lanice conchilega* on denitrifiers: distribution, diversity and composition of *nosZ* transcripts". In the last chapter (**Chapter 5**), the main conclusions are summarized, discussed and compared with other studies and recommendations for future research are provided.

Chapter 2

Variable Importance of Macrofaunal Functional Biodiversity for Biogeochemical Cycling in Temperate Coastal Sediments

Modified from the following publication:

Braeckman, U., Yazdani Foshtomi, M., Van Gansbeke, D., Meysman, F., Soetaert, K., Vincx, M., Vanaverbeke, J., 2014. Variable Importance of Macrofaunal Functional Biodiversity for Biogeochemical Cycling in Temperate Coastal Sediments. Ecosystems. 17**,** 720–737.

ABSTRACT

Coastal marine systems are currently subject to a variety of anthropogenic and climate-change-induced pressures. An important challenge is to predict how marine sediment communities and benthic biogeochemical cycling will be affected by these ongoing changes. To this end, it is of paramount importance to first better understand the natural variability in coastal benthic biogeochemical cycling and how this is influenced by local environmental conditions and faunal biodiversity. Here, we studied sedimentary biogeochemical cycling at ten coastal stations in the Southern North Sea on a monthly basis from February to October 2011. We explored the spatio-temporal variability in oxygen consumption, dissolved inorganic nitrogen and alkalinity fluxes, and estimated rates of nitrification and denitrification from a mass budget. In a next step, we statistically modeled their relation with environmental variables and structural and functional macrobenthic community characteristics. Our results show that the cohesive, muddy sediments were poor in functional macrobenthic diversity and displayed intermediate oxygen consumption rates, but the highest ammonium effluxes. These muddy sites also showed an elevated alkalinity release from the sediment, which can be explained by the elevated rate of anaerobic processes taking place. Fine sandy sediments were rich in functional macrobenthic diversity and had the maximum oxygen consumption and estimated denitrification rates. Permeable sediments were also poor in macrobenthic functional diversity and showed the lowest oxygen consumption rates and only small fluxes of ammonium and alkalinity.

Macrobenthic functional biodiversity as estimated from bioturbation potential appeared a better variable than macrobenthic density in explaining oxygen consumption, ammonium and alkalinity fluxes, and estimated denitrification. However, this importance of functional biodiversity was manifested particularly in fine sandy sediments, to a lesser account in permeable sediments, but not in muddy sediments. The strong relationship between macrobenthic functional biodiversity and biogeochemical cycling in fine sandy sediments implies that a future loss of macrobenthic functional diversity will have important repercussions for benthic ecosystem functioning.

Keywords: benthic ecosystem functioning; macrobenthos; functional biodiversity; oxygen consumption; nutrient fluxes; alkalinity; North Sea; bioturbation potential.

1. Introduction

Coastal seas cover only 10% of the total ocean surface (Jörgensen, 1983; Smith and Hollibaugh, 1993; Wollast, 1998) but provide a wealth of ecosystem goods and services including carbon storage or nutrient availability. The world-wide human pressures on coastal ecosystems (Vitousek *et al*., 1997; Halpern *et al*., 2008) make understanding and predicting the effects of human and climate-change-induced stressors on coastal marine ecosystems nowadays one of the main challenges (Doney, 2010).

Owing to intensive primary production and shallow water depth, a close coupling generally exists between the pelagic and benthic compartments in coastal ecosystems (for example, Marcus and Boero, 1998). The biogeochemical cycling of elements within coastal sediments influences water column processes in multiple ways. For example, primary production and associated phytoplankton dynamics in the water column are crucially dependent on the rate and timing of nutrient release during benthic organic matter (OM) mineralization (Lancelot *et al*., 2005; Soetaert and Middelburg, 2009). Also, anaerobic pathways of benthic mineralization can be an important source of alkalinity generation, which increase the capability of CO_2 uptake in coastal waters (Chen, 2002; Thomas *et al*., 2009; Hu and Cai, 2011). So an important challenge is to predict how benthic biogeochemical cycling in coastal seas will be affected by anthropogenic and climate-change-induced pressures.

In addition to being sites of intense OM mineralization, coastal sediments are also typically inhabited by a rich faunal community, which can exert an important control on sedimentary biogeochemical cycling through their bioturbation and bio-irrigation activities (Aller, 1988; Meysman *et al*., 2006). In search for food, these organisms actively rework and irrigate the sediment matrix. Oxygen and OM are transported into the deeper sediment layers, while the exchange of solutes between pore water and overlying water is enhanced by various burrow flushing activities (Yingst and Rhoads, 1980; Mermillod-Blondin *et al*., 2004). This increases nutrient turnover, and hence, replenishes the nutrient pool available for primary production (Blackburn, 1988). Given the variety in macrobenthic sediment reworking activities (Gérino *et al*., 2003), it is rather the functional biodiversity than taxonomic biodiversity that matters for benthic ecosystem functioning (Emmerson and Raffaelli, 2000; Ieno *et al.,* 2006; Cardinale *et al*., 2012).

The relation between functional biodiversity and biogeochemical cycling in marine sediments has mainly been assessed through manipulative experiments in a laboratory context (Mermillod-Blondin *et al*., 2004; Waldbusser *et al*., 2004; Michaud *et al*., 2005; Ieno *et al*., 2006; Norling *et al*., 2007; Godbold *et al*., 2008; Braeckman *et al*., 2010; Bulling *et al*., 2010; Gilbertson *et al*., 2012). This experimental approach is suitable to examine the effect of a single species or a multispecies assemblage on biogeochemical cycling. The advantage is that possible cause–effect relationships can be uncovered, without the inherently co-varying environmental factors operating in the real world (Benton *et al*., 2007). However, the upscaling of these experimentally derived functional biodiversity–ecosystem functioning relationships to the field situation is difficult because the artificial assemblages used in laboratory experiments are highly simplified models of the in situ community. In addition, experiments are typically only of short duration, whereas the sediment is often strongly manipulated and artifacts may arise (for example, container effects). As an alternative to experimental studies, one can carry out in situ observational studies that make use of proxies for functional biodiversity and/or ecosystem functioning. One such proxy is the bioturbation potential of the community (BPc), an index that involves biomass, abundance, and functional sediment reworking characteristics, as a measure for macrobenthic functional biodiversity (Solan *et al*., 2004). BPc was negatively related to total organic carbon and positively to chlorophyll concentrations in the sediment (Solan *et al*., 2012) and positively to the apparent redox potential discontinuity layer (aRPD) as derived from sediment profile imaging (Birchenough *et al*., 2012). This aRPD was calculated as the mean depth at which the sediment changes color (from light colored to black), and was interpreted as a proxy for the intensity of biogeochemical cycling in the sediment. However, the aRPD is the result of a combination of environmental conditions, food input and faunal activity (Teal *et al*., 2010), and so, to relate macrobenthic functional biodiversity independently to benthic carbon cycling, it is therefore better to use actual measurements of biogeochemical cycling (Van Colen *et al*., 2012). Because of the strong influence of the annual phytoplankton bloom on biogeochemical cycling in coastal seas, such relationships should be investigated over a full seasonal cycle (Teal *et al*., 2010; Provoost *et al*., 2013).

In the light of sustainable ecosystem management and associated monitoring, it is important to understand how OM mineralization and nutrient release are distributed in

space and time and how this carbon mineralization relates to the predominant environmental conditions and local biodiversity. Few attempts have been made to assess the relative importance of functional biodiversity and environmental conditions in affecting biogeochemical processes in natural systems because these ecosystems are structured by multiple and simultaneously operating abiotic and biotic factors that are difficult to disentangle (Godbold and Solan, 2009). In this study, we therefore measured various biogeochemical processes at ten stations in the Southern North Sea over a yearly cycle. These sites are representative of a range of sediment types and macrobenthic communities. We tested for (1) spatial and (2) temporal differences in biogeochemical cycling and functional biodiversity of macrobenthos. Further, we (3) developed models to predict oxygen, ammonium, and alkalinity fluxes, nitrification and denitrification from environmental variables including functional biodiversity (that is, bioturbation potential) of macrobenthos.

2. Materials and Methods

Our approach involved a combination of field measurements and modeling. As rates of sedimentary biogeochemical cycling, we quantified sediment community oxygen consumption (SCOC), the exchange of dissolved inorganic nitrogen (DIN) and alkalinity (A_T) across the sediment–water interface. These fluxes were obtained by incubating undisturbed sediment cores. From the resulting oxygen and DIN fluxes, we modeled nitrification and potential denitrification rates using an integrated mass budget approach (Soetaert *et al*., 2001; Braeckman *et al*., 2010). We quantified functional biodiversity as community bioturbation potential (BPc, Solan *et al*., 2004).

2.1. Sampling

SCOC, DIN, and A_T fluxes were quantified at seven stations (station codes: 120, 130, 230, 145, 700, 710, and 780) on a monthly basis from February to September 2011 (Figure 1; Table S1, Addendum 1) with the *RV* Zeeleeuw. Three additional stations with coarse sediment (ZG02, 330, and 215) were sampled in June and August 2011. In October 2011, representatives of fine sandy (780), muddy (130) and coarse sandy (330) stations were sampled for measurements of denitrification (N_2:Ar method, see below) and DIC flux.

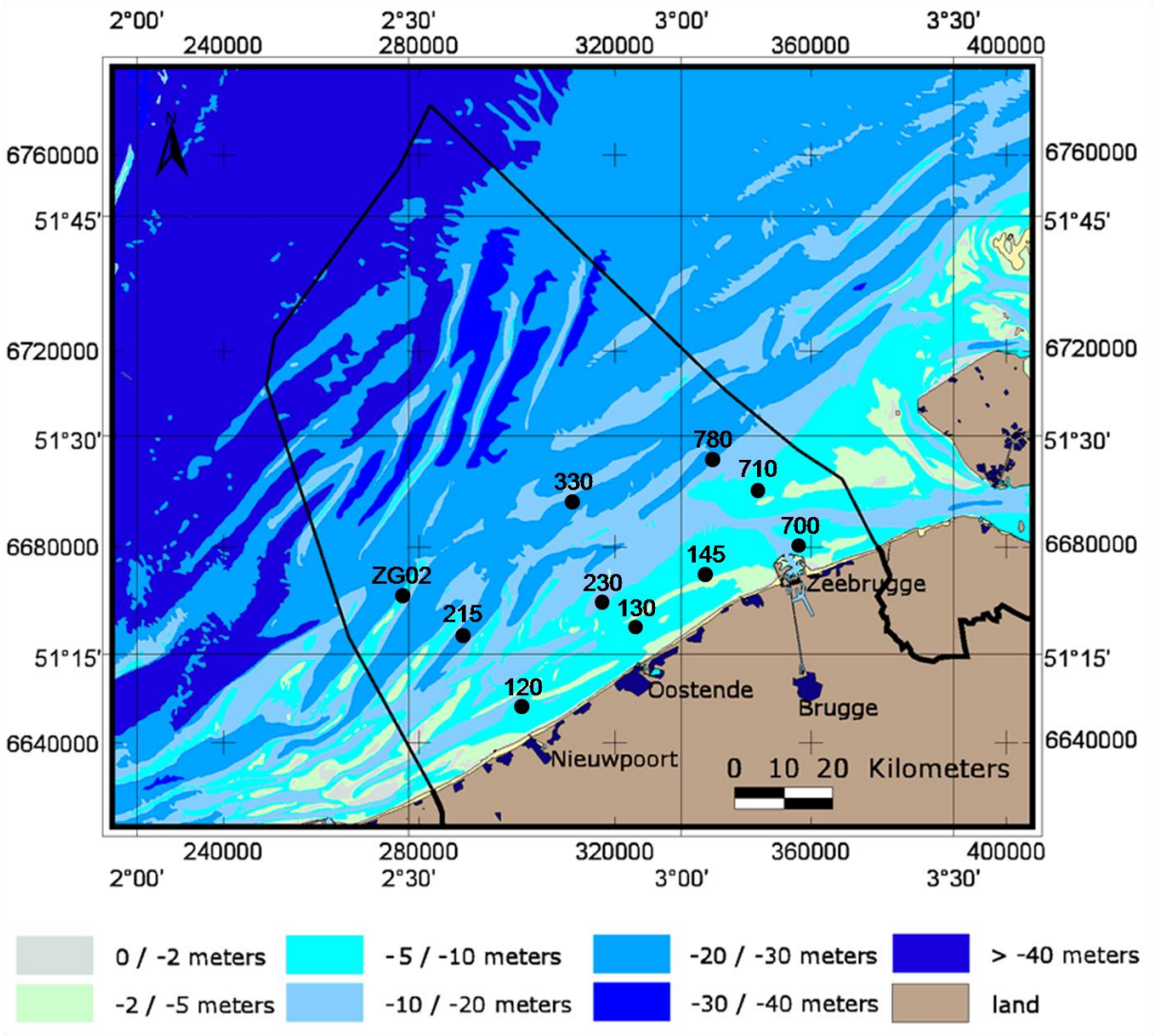


Figure 1: Bathymetry map of the Belgian Part of the North Sea with indication of the sampled stations. Stations 215, ZG02 have been sampled only in June and August. 330 was sampled in June, August, and October (330).

At each station, a CTD cast was performed and the bottom water (±1 m above the sea floor) was sampled with 5-L Niskin bottles. The bottom water was subsampled for oxygen (duplicate 12 ml Winkler bottles) and DIN concentrations (triplicate 10 ml in scintillation vials, samples filtered on Whatman™ GF/F filters). Three additional water samples were filtered on precombusted Whatman™ GF/F filters for the determination of pigments (chl-*a* and its degradation products).

2.2. Sediment Core Incubations

At the seven stations (120, 130, 230, 145, 700, 710, and 780), triplicate small sediment cores (Plexiglass, internal diameter: 10 cm; H: 25 cm) were inserted in a

Reineck Box corer that was deployed several times. A 3-ml sediment subsample of the upper 2 cm of the sediment was used for the determination of pigments (in triplicate) and was stored at -80 ºC until analysis.

At the three additional coarse sediment stations (330, 215, and ZG02), we used centrally stirred chambers (Plexiglass, internal diameter: 19 cm; H: 30 cm) to create an advective pore water flow (Huettel and Rush, 2000). At each station, three chambers were filled up to 10 cm with homogenized sediment from the upper 10 cm of the sediment obtained from a Reineck box corer. Cores were transported within 10 h to a temperature-controlled room at in situ temperature (recorded from the CTD cast) in the lab and immediately submerged in well-aerated sea water. This water is further referred to as ‘‘tank water.’’ Teflon-coated magnets rotated by a central magnet were adjusted 5 cm above the sediment surface of the small cores (internal diameter: 10 cm) to ensure mixing of overlying water. The rate of water circulation was kept well below the resuspension threshold. The centrally stirred chambers had top lids equipped with a flat stirring disk, which was positioned at 5.4 cm above the sediment surface and rotated at 12 rpm. The sediments were left to acclimatize overnight in the dark. The next day, closed sediment incubations were initiated by closing cores airtight. Cores were subsequently incubated in the dark for a period long enough for the fluxes to reach steady-state but ensuring oxygen not to drop below 50% saturation.

The actual incubation period depended on the sediment type and temperature (that is, longer incubation times for coarse sediment and measurements at low temperatures). From April onward, the influence of water column processes was estimated by incubating tank water in cores of half the volume of a normal fine sandy sediment core (that is, internal diameter: 10 cm; H: 12.5 cm). The overlying water in all incubations was subsampled with a glass syringe for O_2 (2 x 12 ml, Winkler), DIN (3 x 10 ml), and A_T (3 x 25 ml), the latter two subsamples filtered through Whatman™ GF/F filters. Oxygen and alkalinity concentrations were determined at the start and the end of the incubation, because their respective decrease and increase are generally linear. Triplicate DIN samples were taken at the start and end of the incubation, as well as half way through the incubation time to check for linearity in DIN exchange.

At the same time, samples of tank water were taken for O_2, DIN, and A_T to correct for concentration changes due to water replacement during subsampling. Oxygen

samples were stored in the same temperature-controlled room in the dark until further analysis (within 3 days); DIN samples were stored frozen (-20 ºC); and alkalinity samples were stored refrigerated (4 ºC).

For the N_2:Ar samples in October, all equipment was stored submerged in the incubation tank 48 h before samples were obtained, to avoid introduction of new surfaces for O_2, N_2, and Ar adsorption. This is pivotal for the correct determination of N_2 production (Kana *et al.,* 1994). Sample collection was performed as described in Na *et al*. (2008). The concentrations of N_2 and Ar were determined by membrane-inlet mass spectrometry (MIMS; GAM; IPI) at the Max Planck Institute for Marine Microbiology in Bremen. DIC samples were analyzed with a Li-Cor® LI 7000 solid state infrared CO_2 detector after acidification (AS-C3 DIC analyzer, Apollo SciTech).

At the end of the incubations, the cores were opened and aerated again. After 24 h of reaeration, depth profiles of oxygen were measured in the sediment with O_2 microsensors (25 and 100 µm tip size, Unisense) in vertical increments of 250 µm (3 replicate profiles in each core). pH depth profiles were also measured using a sturdy pH sensor (Hannah Instruments) in February-May at 1-cm depth intervals down to 10 cm. Hereafter, the sediment surface (upper 2 cm) was subsampled (2 ml) for the analysis of pigments, % organic carbon and nitrogen and grain size. Subsequently, the remainder of the sediment was sieved on a 1-mm mesh to sample the macrofauna, which was preserved in ethanol. The pigment samples were stored at -80 ºC until analysis, whereas the organic C/N and grain size samples were oven-dried at 60 ºC before analysis.

2.3. Macrobenthos Analysis

Macrobenthos specimens were identified to the lowest possible taxonomic level (typically species level) and biomasses were determined (blotted wet weights). Using this macrofauna dataset, the bioturbation potential index (BPi) and the BPc were calculated (Solan *et al*., 2004; Birchenough *et al*., 2012; Queirós *et al*., 2013).

2.4. Laboratory Analyses and Flux Calculation

Pigments were determined by HPLC analysis according to Wright and Jeffrey (1997). Total organic C and N content was analyzed with a FLASH 2000 NC Elemental Analyzer (0.01 % detection limit) and sediment granulometry by laser diffraction (Malvern Instruments, 2 µm detection limit). Sediment permeability was calculated

according to Hazen (k_H = 1.1019 x 10^3 m^{-2} s x d^2_{10} x v; with k_H = permeability; d_{10} = first decile of the grain size distribution (m); and v = kinematic viscosity (in m^2 s^{-1})) (Eggleston and Rojstaczer, 1998). Obtained permeabilities were corrected according to Rusch *et al*. (2001). Sediments with a k_H above 2.5 x 10^{-12} m^2 are considered permeable (Forster *et al*., 2003; Wilson *et al*., 2008). Oxygen was analyzed by automated Winkler titration (Parsons *et al*., 1984; detection limit ~2 µmol l^{-1}), DIN was analyzed using automated colorimetric techniques (SKALAR) and alkalinity was determined by automated end-point titration (to pH 4.2) (G20 Mettler-Toledo). Oxygen and alkalinity fluxes were calculated from the difference between initial and final concentrations in the overlying water compensating for concentration changes during subsampling. DIN, N_2:Ar, and DIC fluxes were calculated, after correction for dilution by refill water, from the significant regression slopes ($P < 0.05$) of concentration over time. Finally, a correction for the processes occurring in the water column was made in the fine sandy sediment cores from April to October, by subtracting the rates in the water column from the total measured rate.

2.5. Mass Budget Modeling

The fluxes of O_2, NO_x, and NH_x across the sediment–water interface were used to estimate rates of denitrification, nitrification, and total carbon and nitrogen mineralization. This was done by constructing an integrated mass balance of oxygen, nitrate, and ammonium over the entire sediment column (Soetaert *et al*., 2001) (see Text S1).

It must be noted that the DIN concentrations of the ‘‘tank water’’ used in the core incubations (see further) differed from the field DIN concentrations (Table S2, Addendum 1): although NH_x concentrations were rather similar in the field and in the lab, NO_x concentrations were one order of magnitude higher in the tank than in the field. The reported NO_x fluxes and modelled denitrification estimates should therefore be considered as ‘‘potential rates.’’

2.6. Statistical Analyses

Two-way ANOVAs with type III sums of squares for unbalanced designs, followed by Tukey post-hoc tests were carried out to investigate whether the variation in environmental variables, univariate macrobenthic measures (species density, richness, biomass, BPc), and measured fluxes (SCOC, DIN, and alkalinity fluxes)

and modelled processes (nitrification, denitrification) depended on the categorical predictors station and month and their interaction station x month. To ensure homogeneity of variances, some variables were square root transformed (Table 1). Multivariate differences in macrobenthic communities among station and month were tested with a two-way crossed ANOSIM. Subsequent SIMPER analysis indicated the macrobenthic species that contributed most to the community composition of each station. Second, we constructed linear models to predict SCOC, NH_x, and alkalinity fluxes and estimated denitrification and nitrification, as a function of the abiotic and biotic (macrobenthic) environmental variables. Only uncorrelated environmental variables were selected, based on the variance inflation factor less than 5 (Heiberger and Holland, 2004): median grain size, rather than silt percentage, macrobenthic density rather than species richness and biomass, water column chl-*a*, N and C in the sediment, temperature, and BPc. Graphical exploratory techniques were used to check the assumptions for linear regression prior to analysis. However, plots of residuals versus fitted values clearly indicated heterogeneity of variances. Therefore, we adopted a linear regression with a generalized least-squares extension (West and Welch, 2006; Pinheiro and Bates, 2009; Zuur *et al*., 2009), which allows unequal variances among treatment combinations to be modeled as a variance covariance matrix (West and Welch, 2006; Pinheiro and Bates, 2009). Following West and Welch (2006) and Zuur *et al*. (2009), the most appropriate variance covariate matrix was determined using AIC scores in conjunction with plots of fitted values versus residuals with different variance covariate terms relating to the independent variables, using restricted maximum likelihood (ML) (REML, West and Welch (2006)). This procedure resulted in the use of a variance structure that allowed for different variances per stratum for station (varIdent function, R package nlme). Violation of independence of the residuals through temporal autocorrelation between sampling events was investigated by plotting the autocorrelation factor (ACF) versus the time lag. In case these plots indicated temporal autocorrelation, we re-ran the model with an auto-regressive model of order 1 (AR-1) autocorrelation structure, specifying the ‘‘corAR1’’ correlation option with respect to sampling event.

We compared the models with and without autocorrelation structure using Akaike’s information criterion (AIC). Once the appropriate random component had been determined, the fixed component of the model was refined by manual backwards

stepwise selection using ML to remove insignificant variable terms. The minimal adequate model was presented using REML (West and Welch, 2006).

Following Underwood (1997), the highest order significant interactions in the minimal adequate model were examined, but the nested levels within these were not. The importance of the highest order term was estimated using a likelihood ratio (*L* ratio) test to compare the full minimal adequate model with a model in which the relevant variable and all the interaction term that it was involved in, was omitted. Fluxes were expressed as a function of the independent environmental variables and macrobenthic descriptors. Only cases with information on all variables (n = 165) were used in the models.

Models with different combinations of variables and their centered (to reduce collinearity) quadratic terms were evaluated. Adding a temporal autocorrelation component to the models of SCOC and NH_x decreased the temporal pattern in the residuals and lowered the AIC. The importance of faunal predictor's density and BPc was assessed by comparing nested models with and without faunal predictors, using ML (*L* ratio) tests. In addition, models including BPc as predictor were evaluated for muddy, fine grained, and permeable sediment types separately. All analyses were performed in the free statistical environment R, except for the multivariate analyses carried out in Primer v6 (Clarke and Gorley, 2006). Unless indicated differently, results are expressed as mean ± standard deviation.

3. Results

3.1. Spatial and Temporal Variability in Benthic–Pelagic Coupling

Chl-*a* concentrations in the water column of the Southern North Sea followed clear temporal and spatial patterns (Figure 2; two-way ANOVA see Table 1). In the near-shore stations 120, 130, 145, 700, 230, 710 and the more offshore located station 780, concentrations were low from February to April (0.02 ± 0.02 µg l^{-1}), followed by a clear peak in chl-*a* at the end of May (12.18 ± 4.07 µg l^{-1}). After this spring bloom peak, chl-*a* concentrations declined sharply (5.88 ± 2.71 µg l^{-1}). A certain portion of the peak bloom arrived at the sediment from May onward (Figure 2). In the offshore stations 215, 330, and ZG02, chl-*a* concentrations in the overlying water in August (9.82 ± 3.76 µg l^{-1}) were double those in June (3.94 ± 1.08 µg l^{-1}). Owing to strong benthic–pelagic coupling, a pattern of spatio-temporal differences in the water

column pigments is also reflected in the chl-*a* concentrations in the sediment (two-way ANOVA, Table 1; Figure 2).

Sediment median grain size, silt content, % organic N, and permeability differed among stations and time (Table 1), but a Tukey HSD test indicated mostly differences among stations. Based on the Wentworth granulometry scale (Wentworth, 1922) and calculated permeability (Table S3 and Figure S1, Addendum 1), the sampled stations can be classified into ‘‘muddy’’ (St. 130, 145, and 700), ‘‘fine sandy’’ (St. 120, and 780), and ‘‘permeable’’ sediments (near-shore St. 230 and 710 and offshore St. 330, 215, and ZG02) and will be named as such henceforth.

Table 1: Results of ANOVA for water column and sediment characteristics, fluxes, and macrobenthic descriptors

Variable	Factor	*F*	df_{term}	df_{error}	*P*	Transformation
Water chl-*a*	St * M	8.42	36	110	***	√
Sediment chl-*a*	St * M	18.54	34	103	***	√
Median grain size	St * M	1.89	36	110	**	
% silt	St * M	2.49	36	110	***	
% N	St * M	3.58	36	110	***	√
% C	St * M	4.02	36	110	***	
Permeability	St * M	3.05	36	110	***	
SCOC	St * M	2.64	36	110	***	
NH_X	St * M	4.40	36	110	***	
NO_X	St * M	5.09	36	110	***	
TA	St * M	1.77	36	110	*	
DIC	St	45.52	2	9	***	
Nitrification	St * M	5.16	36	110	***	
Denitrification	St * M	4.88	36	110	***	
Max. oxygen penetration depth = max. depth where oxygen is present	St * M	10.82	29	81	***	
Macrobenthic density	St	38.50	9	110	***	√
Macrobenthic species richness	St	35.13	9	110	***	
	M	4.35	9	110	***	
Macrobenthic biomass	St	24.78	9	110	***	√
Bioturbation potential	St	36.42	9	110	***	√

*Single factor results are only given where the interaction station * month (St * M) is not significant. Significance levels are indicated with * (0.01 < P < 0.05), ** (0.001 < P < 0.01), or *** (P < 0.001).*

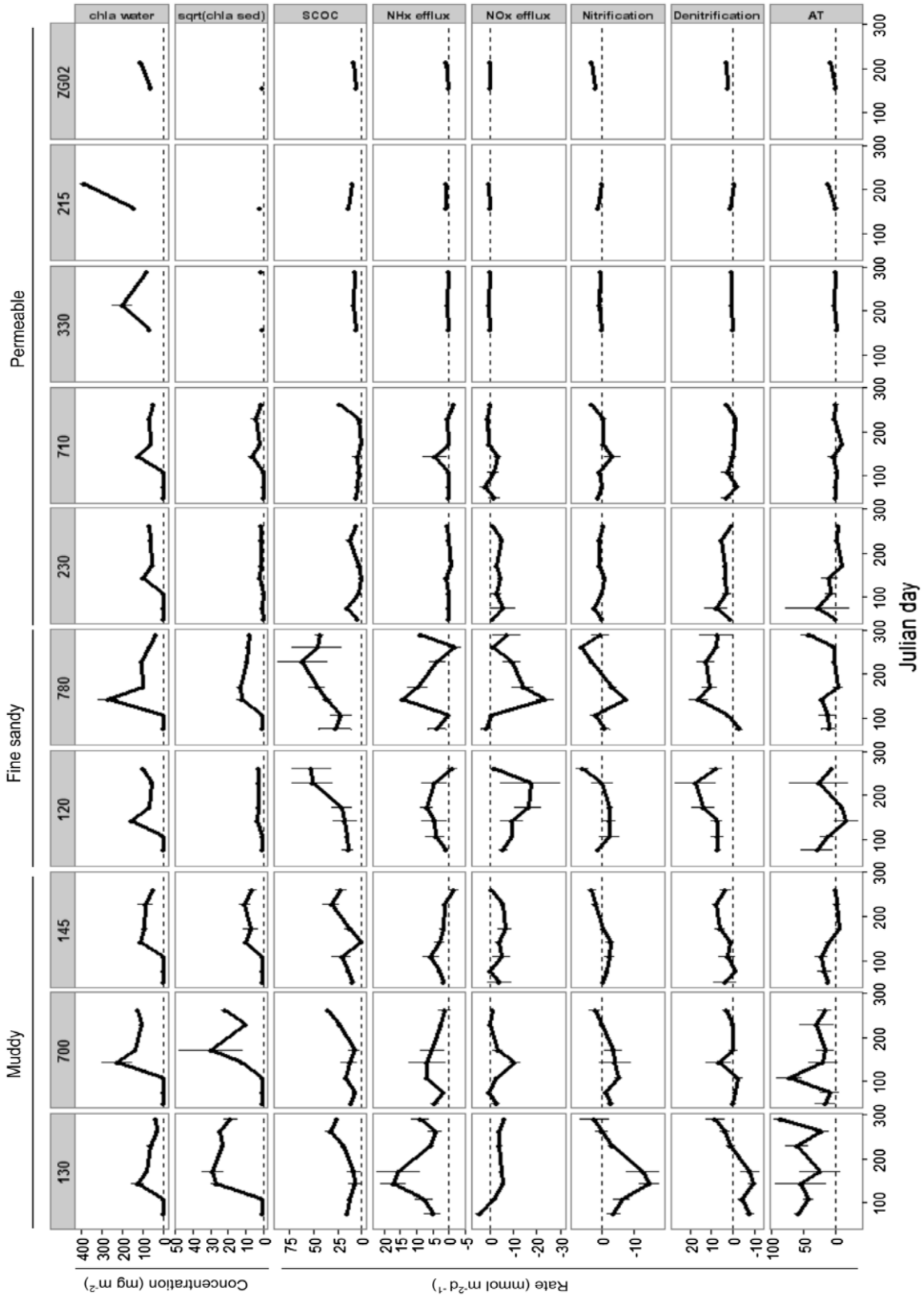


Figure 2: Chlorophyll *a* in overlying water (1 m above the sea floor, integrated over water depth) and in the surface sediment (upper 2 cm; sqrt transformed for better visualization), measured SCOC (mmol O_2 m^{-2} d^{-1}), NH_x effluxes, potential NO_x effluxes (mmol N m^{-2} d^{-1}), estimated nitrification and denitrification (mmol N m^{-2} d^{-1}), and measured alkalinity flux (meq m^{-2} d^{-1}) in all stations over time (mean ± SD). No data for St. 780, 120, and 130 in February. Note the different scales on y-axes.

3.2. Spatial and Temporal Variability in Biogeochemical Cycling and Macrobenthic Structural and Functional Biodiversity

All measured fluxes differed among stations and time (two-way ANOVA, Table 1), but again a Tukey HSD test indicated mostly differences among stations. Macrobenthic characteristics varied mainly among station (two-way ANOVA, Table 1) and separate macrobenthic communities could be distinguished. Two-way-crossed ANOSIM revealed differences in community composition between stations ($R = 0.582$, $P < 0.001$) and sampling months (0.207, $P < 0.001$). Pairwise tests indicated that macrobenthic community composition was only similar between stations within muddy (St. 130, 700 and 145) and permeable (St. 230 and 710) sediments ($P > 0.05$). The temporal effect consists of a shift in composition from spring to autumn. The SIMPER results that characterize each station by the most abundant species are shown in Table S4 (Addendum 1).

3.2.1. Muddy Sediments

St. 130, 145, and 700 showed the maximum OM content (averages of the muddy stations ranged between 1.10-1.91 % of organic carbon and 0.12–0.30 % organic nitrogen, Table S3 and Figure S1, Addendum 1) and a stable annual maximum oxygen penetration depth (‘‘OPD,’’ that is, the maximum depth where oxygen is still present) ranging between 3.4 ± 0.2 mm (St. 130) and 3.7 ± 0.2 mm (St. 145 and 700). Despite the substantial OM content, these stations displayed only moderate SCOC rates (Figure 2). St. 145 had a variable SCOC (0–41.92 mmol O_2 m^{-2} d^{-1}), with a minimum in May (Tukey HSD $P < 0.05$). SCOC at St. 130 and 700 showed a more regular pattern, with low values early in the year (12.10 ± 3.36 mmol O_2 m^{-2} d^{-1}), gradually increasing to maximum rates in September (33.99 ± 2.81 mmol O_2 m^{-2} d^{-1}). These elevated summer SCOC rates coincided with a high water column temperature (16 °C), and showed a time lag of 3-4 months with respect to the deposition of the phytoplankton bloom in May-June (Figure 2). In contrast, increased ammonium effluxes (average of St. 130 and 700 in May and June, respectively, 16.34 ± 5.00 and 5.94 ± 4.44 mmol N-NH_x m^{-2} d^{-1}) did immediately follow the phytoplankton bloom deposition, suggesting an offset in the timing of OM mineralization and O_2 consumption. These high mineralization rates also appear associated with a sharp decline in pH of up to 1 pH unit in the subsurface sediment layers (Figure 3).

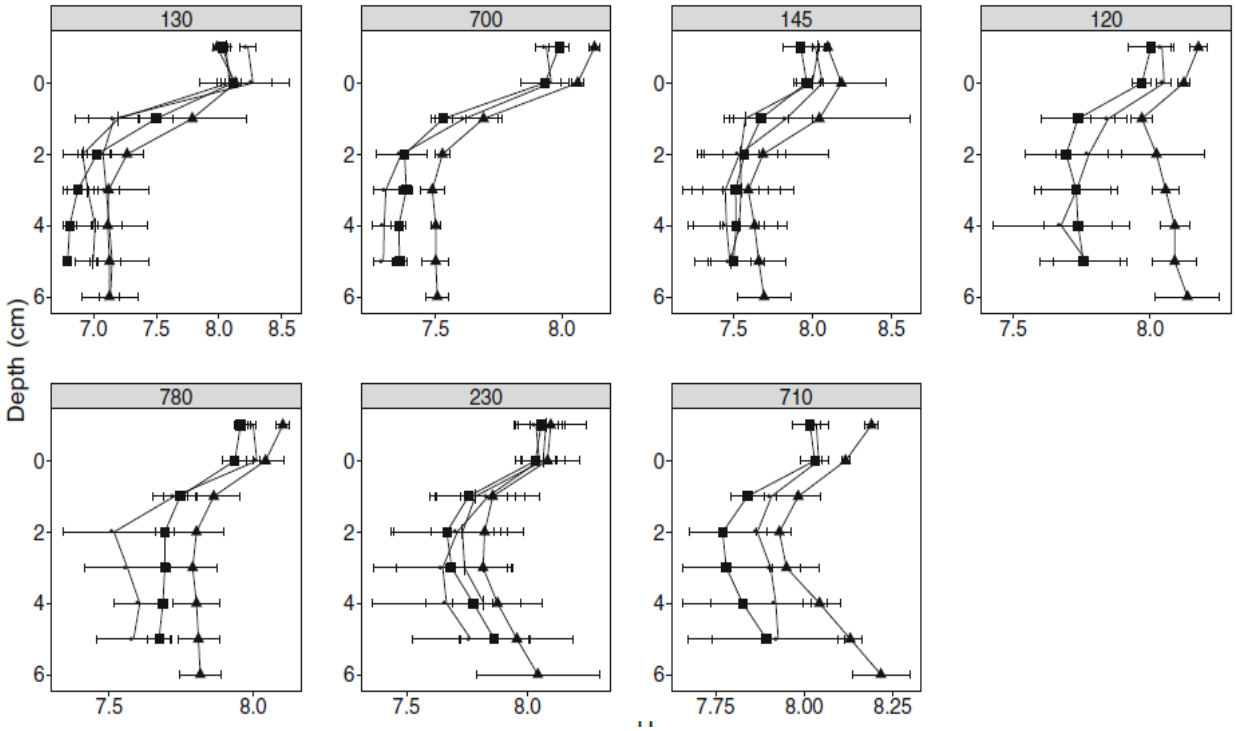


Figure 3: Sediment pH depth profiles (average ± SD) in each station in February (filled triangle), March (filled circle), April (filled square), and May (plus). In May, only St. 130, 145, and 230 were profiled.

No pronounced nitrate exchange was observed, except for a nitrate efflux in March at St. 130 (4.48 ± 1.45 mmol N-NO_x m^{-2} d^{-1}) and the very variable nitrate effluxes in April (range -1.73 to +33.34 mmol N-NO_x m^{-2} d^{-1}) and August (range -0.31 to +42.49 mmol N-NO_x m^{-2} d^{-1}) at St. 700. These two replicates at St. 700 with extremely high NO_x effluxes were considered outliers and are not further used in models and also not shown in Figure 2. The maximum alkalinity effluxes of all stations were measured at St. 130 and 700. At St. 130, alkalinity effluxes were higher later in the year (maximum in October: 86.88 ± 8.75 meq A_T m^{-2} d^{-1}), associated with elevated DIC effluxes (St. 130: 66.49 ± 18.14 mmol DIC m^{-2} d^{-1} in October), which seem indicative of carbonate dissolution. No significant denitrification was measured using N_2:Ar technique at St. 130 in October, which is in accordance with the very low denitrification values deduced from our mass budget approach at this station. Nitrification estimates based on the mass budget approach resulted in (unphysical) negative values until late summer for all muddy stations (September: 2.05 ± 2.68 mmol N m^{-2} d^{-1}), whereas denitrification estimates were low but positive at St. 145 in all months but March, and positive from September on at St. 700 and 130 (6.27 ±

4.54 mmol N m^{-2} d^{-1}) (Figure 2). Negative nitrification and denitrification estimates are not realistic. Rather, in these cases, the nitrification and denitrification processes are either zero or some of the assumptions underlying the mass budget were not justified (steady-state assumption; assumption that all reduced substances are reoxidized within the sediment). The latter explanation is corroborated by the low respiratory quotient (SCOC/DIC production) for St. 130 (= 0.39), which implies that reduced compounds in the sediment are not efficiently reoxidized or that intensive carbonate dissolution occurs.

Although the mass budget did not perform well in these muddy sediments, it highlights the need for data on sulfate reduction, the accumulation of reduced substances (for example, iron sulfides) and the dissolution of carbonate to effectively predict nitrogen cycling rates. This was beyond the scope of this study, but is crucial as guidance to future studies.

The muddy stations harbored little fauna, hence a low macrobenthic density (527 ± 795 ind. m^{-2}), biomass (48.72 ± 66.64 g WW m^{-2}), and species richness (1.67 ± 1.38 species core-1). The fauna that is present is characteristic of the *Macoma balthica* community (Degraer *et al*., 2009) (SIMPER results in Table S4, Addendum 1). The muddy sediments harbor mainly surficial modifiers (*Macoma balthica* and small polychaetes, Tables S4 and S5) that in addition to their low densities and biomass in these sediments have a limited influence on sediment reworking. This is reflected in a low BPc (416.13 ± 494.21 m^{-2}) (Figure 4).

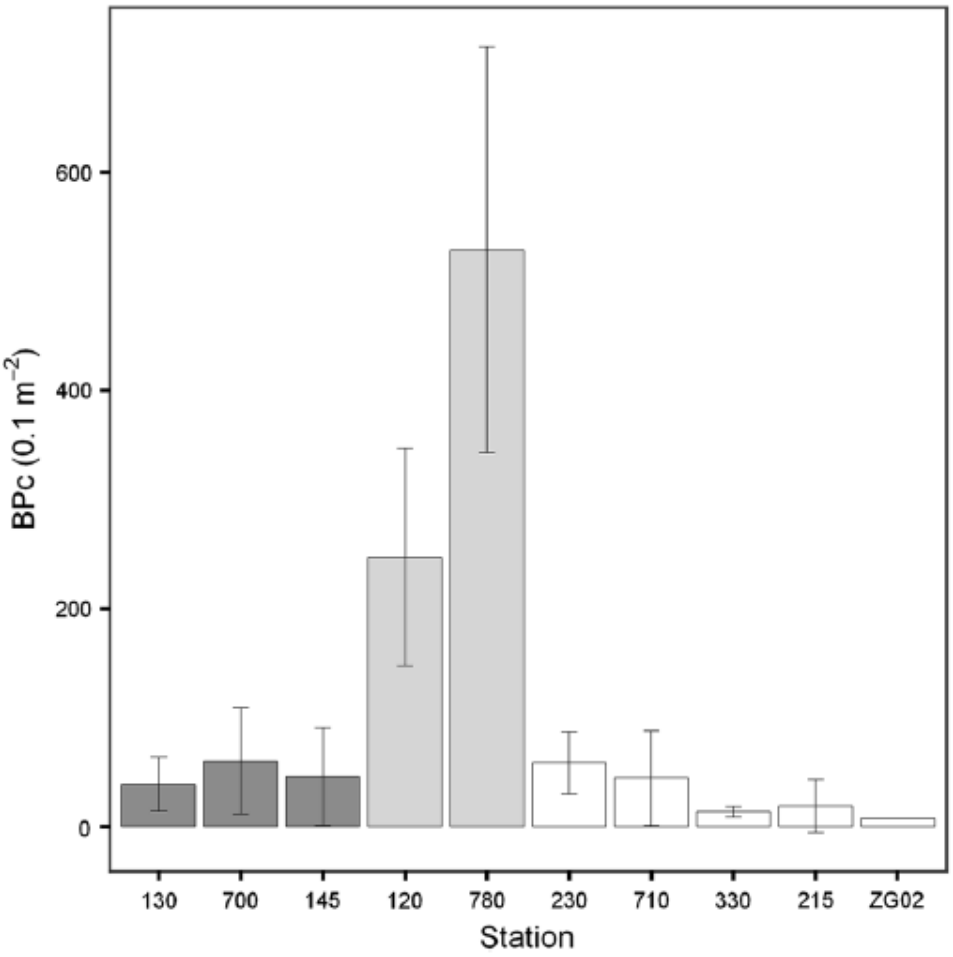


Figure 4: Bioturbation potential of the community (BPc) in each station averaged over the year (mean ± SD). Muddy sediments are indicated in dark gray bars, fine sandy sediments in light gray, and permeable sediments in white. Fine sediment stations (St. 780 and 120) display highest BPc ($P < 0.05$) while permeable and muddy stations have comparably low BPc values.

3.2.2. Fine Sandy Sediments

St. 780 and 120 displayed an intermediate organic carbon (respectively, 0.40 and 0.52 %) and nitrogen (0.04-0.07 %) content (Table S3 and Figure S1, Addendum 1) and an annual average maximum OPD of 3.9 ± 0.2 and 3.7 ± 0.2 mm, respectively. This relatively shallow OPD corroborates the maximum oxygen consumption rates (average in August: 56.31 ± 22.12 mmol O_2 m^{-2} d^{-1}), which coincides with the highest annual sea water temperature (18 °C), which is also a time lag of 3 months with respect to phytoplankton deposition (Figure 2). Moderate to high ammonium effluxes (4.45 ± 4.87 mmol N-NH_x m^{-2} d^{-1}) and clear nitrate influxes (8.67 ± 8.42 mmol N-NO_x m^{-2} d^{-1}) characterized these stations (Figure 2). At St. 780, maximum NH_x effluxes

(14.37 ± 0.51 mmol N-NH_x m^{-2} d^{-1}) and NO_x influxes (23.49 ± 3.74 mmol N-NO_x m^{-2} d^{-1}) coincided with deposition of the phytoplankton bloom (Figure 2). Moderate alkalinity fluxes (10.70 ± 21.20 meq A_T m^{-2} d^{-1}) typify these fine sandy stations (Figure 2). At both stations, nitrification estimates in summer (3.35 ± 3.43 mmol N m^{-2} d^{-1}) and denitrification estimates throughout the year (11.01 ± 8.16 mmol N m^{-2} d^{-1}) were higher than at all other stations and a clear increase in modeled denitrification at the time of phytoplankton deposition was observed (Figure 2). Although the NO_x concentrations in the water column of the lab incubations were higher than those of the overlying water in the field, observed and modeled denitrification at St. 780 in October (measured: 5.54 ± 1.02 mmol N m^{-2} d^{-1}; modeled: 7.43 ± 8.27 mmol N $m^{-2}d^{-1}$) agreed reasonably well. This indicates that the mass budget model results are within realistic ranges for these stations. Sediment pH profiles showed an initial decline of about 0.25 pH units in the first 1-2 cm, after which they were stable in depth (Figure 3). The DIC efflux at St. 780 in October was comparable to the one measured at St. 130 (62.70 ± 13.24 mmol DIC m^{-2} d^{-1}), but taking into account the increased SCOC (43.88 ± 4.84 mmol O_2 m^{-2} d^{-1}), this results in a more elevated respiratory quotient (= 0.70) at this fine sandy station, which implies that reduced compounds in the sediment are more efficiently reoxidized, and/or that carbonate dissolution is less important.

St. 780 and 120 displayed macrobenthic abundances (4,608 ± 3,527 ind. m^{-2}), biomass (411.69 ± 488.32 g WW m^{-2}), and BPc (3,952.89 ± 2,813.37 m^{-2}) (Figure 4) that are an order of magnitude higher than the muddy and permeable sediments (Tukey HSD $P < 0.05$). Maximum values of species richness (7.44 ± 2.74 species $core^{-1}$) were displayed in this sediment type (Tukey HSD $P < 0.05$). The fauna present belongs to the species rich and abundant *Abra alba* community (Van Hoey *et al*., 2004) (SIMPER results in Table S4, Addendum 1).

The very high BPc in the fine sandy stations are mainly made up by the BPc of the biodiffusers *Echinocardium cordatum*, *Sagartia troglodytes*, and *Abra alba* (Table S5, Addendum 1). The total proportion of biodiffusers in the community is not that different from the biodiffuser proportion in other stations, but the biomass is the highest of all stations. At St. 780, also increased densities and biomass of surficial modifiers (mainly *Owenia fusiformis*) contribute to a higher BPc (Tables S4 and S5).

3.2.3. Permeable Sediments

Near-shore St. 230, 710 and offshore St. 215, 330, and ZG02 displayed the lowest organic carbon (averages of the permeable stations ranged between 0.10 and 0.25%) and nitrogen content (0.02-0.09%; Table S3 and Figure S1, Addendum 1) and deeper oxygen penetration (annual average maximum OPD, respectively, 4.6 ± 0.4 and 7.8 ± 1.2 mm at St. 230 and 710), which correspond to a lower oxygen demand (6.73 ± 5.54 mmol O_2 m^{-2} d^{-1}), lower DIN exchange (average NH_x efflux of 0.44 ± 1.26 mmol N-NH_x m^{-2} d^{-1} and an average NO_x influx of 1.00 ± 2.41 mmol N-NO_x m^{-2} d^{-1}) and lower alkalinity effluxes (2.58 ± 12.24 meq A_T m^{-2} d^{-1}) (Figure 2). The maximum OPD at St. 710 decreased with higher temperatures to 2.58 ± 0.58 mm (Tukey HSD $P < 0.05$); this increased diffusive oxygen uptake coincided with higher SCOC in September (22.94 ± 1.87 mmol O_2 m^{-2} d^{-1}).

Denitrification was only observed in October in one core at St. 330 (measured: 1.80 mmol N m^{-2} d^{-1} vs. modeled: 0.62 ± 0.05 mmol N m^{-2} d^{-1}). Low SCOC (6.63 ± 2.18 mmol O_2 m^{-2} d^{-1}) was associated with an equally low DIC efflux (6.95 ± 2.41 mmol DIC m^{-2} d^{-1} at St. 330) in October, resulting in a respiratory quotient of 0.97. This implies little mineralization and a reoxidation of reduced mineralization products. Very low nitrification (0.68 ± 1.55 mmol N m^{-2} d^{-1}) and denitrification (2.13 ± 3.39 mmol N m^{-2} d^{-1}) rates were estimated throughout the year (Figure 2). Beyond an initial decrease of about 0.25 U in sediment pH in the first centimeter, the pH stabilized, or even increased again at depth (St. 230 and 710 in all months) (Figure 3).

The fauna present in these permeable sediments belongs to the species-poor *Nephtys cirrosa* and *Ophelia limacina* communities (Van Hoey *et al*., 2004) (see SIMPER results in Table S4, Addendum 1). Macrobenthic density (548 ± 775 ind. m^{-2}), biomass (17.33 ± 40.14 g WW m^{-2}), species richness (2.28 ± 2.00 species $core^{-1}$), and BPc (303.34 ± 376.90 m^{-2}) in these stations are low, but comparable to the very muddy stations (Figure 4). However, other than surficial modifiers (bivalves such as *Ensis directus*), there were also biodiffusers (small *Echinocardium cordatum* and *Glycera alba*) and upward (/downward) conveyors present (different Capitellidae and Spionidae species), that nevertheless contributed little to BPc because of low biomass and density (Tables S4 and S5).

3.3. Statistical Modeling of Fluxes from Abiotic and Biotic Environmental Variables

The statistical models that best predicted SCOC, NH_x, alkalinity efflux, and denitrification included strong effects of BPc and were significantly better performing (that is, had a lower AIC and a higher *L* ratio) than similar models with macrobenthic density as the faunal component (Table 2). The best model predicting denitrification included only a strong effect of BPc and its quadratic term (Denitrification 2, Table 2). The best model predicting nitrification was the only model that included medium effects of both BPc and density. Median grain size was further an important explanatory variable for all modeled processes but denitrification.

Temperature was significantly contributing to the explained variance in SCOC and nitrification and to a lesser extent also NH_x and alkalinity efflux, whereas chl-*a* in the water column was only important for NH_x and alkalinity effluxes. When fluxes were modeled per sediment type, BPc appeared as an important explanatory variable in predicting fluxes in fine sandy sediments (SCOC, NH_x, denitrification, and alkalinity model, Table S6, Addendum 1) and to a lesser extent in permeable sediments (SCOC, nitrification, and denitrification model). In contrast, BPc was never significantly contributing to explaining fluxes in muddy sediments; macrobenthic density appeared more important in this case (NH_x, nitrification, and alkalinity model).

Table 2: Oxygen consumption (SCOC), ammonium (NH_x) and alkalinity (AT) effluxes and nitrification and denitrification estimates as a function of environmental variables temperature (Temp), median grain size (MGS), chlorophyll *a* concentration in the water column (chl-*a*), macrobenthic density (Dens) and bioturbation potential of the community (BPc), and their centered quadratic terms.

Model	Model formula	AIC	*L* ratio fauna term
SCOC1	$SCOC = 2.35 + 0.003\ Dens^{***} - 0.034\ MGS^{***} + 6.93e^{-05}\ MGS^{2***} + 0.787\ Temp^{***} + 0.086\ Temp^{2*}$	1,254.063	17.35
SCOC2	$SCOC = 0.75 + 0.005\ BPc^{***} - 0.033\ MGS^{***} + 7.38e^{-05}\ MGS^{2***} + 0.839\ Temp^{***} + 0.092\ Temp^{2**}$	1,221.676	49.94
NH_x1	$NH_x = 2.15^{***} + 2.1e^{-04}\ Dens^{***} - 0.01\ MGS^{***} + 3.6e^{-05}\ MGS^{2***} + 0.04\ Temp + 0.01\ Temp^{2*} + 0.09$ chl-*a***	785.5602	2.67
NH_x2	$NH_x = 1.75^{**} + 6.37e^{-04}\ BPc^{***} - 0.01\ MGS^{***} + 3.1e^{-05}\ MGS^{2***} + 0.07\ Temp^{*} + 0.01\ Temp^{2*} + 0.09$ chl-*a****	771.6502	16.58
Nitrification1	$Nitrification = -5.69^{***} - 5e^{-04}\ Dens^{**} + 6.03e^{-08}\ Dens^{2*} + 9.5e^{-04}\ BPc^{**} - 1.5e^{-07}\ BPc^{2**} + 0.006\ MGS^{***} - 3.4e^{-05}\ MGS^{2**} + 0.36\ Temp^{***} + 0.05\ Temp^{2***} - 0.21$ chl-*a****	920.3118	Density: 7.95 BPc: 9.71
Denitrification1	$Denitrification = 0.59^{**} + 0.002\ Dens^{***} - 2.00e^{-07}\ Dens^{2**}$	1,050.058	$Dens + Dens^2$: 41.14
Denitrification2	$Denitrification = 0.68^{***} + 0.004\ BPc^{***} - 3.00e^{-07}\ BPc^{2***}$	1,035.271	$BPc + BPc^2$: 54.49
Alkalinity1	$Alkalinity = -8.94 + 0.002\ Dens^{***} - 0.04\ MGS^{***} + 2.0e^{-04}\ MGS^{2***} + 1.18\ Temp^{*} + 0.16\ Temp^{2*} - 0.83$ chl-*a* $+ 0.16$ chl-a^{2**}	1,415.762	10.83
Alkalinity2	$Alkalinity = -8.93 + 0.002\ BPc^{***} - 0.04\ MGS^{***} + 2.0e^{-04}\ MGS^{2***} + 1.16\ Temp^{*} + 0.15\ Temp^{2*} - 0.81$ chl-*a* $+ 0.17$ chl-a^{2**}	1,413.818	12.41

Significance of terms (P < 0.05, ** P < 0.01, *** P < 0.001) and the AIC as determined with restricted maximum likelihood of the best models are given; bold AIC values indicate the best model.*

4. Discussion

The Southern North Sea harbors a variety of benthic habitat types, both in terms of sedimentology (Verfaillie *et al*., 2006) and macrobenthic biodiversity (Van Hoey *et al*., 2004; Degraer *et al*., 2008). This study also showed that biogeochemical cycling clearly differs among habitat types and throughout the year. The annual spring phytoplankton bloom, deposited on the sea floor in May, results in a substantial input of OM to the benthic ecosystem. The observed patterns in chl-*a* in sediment and overlying water are consistent with the phytoplankton dynamics previously described for the Southern Bight of the North Sea (for example, Rousseau *et al*., 2002; Lancelot *et al*., 2005). Benthic–pelagic coupling was strong at most stations: the peak in sediment chl-*a* coincided with the peak in the overlying water and the deposition event triggered OM mineralization processes at the sea floor, which differed according to sediment type.

Whereas the deposition of the phytoplankton bloom in spring to muddy sediments was immediately evidenced by high ammonium effluxes, the highest oxygen consumption was delayed until summer when temperatures reached a maximum. The deposition event has been shown to trigger the microbial loop in the muddy sediments of the Southern North Sea (Pede, 2012), which can indeed initiate the first degradation of particulate OM to ammonium (Jensen *et al*., 1990; Grenz *et al*., 2000). However, in other shallow coastal seas as well, the time lag in oxygen consumption has been demonstrated (Kanneworff and Christensen, 1986; Rudnick, 1989), revealing the temperature dependency of sedimentary biogeochemical processes (Provoost *et al*., 2013). Because of the increased OM content and bacterial abundances (Gillan *et al*., 2012), the cohesion of the sediment matrix and the lack of bioturbating fauna in the muddy stations, oxygen rapidly becomes depleted in the upper millimeters. The small amount of oxygen present in these muddy sediments is probably mostly used for oxic mineralization and sulfide re-oxidation, because the estimated nitrification rates are low throughout most of the year. In addition, estimated and measured denitrification rates were small to non-detectable. At sites with elevated OM mineralization rates, denitrification is often reduced through sulfide inhibition of nitrifying bacteria or by inhibition of coupled nitrification-denitrification in oxygen-deficient sediment (Joye and Hollibaugh, 1995; Eyre and Ferguson, 2002). In this case, most of the nitrogen returns to the water column as NH_4^+, especially after the phytoplankton bloom deposition, which creates a positive feedback to

eutrophication in an area that is already sensitive to oxygen stress (Van Hoey *et al*., 2009). Alternatively, nitrate can be turned into NH_x through dissimilatory nitrate reduction to ammonium (DNRA) (Hulth *et al*., 2005), again stimulating a release of NH_4^+ to the water column.

The muddy sediments displayed the highest alkalinity effluxes of all sampled stations, with a maximum in summer at St. 130. This corroborates the earlier observed seasonal variability in sediment-water alkalinity fluxes in the North Sea (Thomas *et al*., 2009). Important sources of alkalinity generation in coastal areas include sulfate reduction with the formation of metal sulfides and to a lesser extent denitrification (Chen and Wang, 1999; Thomas *et al*., 2009). Because denitrification estimates were low at the muddy stations, it is probably either sulfate reduction (or the subsequent accumulation of pyrite) or the dissolution of carbonate that generates the high alkalinity effluxes at this station. Increased sulfide accumulation at depth has been observed at St. 130 (Gao *et al*., 2009; Malkin *et al*., 2014), which indicates that intensive sulfate reduction is indeed taking place. However, electrogenic filamentous bacteria have recently been discovered at St. 130 (Malkin *et al*., 2014). Although Hu and Cai (2011) argue that only net sulfate reduction (with the formation of metal sulfides) contributes to alkalinity generation in sediments, this novel electrogenic pathway of sulfide oxidation may generate high alkalinity effluxes. Electrogenic sulfur oxidation strongly consumes protons within the upper layer of the sediment (Nielsen *et al*., 2010), and this way, it creates a zone of high alkalinity generation in the upper millimeters of the sediment, potentially driving the observed efflux of alkalinity out of the sediment. In addition, electrogenic sulfur oxidation has been associated with strong carbonate dissolution at depth (Risgaard-Petersen *et al*., 2012), thus providing a second pathway for sedimentary alkalinity generation.

The response to phytoplankton bloom deposition in the fine sandy sediments in terms of ammonium effluxes and oxygen consumption was very similar to the one described for muddy sediments. Although the OM content of these fine sandy sediments is substantial, the abundant bioturbating and bio-irrigating fauna keep the sediment sufficiently oxygenated to stimulate nitrification. The produced nitrate can then serve as a substrate for denitrification. The importance of bioturbation and bio-irrigation processes has been shown to enhance coupled nitrification–denitrification (Pelegri *et al*., 1994; Rysgaard *et al*., 1995). Maximum estimates of both nitrification (in summer) and denitrification (throughout the year) were indeed found at the fine

sandy stations, which suggest intensified coupled nitrification–denitrification (Cornwell *et al*., 1999). Denitrification is an important process, because it effectively removes fixed nitrogen from the system (Seitzinger, 1988). However, increased denitrification would lead to higher alkalinity fluxes, which is not supported by our observations. The lower alkalinity effluxes at St. 780 and 120 can probably be attributed to enhanced reoxidation of reduced substances by irrigating fauna, and/or reduced rates of carbonate dissolution. The same accounts for the temporal trend in alkalinity fluxes: because of oxygen deficiency, it is supposed that more anaerobic processes take place in summer, but because fauna are also more active at this time (Maire *et al*., 2007; Braeckman *et al*., 2010), enhanced reoxidation of the reduced substances takes place (Eyre and Ferguson, 2002). These observations clearly confirm the importance of bioturbating and bioirrigating fauna for benthic carbon and nitrogen mineralization.

Overall, very small benthic fluxes were observed in the permeable sediments. Because of the efficient reoxidation of reduced substances, alkalinity effluxes are also very low. The near-shore St. 230 and 710 evidenced more pronounced fluxes than the offshore permeable stations, although the latter were subjected to an experimentally induced advective current into the sediment. However, because SCOC observed at St. 330 is similarly low as the measurements without the centrally stirring disk in Franco et al. (2010) at the same station, the advective flow here applied was probably too weak to really simulate the hydrodynamic forces experienced in the field. On the other hand, there might be no OM left in these permeable sediments because they recycle fresh OM quickly (Huettel *et al*., 2003; Rasheed *et al*., 2003). Taking into account the substantial inflow of oxygenated sea water into permeable sediments under in situ conditions, it is possible that these sediments are equally if not even more important than muddy and fine sandy sediments in terms of carbon mineralization (Gao *et al*., 2012), especially when OM is deposited on the sea floor (Huettel *et al*., 2003; Rasheed *et al*., 2003). Non-invasive in situ measurements such as the Eddy correlation technique (Berg *et al*., 2003) should be compared to core incubations in the near future.

For the development of the statistical models predicting measured or estimated processes, not all the measured environmental variables were included. Preliminary models also showed the importance of sediment chl-*a* and organic nitrogen content of the sediment. However, to facilitate future spatial extrapolation of the measured

biogeochemical cycling processes, we chose to use only those environmental parameters for which full coverage maps exist [sediment median grain size (Verfaillie *et al*., 2006), temperature and chl-*a* in the water column (MODIS and MERIS satellite data, http://www2.mumm.ac.be/remsem/index.php)], macrobenthic density (Degraer *et al*., 2006) or will be available in the near future (BPc).

Several studies have estimated fluxes from environmental variables, extrapolating from, for example, sediment porosity, chl-*a* content and bottom water nutrient concentration (Grenz *et al*., 2000), sediment permeability (Gao *et al*., 2012), granulometry, sediment oxygen content and organic carbon content (Deutsch *et al*., 2010), depth and primary production (Wenzhöfer and Glud, 2002), and nitrate availability in the overlying water and granulometry (Deek *et al*., 2012). None of these studies take into account biotic parameters.

Effects of biodiversity can be hard to detect in natural systems, because variability of environmental variables outweighs the mediating effects of biota (Raffaelli, 2006; Godbold and Solan, 2009). For our study area however, significantly better statistical models were obtained when including biotic information: in all models, BPc or macrobenthic density was essential in explaining spatio-temporal variability in fluxes. The importance of BPc for biogeochemical processes depended also on sediment type: BPc was a crucial predictor variable for SCOC, NH_x efflux, and alkalinity efflux and denitrification in fine sandy sediments and to a lesser extent for permeable sediments, whereas fluxes in muddy sediments depended on abiotic variables and/or macrobenthic density. This makes clear that the importance of functional biodiversity for carbon cycling is not equal across all habitat types.

The unequal importance of BPc across habitats can be explained by the inherent biotic and abiotic characteristics of each habitat: macrobenthic abundance and biomass, hence also BPc is typically low in cohesive and permeable sediments. If any macrofauna at all are present in the oxygen depleted muddy sediments, they have to stay in contact with the oxygen-rich overlying water, either by dwelling at the surface (surficial modifiers that have limited impact on sediment reworking) or by using siphons or by ventilation of their burrows, hence bio-irrigation, a mechanism that is not accounted for in BPc. In permeable sediments, advective pore water transport overrules the effect of bioturbating fauna (Kristensen and Kostka, 2005). In the fine sandy sediments, macrofauna densities and biomass are highest, but still, the elevated BPc values are mainly made up by biodiffusers: a few but large sea

urchins (*Echinocardium cordatum*) and many but small bivalves (*Abra alba*). Where present, dense aggregations of surface modifying tube worms (*Owenia fusiformis*) can also contribute to nearly half of the BPc. So in areas where functional diversity is important, it stands out compared to species richness, density, or biomass alone.

Although the goodness-of-fit was higher when models included BPc compared to models with only abiotic variables, we have to keep in mind that BPc has some other drawbacks. The bioturbation potential of a species is assumed to be context independent; the activity of an organism is independent of the ruling environmental conditions such as temperature and disturbance (Queirós *et al*., 2013). In addition, BPc is a summation of single species effects, biomass, and abundance (Solan *et al*., 2004), whereas many laboratory experiments with artificial communities have shown that the effect of multispecies assemblages on biogeochemical processes are not per se the summation of the single species effects (Waldbusser *et al*., 2004; Mermillod-Blondin *et al*., 2005; Godbold *et al*., 2008). This again shows that our understanding of how fauna and environment interact with each other while influencing OM cycling is still limited (Teal *et al*., 2013). In summary, there is room for improving the proxy for functional biodiversity (BPc), by including measures of interaction between species and their biotic and abiotic environment and by introducing a measure for bio-irrigation.

Notwithstanding its above-mentioned drawbacks, the BPc metric encompassing density, biomass, and functional trait parameters such as bioturbation fashion and intensity appears to explain the variability in fluxes much better than macrobenthic density or biomass alone (this study, Van Colen *et al*., 2012). Especially for monitoring of marine habitats in the framework of the European Marine Strategy, this functional biodiversity index proves valuable for establishing a comprehensive link with carbon mineralization (Birchenough *et al*., 2012; Queirós *et al*., 2013), mainly for fine sandy sediments. Here, a loss of macrobenthic functional diversity might bring along a loss in OM cycling. Because changes in biota can have greater effects on ecosystem properties than changes in abiotic conditions (Hooper *et al*., 2005), it is crucial to maintain this macrobenthic functional diversity.

Conclusions

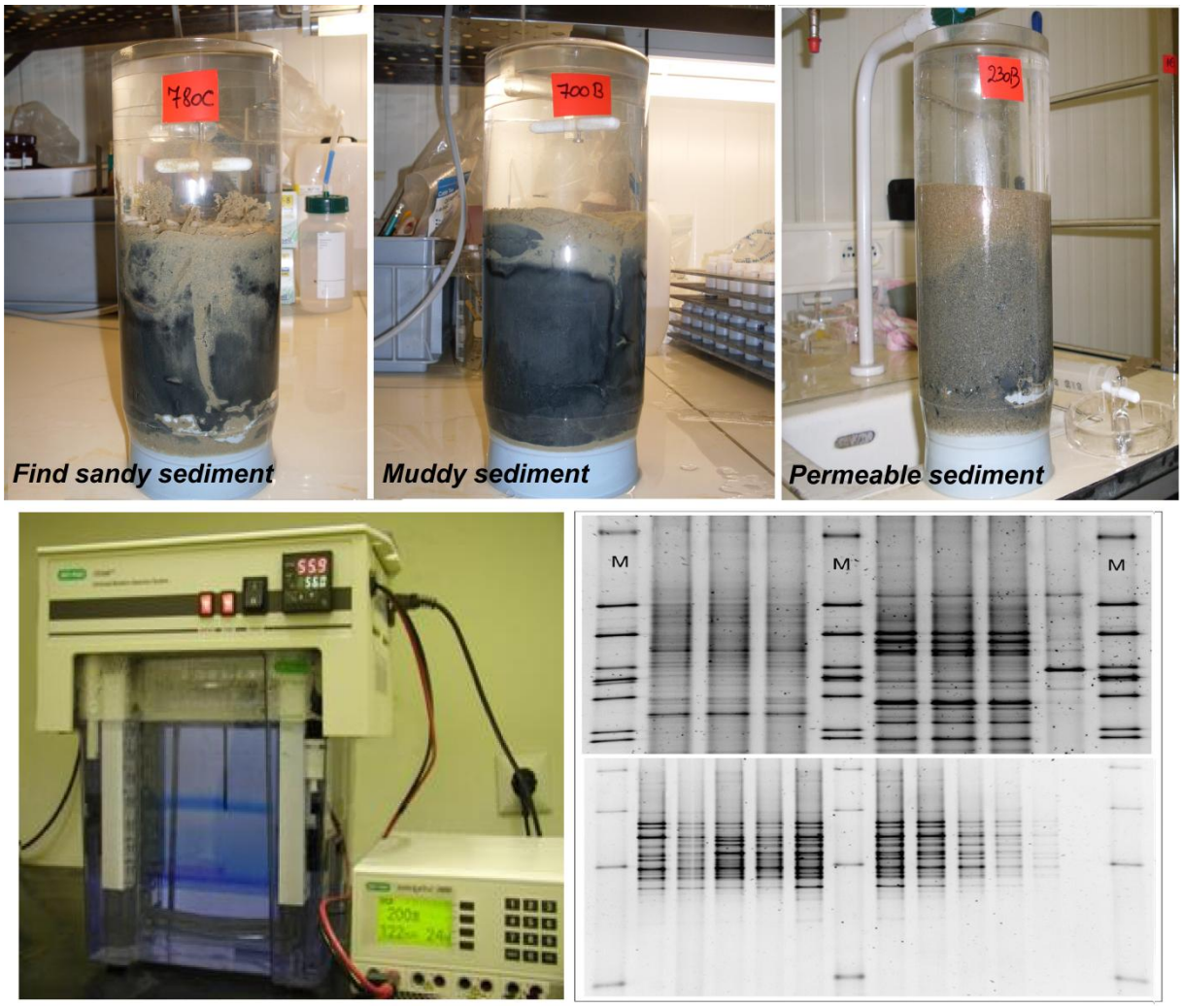
This study shows that a wide diversity in habitat types exists in the Southern North Sea, not only in terms of macrobenthic functional biodiversity, but also in terms of biogeochemical cycling. The muddy sediments in our study area appear especially important in alkalinity generation. Biogeochemical cycling in the fine sandy sediments was strongly influenced by macrobenthic functional biodiversity, where BPc was shown to be an important metric in explaining SCOC, denitrification, alkalinity fluxes, and NH_x fluxes. Our results pinpoint the strong link between macrobenthic functional biodiversity and carbon and nitrogen mineralization, especially in fine sandy sediments. This has the important implication that a loss of macrobenthic functional diversity entails a loss in benthic ecosystem functioning.

Acknowledgements

We are very grateful to the two anonymous reviewers who considerably helped us to improve this manuscript. We are further indepted to the crew of R.V. Zeeleeuw for help with sampling at sea, to Yves Israeël for the development of the experimental set-up, to Sofie Jacob, Bart Beuselinck, Niels Viaene, Liesbet Colson, Hannah Marchant, Dr. Gaute Lavik, and Jurian Brasser for help with sample processing and to Heiko Brenner, Sairah Malkin, Alexandra Rao, and attendants of the Wadden Sea Processes Mini-Workshop at Hamburg University for fruitful discussions. Kristof Van Steelandt from FIRE Statistical Consulting is acknowledged for statistical support. U.B. was financially supported by FWO Project No. G.0033.11. Additional funding was provided by the Special Research Fund of Ghent University (BOF-GOA 01GA1911W).

Chapter 3

The Link between Microbial Diversity and Nitrogen Cycling in Marine Sediments is Modulated by Macrofaunal Bioturbation



Modified from the following publication:

Yazdani Foshtomi, M., Braeckman, U., Derycke, S., Sapp, M., Van Gansbeke, D., Sabbe, K., Willems, A., Vincx, M., Vanaverbeke, J., 2015. The Link between Microbial Diversity and Nitrogen Cycling in Marine Sediments Is Modulated by Macrofaunal Bioturbation. PLoS ONE. 10(6), e0130116.

ABSTRACT

Objectives

The marine benthic nitrogen cycle is affected by both the presence and activity of macrofauna and the diversity of N-cycling microbes. However, integrated research simultaneously investigating macrofauna, microbes and N-cycling is lacking. We investigated spatio-temporal patterns in microbial community composition and diversity, macrofaunal abundance and their sediment reworking activity, and N-cycling in seven subtidal stations in the Southern North Sea.

Spatio-Temporal Patterns of the Microbial Communities

Our results indicated that bacteria (total and β-AOB) showed more spatio-temporal variation than archaea (total and AOA) as sedimentation of organic matter and the subsequent changes in the environment had a stronger impact on their community composition and diversity indices in our study area. However, spatio-temporal patterns of total bacterial and β-AOB communities were different and related to the availability of ammonia for the autotrophic β-AOB. Highest bacterial richness and diversity were observed in June at the timing of the phytoplankton bloom deposition, while richness of β-AOB as well as AOA peaked in September. Total archaeal community showed no temporal variation in diversity indices.

Macrofauna, Microbes and the Benthic N-cycle

Distance based linear models revealed that, independent from the effect of grain size and the quality and quantity of sediment organic matter, nitrification and N-mineralization were affected by respectively the diversity of metabolically active β-AOB and AOA, and the total bacteria, near the sediment-water interface. Separate models demonstrated a significant and independent effect of macrofaunal activities on community composition and richness of total bacteria, and diversity indices of metabolically active AOA. Diversity of β-AOB was significantly affected by macrofaunal abundance. Our results support the link between microbial biodiversity and ecosystem functioning in marine sediments, and provided broad correlative support for the hypothesis that this relationship is modulated by macrofaunal activity. We hypothesized that the latter effect can be explained by their bioturbating and bio-

irrigating activities, increasing the spatial complexity of the biogeochemical environment.

Keywords: microbial diversity; nitrifying organisms; macrofauna; marine sediment; bioturbation; N-cycle processes; North Sea

1. Introduction

Coastal marine sediments play a pivotal role in the ecology of shallow marine ecosystems. They receive up to 30% of the pelagically produced organic matter (Provoost *et al*., 2013), which is mineralised and returned to the water column as inorganic nutrients (Blackburn, 1988), further supporting primary and secondary production.

As mineralization is essentially a microbial process, investigations on how microbial diversity affects benthic ecosystem functioning are called for. However, while a positive biodiversity-ecosystem functioning link has now been established for many ecosystems (Wohl *et al*., 2004; Danovaro and Pusceddu, 2007; Jiang, 2007; Cardinale *et al*., 2012; Lyons and Dobbs, 2012; Naeem *et al*., 2012), negative (Obernosterer et al., 2010; Peter *et al*., 2011a) or non-significant (Jiang, 2007; Szabo *et al*., 2007; Peter *et al*., 2011b; Lyons and Dobbs, 2012) relationship was also reported in natural microbial systems. Hence, yet our knowledge about the biodiversity-ecosystem functioning relationship is not enough. This biodiversity-ecosystem functioning relationship can be modulated by environmental factors (Langenheder *et al*., 2010) such as sediment nitrogen content, carbon stable isotope ratios and sediment chlorophyll *a* concentrations (Abell *et al*., 2013).

While the quantity and quality of organic matter in coastal sediments, and the intensity of the mineralization are often related to water column processes (i.e. timing and extent of phytoplankton bloom and water temperature [Provoost *et al*., 2013]), the distribution of the organic matter in the sediment, and factors affecting mineralization are locally affected by the activities of the sediment-inhabiting larger macrofaunal organisms. While foraging for food, these animals rework and irrigate the sediment, transporting organic matter and oxygen to deeper layers, and enhancing the exchange of solutes between the water column and pore waters (Yingst and Rhoads, 1980; Mermillod-Blondin *et al*., 2004; Mermillod-Blondin, 2011). Hence, the activities of the macrofauna result in additional complexity within the sediment matrix. This affects microbial abundance (Papaspyrou *et al*., 2005; Bertics and Ziebis, 2009), diversity (Laverock *et al*., 2010) and activity – mineralization, nitrification and denitrification (Papaspyrou *et al*., 2006; Braeckman *et al*., 2010 and 2014; Laverock *et al*., 2011; Stief, 2013). However, the impact of bioturbation on microbial communities was mainly derived from the relation between single species of large burrowing macrofauna (ecosystem engineers) and total bacterial community

while less attention was paid to total archaeal community (Stauffert *et al*., 2014) and nitrifying organisms (Satoh *et al*., 2007; Gilbertson *et al*., 2012). In addition, integrated studies including different sediment types, repeated over time, investigating the link between natural macrofaunal communities, microbial communities and rates of ecosystem functioning are not available yet.

In a previous companion paper (Braeckman *et al*., 2014c) the effect of local environmental conditions and the presence and activity of macrofauna on the benthic nitrogen cycle was investigated, given the high importance of nitrogen as a key limiting factor for pelagic primary production (Herbert, 1999). However, as it is well-known that these processes are driven by microbial activities, it is of importance to close the existing gap between marine 'macro-ecologists' (focusing on the link macrobenthos-ecosystem functioning) and marine 'micro-ecologists' (focusing on the link microbial communities-ecosystem functioning). In the present study, we focused on microbiota involved in N-cycling processes. Ammonia oxidation is the first step in nitrification, central to the cycling of nitrogen in the environment and when coupled with denitrification results in loss of nitrogen from marine environments, and can be performed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Canfield et al., 2005). New methods targeting functional genes encoding enzymes involved in specific N transformations now allow direct identification and quantification of the microorganisms involved in N-cycling. Here, we focused on ammonia-oxidizing Beta-Proteobacteria (β-AOB) and AOA, by specifically targeting characteristic functional genes, respectively the bacterial *amoA* and archaeal *amoA* genes.

The main objective of this study is to investigate *(i)* whether there is a link between microbial diversity and benthic ecosystem functioning, i.c. the N-cycle (nitrification, denitrification and N-mineralization) in shallow subtidal marine sediments; and *(ii)* if it is modulated by macrofaunal density and/or activity. To this end, we first investigated the link between composition and diversity of microbial communities (active β-AOB and AOA as well as total bacteria and archaea) with environmental variables (i.c. sediment grain size and organic matter content and quality) and macrofaunal density and functional diversity. In a second step, we investigated whether nitrification, denitrification and N-mineralization rates were related to the diversity of the microbial communities and whether this was affected by macrofaunal density and functional

diversity. We used the Bioturbation Potential of a macrofaunal community (BPc) as proxy for macrobenthic activity (Solan *et al*., 2004; Queirós *et al*., 2013).

2. Materials and Methods

2.1. Study Site, Sampling and Experimental Set-up

In 2011, sediment was collected monthly (February-October) from seven subtidal stations (Figure 1) in the Belgian Part of the North Sea (BPNS).

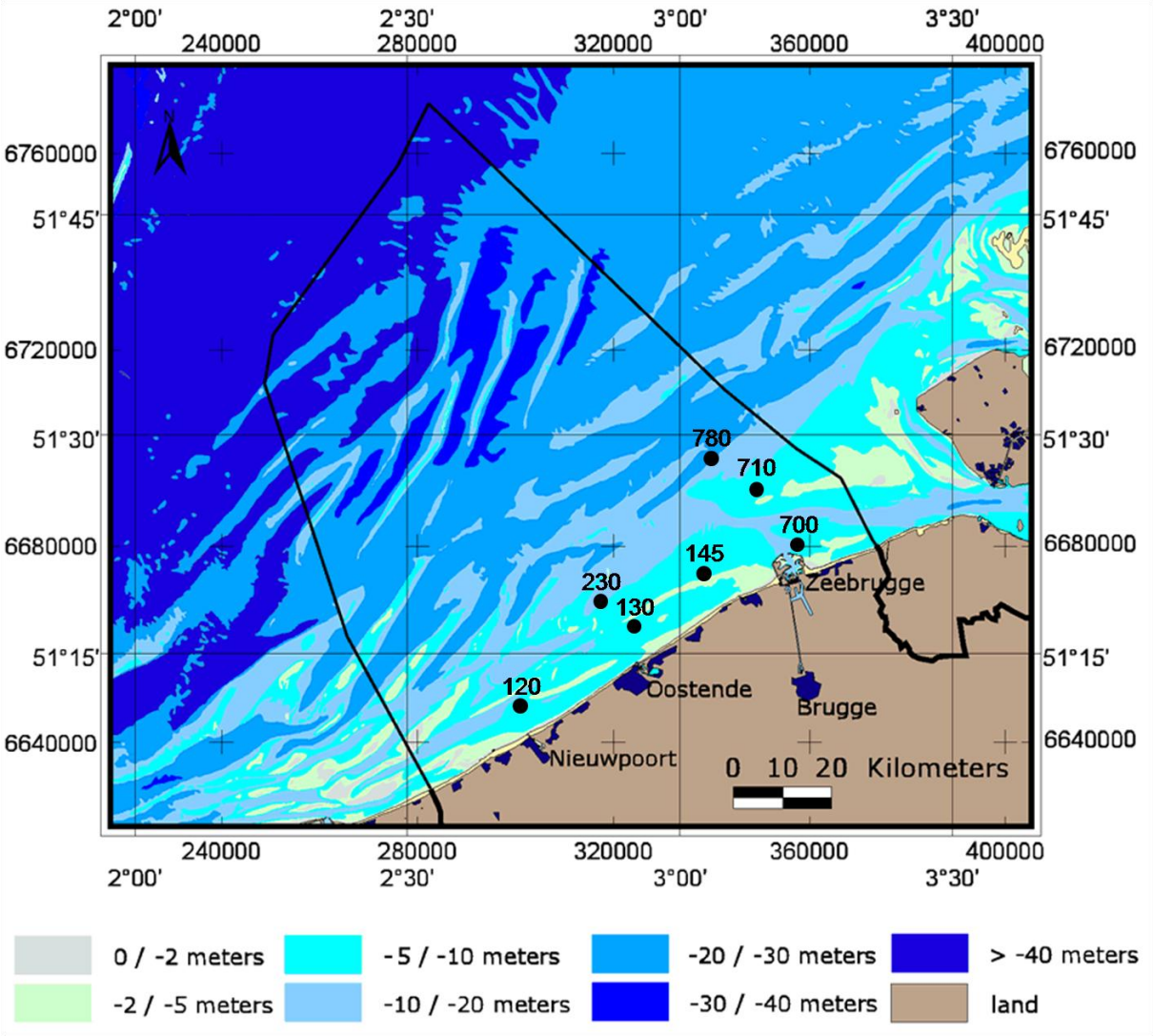


Figure 1: Bathymetry map of the Belgian Part of the North Sea with indication of the sampled stations

A description of the spatial and temporal patterns (8 months) of environmental variables, macrofaunal community characteristics, N-cycling and details about lab incubations are provided in a twin paper (Braeckman *et al*., 2014c). Here, we use a

subset of the environmental and macrofaunal data for statistical analysis (Table S1, Addendum 2). In short, sediments in the different stations could be classified as “muddy” (St. 130, 145 and 700), “fine sandy” (St. 120 and 780) and “permeable” sediments (St. 230 and 710). The different sediments were inhabited by different macrofaunal communities. Muddy sediments were inhabited by the *Macoma balthica* community (with an average of 9 species/0.1 m²), the species rich and abundant *Abra alba* community (with an average of 21 species/0.1 m²) was found in the fine sandy sediments, whereas the species poor *Nephtys cirrosa* community (with an average of 5 species/0.1 m²) prevailed in the permeable sediments (Van Hoey *et al*., 2009). These differences in communities were reflected in the BPc values: highest values were always found in the fine sandy sediments, the macrofaunal communities from the permeable and muddy sediments had comparably low BPc values.

In the water column, chl-*a* concentrations followed clear temporal and spatial patterns previously described in the same area (Rousseau *et al*., 2002; Lancelot *et al*., 2005). In the nearshore stations (St. 120, 130, 145, 700, 230 and 710), a spring phytoplankton bloom was reflected in strongly elevated chl-*a* concentrations in the water column. In the more offshore stations (St. 780), peak chl-*a* concentrations were observed in late summer. Benthic-pelagic coupling was strong at most stations: highest chl-*a* values in the sediment were always observed shortly after peak values in the water column were observed. This deposition of organic matter triggered mineralization processes in the sediment, which were different according to sediment type. Denitrification rates were highest in the fine sandy sediments throughout the year. During summer, nitrification rates in fine sandy sediments were also higher than in muddy and permeable sediment. In general, nitrification and denitrification rates in permeable and muddy sediments were low throughout the year (Braeckman *et al*., 2014c).

Samples for microbial analysis were collected in April (phytoplankton bloom), June (shortly after mass sedimentation of the spring bloom) and September (high mineralization rate; [Provoost *et al*., 2013]). No specific permits for sampling and ethics requirements were needed since our research was linked to microbiota, and approved by the FWO-research proposal (G.0033.11). The field study did not involve endangered or protected species. Triplicate sediment cores (Plexiglas, internal diameter: 10 cm; height: 25 cm; 3×7 cores per month) were gently inserted in a Reineck Box corer (surface area 180 cm^2) deployed several times at every station

and were half filled with sediment to have an equal proportion of sediment and water inside the cores. The intact sediment cores were transported to a temperature-controlled room on the day of sampling and submerged uncapped in tanks containing continuously aerated seawater at *in situ* salinity. The temperature of the climate room was adjusted to the temperature of seawater recorded by CTD 1 m above the sea floor ranging from 11 °C in April to 16 °C in June and September. To create water circulation inside the cores, teflon-coated magnets were inserted at appropriate distance from the sediment surface and rotated by a central magnet in the tanks at a speed below the resuspension limit (Braeckman *et al*., 2010). Every core was aerated separately. Within two days after sampling, the exchanges of dissolved inorganic nitrogen (DIN= NO_3^-, NO_2^-, NH_4^+) and O_2 across the sediment-water interface were measured during a series of dark incubations (cores were incubated as an average 11h in April, 7h in June and 6h in September to reach steady-state but ensuring oxygen did not drop below 50 % saturation) in airtight closed cores as reported in (Braeckman et al., 2014c). The sediment-water exchange fluxes of O_2 and DIN were used to estimate denitrification, nitrification and N-mineralization rates resulting from microbial activities in the sediment using the models described by (Braeckman *et al*., 2010).

At the end of the incubations, cores were sliced. As the annual average maximum oxygen penetration depth (OPD) in all sediment types was less than 1 cm (Braeckman *et al*., 2014c), the top 1 cm of sediment cores was homogenized, and subsampled for microbial analyses (using sterilized tools and stored in sterile 50-ml falcon tubes) and environmental variables (using a cut-off syringe to sample 3 to 5 ml of the sediment for the analysis of chl-*a*, phaeophytin, phaeophorbid, % organic carbon [OrgC], % organic nitrogen [OrgN] and grain size). Pigment samples were immediately frozen at -80 °C, whereas the samples for grain size and % OrgC and OrgN were dried at 60 °C before analysis. Pigments (chl-*a*, phaeophorbid and phaeophytin) were determined by HPLC (Gilson, Middleton, Wisconsin, USA) analysis according to (Wright and Jeffrey, 1997). Total OrgC and OrgN content was analyzed with an Organic Element Analyser (Flash 2000, Thermo Scientific, Wilmington, Delaware, USA) and sediment granulometry by laser diffraction (Malvern Instruments, Malvern, UK). Oxygen samples from the core incubations were analyzed by automated Winkler titration (Parsons, 1984), DIN samples were

analyzed using automated colorimetric techniques. Oxygen and DIN fluxes were calculated using a mass-balance model (Braeckman *et al*., 2014c).

The remaining sediment from the entire core was sieved on a 1mm mesh to retrieve the macrofauna. Macrofauna was sorted, identified to the lowest possible taxonomic level (typically species level), and weighed (as blotted wet weights to determine biomasses) (Braeckman *et al*., 2014c). BPc, calculated taking into account biomass and abundance of each species as well as mobility and sediment reworking traits, was applied as an index to estimate the extent to which macrofaunal communities can affect important ecosystem properties that can affect ecosystem functioning (Solan *et al*., 2004; Queirós *et al*., 2013). The ratio of phaeopigments to the sum of chl-*a* + phaeopigments (PAP ratio; [Boon and Duineveld, 1998]) and C:N ratio were calculated as an indication of the freshness of the material deposited on the sediment, and was not part of the dataset of (Braeckman *et al*., 2014c).

2.2. DNA and RNA Extraction, PCR and RT-PCR, DGGE

Extracellular DNA was removed from sediment samples (2.5 g wet weight) as described by Corinaldesi *et al*. (2005). Intracellular DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, California, USA). The V3 region of the bacterial 16S rDNA gene was amplified for DGGE using universal bacterial primers (Table S2, Addendum 2). A nested PCR design was used for 16S rDNA amplification of total archaea due to very low yield from direct PCR in some samples (Benlloch *et al*., 2002) (see Text S1 for more details).

To analyze active β-AOB and AOA communities, RNA was extracted from 3.5 g sediment (wet weight) using the RNA Power Soil Total RNA isolation kit (MO BIO Laboratories). RNA samples were reverse transcribed by Omniscript and Sensiscript Reverse Transcriptase Kits (Qiagen, Hilden, Germany) respectively for samples containing ≥ or < 50 ng RNA per reaction. The *amoA* gene was amplified for DGGE using AOA and β-AOB specific primer sets (Table S2; see also Text S1, Addendum 2).

Denaturating Gradient Gel Electrophoresis (DGGE) is a commonly used technique to characterize microbial community composition and diversity (Frossard *et al*., 2012; Graue *et al*., 2012; Tang *et al*., 2013; Tiodjio *et al*., 2014; von Scheibner *et al*., 2014). DGGE analysis (Figure S1, Addendum 2) of PCR and RT-PCR amplicons was performed using the DCode Universal Mutation Detection System device (Bio-Rad,

Hercules, California, USA). The gels were stained with SYBR gold (Molecular Probes, Invitrogen, Life Technologies) for 30 min followed by visualization and digital capturing of the profiles via the Molecular Imager Gel Doc XR System (Bio-Rad). Digital images were normalized and processed with BioNumerics (version 5.10, Applied Maths). Band analysis was performed by setting background subtraction and least squares filtering according to the instructions of the manufacturer. Each DGGE band was considered to be an operational taxonomic unit (OTU) (Franco *et al*., 2007; Duarte *et al*., 2012). The relative intensities of the bands in each lane to the total intensity of the lane were used to estimate relative abundance of OTUs (Franco *et al*., 2007; Duarte *et al*., 2012). Bands were not excised and sequenced as the main focus of the present study was on the effects of macrofauna on microbial taxonomic and functional diversity per se, and not on the identity of the OTU's.

2.3. Data Analysis

We used permutational multivariate ANOVA (Anderson *et al*., 2008) to assess temporal and spatial differences in community composition (based on relative abundance of OTUs), diversity (species richness [S, 'richness', number of OTUs] and Shannon-Wiener [H', log e, 'diversity', number and relative abundance of OTUs]) of total bacteria, total archaea and metabolically active β-AOB and AOA. The data set was analyzed using a two-way fixed factor model design. The factors 'month' (three levels: April, June, September), 'station' (seven levels: 120, 130, 145, 230, 700, 710, 780) and their interactions were tested. Pairwise tests were performed for significant (interaction) terms. Variation in microbial community structure was visualized using Principal Coordinates Analysis (PCO) based on square root transformed data (relative abundance of the band intensity) to remove the contribution of only common species to the similarity (Clarke and Warwick, 2001). Second-stage MDS, derived from Spearman correlations between pairs of similarity matrices, was applied to visualize interrelationships between multivariate patterns of the different microbial communities, and the macrofaunal communities.

We used Distance based Linear Models (DistLM) to investigate the role of measured environmental factors, total macrofaunal density and functional diversity (BPc) in explaining the variation in richness, diversity and community composition of total bacteria and archaea, and active β-AOB and AOA. In a next step, we established the link between abiotic and biotic factors (macrofaunal density and BPc) and attributes

of the microbial communities on the one hand and nitrification, denitrification and N-mineralization. All DistLM analyses were performed using the step-wise selection procedures (see Text S1).

As microbial richness and diversity were highly correlated, they were not incorporated in a single model. Therefore, we ran two different DistLM analyses for each process in the N-cycle. In addition, to test the relationships between organisms and their activities, the nitrification model was run with diversity indices of AOA and β-AOB. As we did not specifically investigate organisms involved in the denitrification process, total communities (bacteria and archaea) were used to construct the best fitted model of denitrification and N-mineralization. Statistical analyses were performed using Primer v6.1.10 (Primer-E Ltd., Plymouth, United Kingdom) with the PERMANOVA + add-on package (Anderson *et al*., 2008).

3. Results

3.1. Microbial Community Composition and Diversity Indices

OTU richness (S) ranged between 13-32 for bacteria, 6-20 for archaea, 0-30 for β-AOB and 0-22 for AOA, while Shannon-Wiener's index (H') fluctuated between 2.08-3.24 in bacteria, 1.21-2.67 in archaea, 0-2.97 in β-AOB and 0-2.71 in AOA.

Community composition of total bacteria, total archaea and the β-AOB and AOA were all significantly affected by an interaction between months and stations (term "MoxSt", Table S3, Addendum 2).

Pairwise tests (Tables S4 and S5) and the PCO plots (Figure 2) revealed that the different microbial groups showed different spatial and temporal patterns.

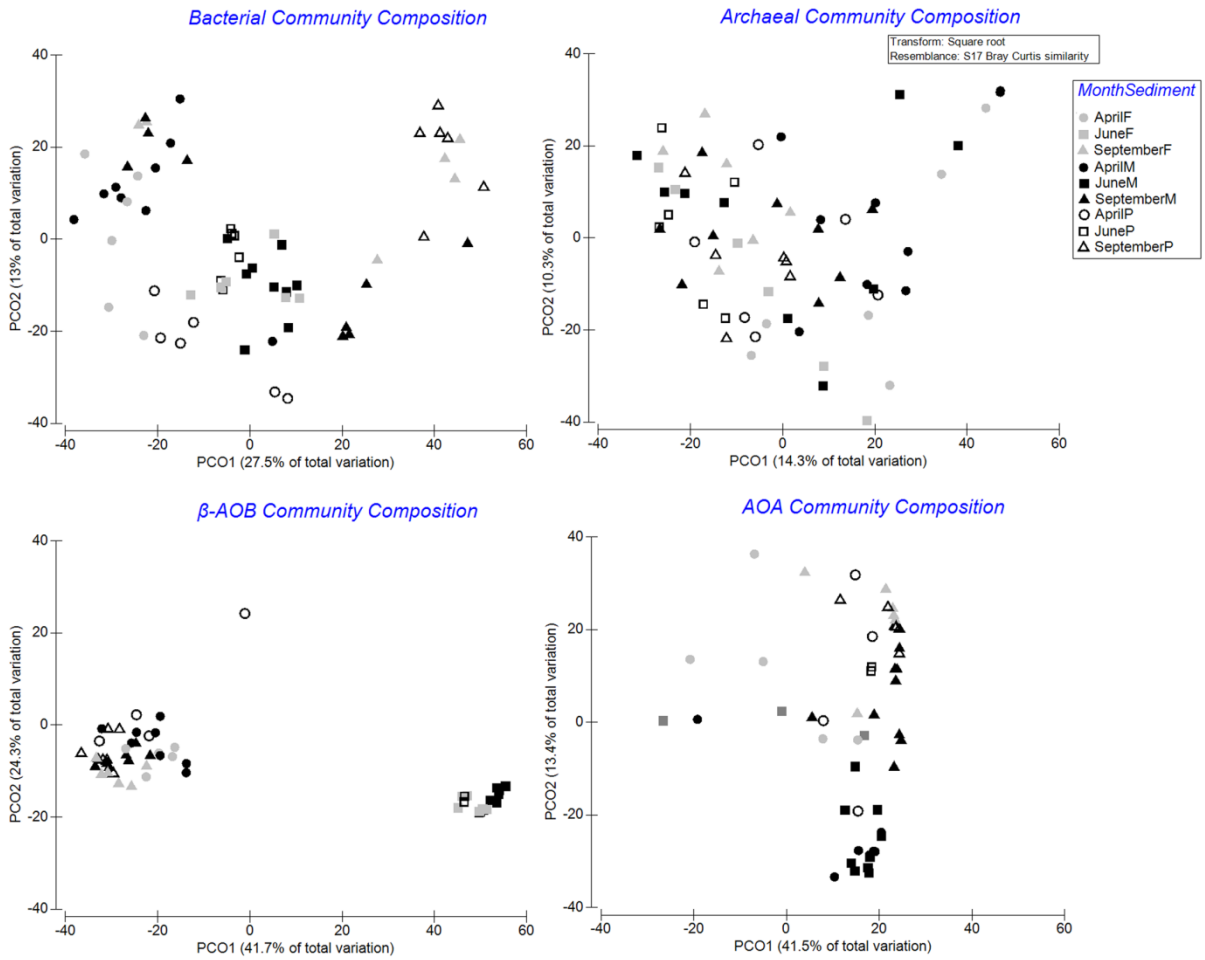


Figure 2: Principal Coordinates Analysis (PCO) of relative abundance data of microbial communities. Data are square root transformed and based on Bray-Curtis similarities. Symbols: April (circle), June (square), September (triangle), muddy stations (black-filled shapes), fine stations (grey-filled shapes), and permeable stations (open shapes). Total microbial (bacterial and archaeal) and nitrifying (ammonia-oxidizing Beta-Proteobacteria: β-AOB and ammonia-oxidizing archaea: AOA) communities were investigated based on, respectively, 16S rDNA gene and *amoA* transcripts.

While total archaeal community composition showed limited temporal patterns only in muddy stations, the AOA community in September was separated from June along the second PCO axis (PCO2; 13.4% of the total variation) (pairwise tests, all $P < 0.05$). A seasonal transition was observed in total bacterial and β-AOB community composition (PCO1), which was supported by PERMANOVA (Table S4, Addendum 2). Visualization by PCO also revealed a clear separation in β-AOB community in June, and within-station (muddy St. 130 and 145 and fine sandy St. 780) variation in the community composition of total bacteria in September.

Spatial differences of community composition were present for all investigated groups (total archaea showing limited differences), and mainly in June and September (pairwise tests, all $P < 0.05$). However, consistent pairwise differences were not observed (Table S5, Addendum 2). As stations within each season differed in bacterial and β-AOB community composition mainly along the second axis (PCO2) explaining a lower portion of the total variation than PCO1, it seems community composition in these two groups were more seasonally structured than spatially as evidenced also by a clear separation in June in β-AOB.

Bacterial and AOA richness, and β-AOB and AOA diversity were significantly affected by the interaction term MoxSt. Total archaeal richness and diversity were only affected by “Station”. Richness of β-AOB and diversity of total bacterial community were affected by “Month” and “Station”, however not by their interaction (Table S3, Addendum 2).

Total bacterial richness and diversity were significantly highest in June in all sediment types while no seasonal difference was observed in total archaea (pairwise tests, all $P > 0.05$; Tables S6 and S7; Figures 3 and 4).

Investigating spatial differences, generally highest and lowest bacterial richness (mostly present in June) and diversity were observed in muddy and permeable sediments, respectively. Differences between sediment types in archaeal richness and diversity were limited to muddy and permeable sediments and in contrast to bacteria, permeable stations showed generally higher values than muddy stations (pairwise tests, all $P < 0.05$; Tables S8 and S9; Figures 3 and 4).

In both nitrifying groups (β-AOB and AOA), significantly highest richness values were observed in September in almost all stations. However, timing of highest values for diversity was not consistent for different stations (Tables S6 and S7; Figures 3 and 4).

Spatial differences in β-AOB richness were lowest in permeable sediments and generally highest in fine sediments. In the latter sediment type, high β-AOB diversity was also obtained in September. Spatial differences per sampling month in AOA richness and diversity were mainly detected in June, when highest values were generally recorded in muddy sediments (pairwise tests, all $P < 0.05$; Tables S8 and S9; Figures 3 and 4).

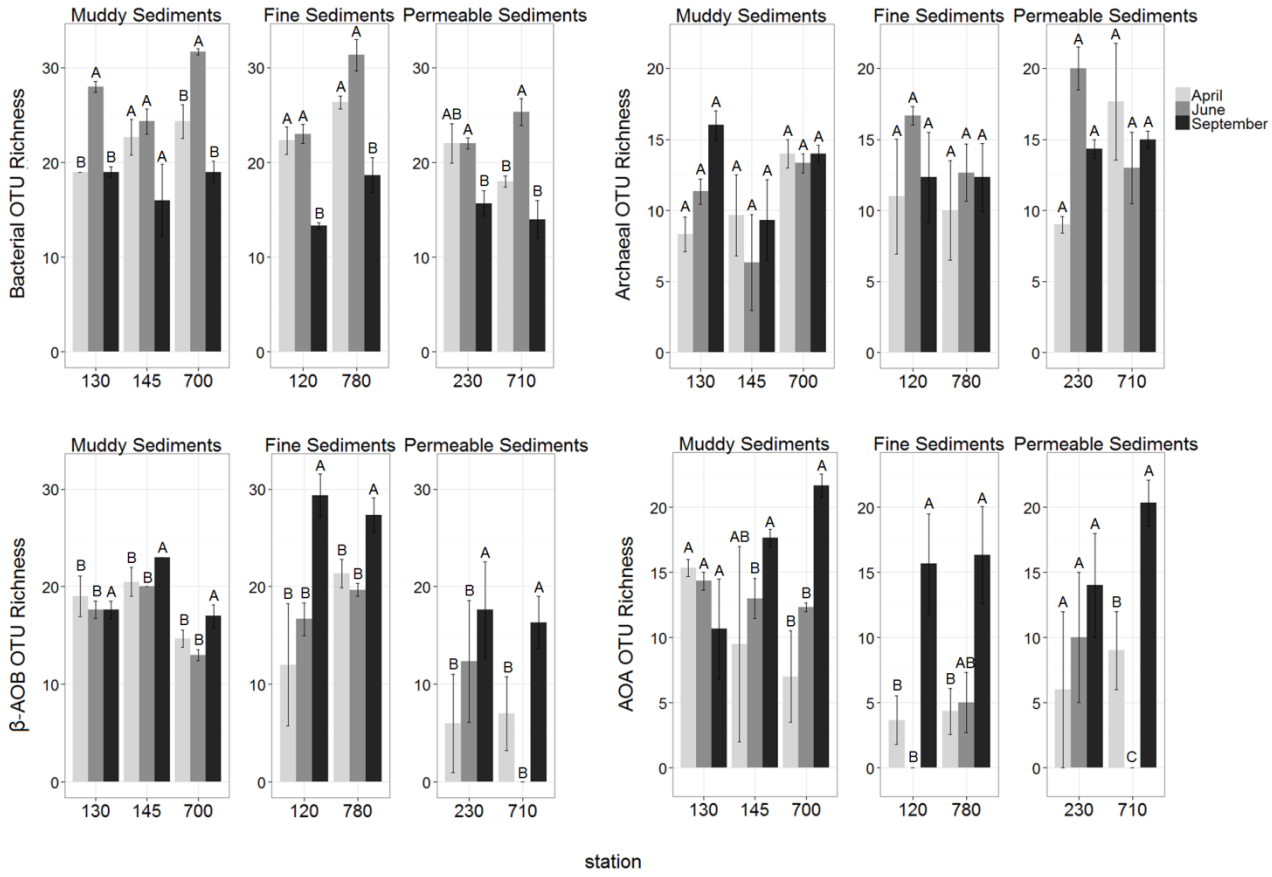


Figure 3: Spatial and temporal variations of OTU richness (mean ± se, n=3) of all investigated microbial communities. Total microbial (bacterial and archaeal) and nitrifying (ammonia-oxidizing Beta-Proteobacteria: β-AOB and ammonia-oxidizing archaea: AOA) communities were investigated based on, respectively, 16S rDNA gene and *amoA* transcripts. Different capital letters above the columns indicate statistically significant results (A > B > C) of pairwise tests ($P < 0.05$) of temporal differences in the microbial richness (see Table S8, Addendum 2 for spatial differences).

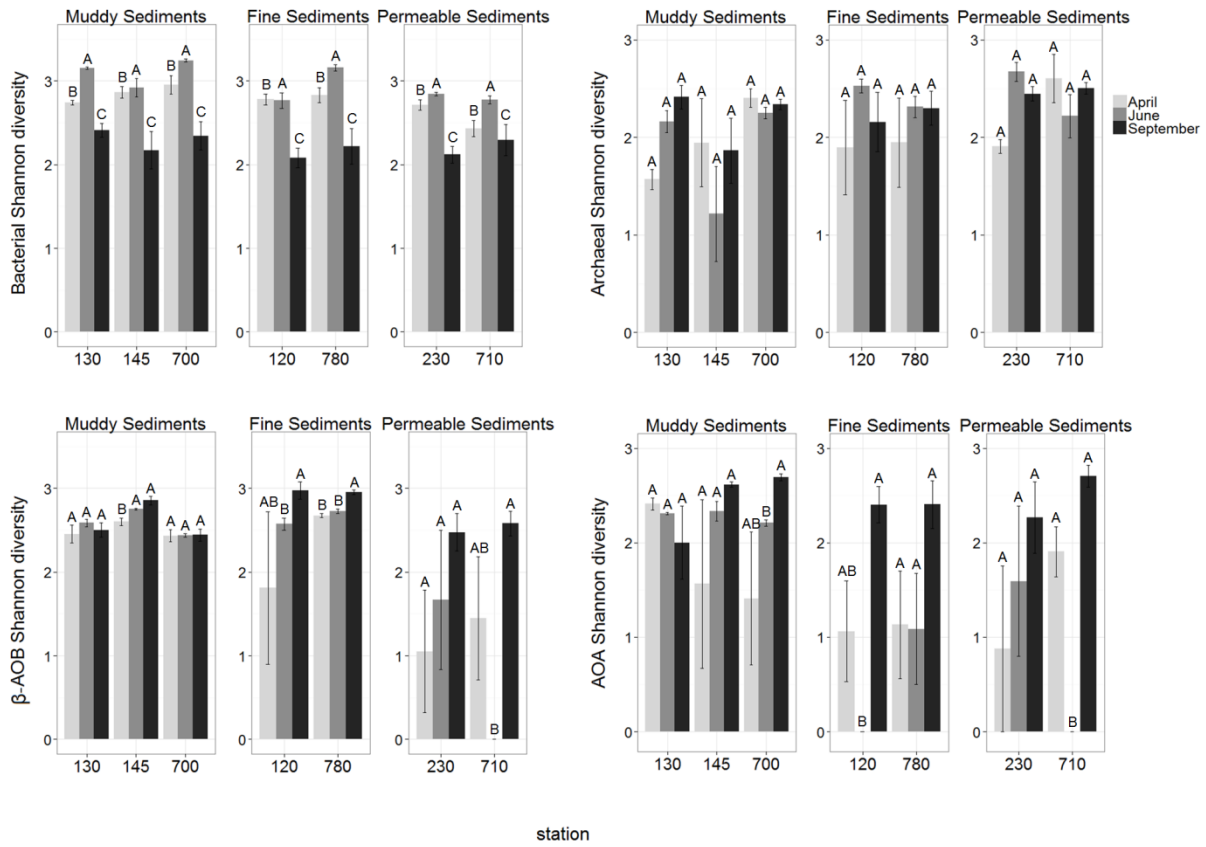


Figure 4: Spatial and temporal variations of Shannon diversity (mean ± se, n=3) of all investigated microbial communities. Total microbial (bacterial and archaeal) and nitrifying (ammonia-oxidizing Beta-Proteobacteria: β-AOB and ammonia-oxidizing archaea: AOA) communities were investigated based on, respectively, 16S rDNA gene and *amoA* transcripts. Different capital letters above the columns indicate statistically significant results (A > B > C) of pairwise tests ($P < 0.05$) of temporal differences in the microbial Shannon diversity (see Table S9, Addendum 2 for spatial differences).

Visualization of the similarity of the multivariate patterns of the different microbial groups by second-stage MDS showed that the multivariate patterns for all investigated groups were different but showing more similar patterns in AOA and β-AOB in comparison with those for total bacteria and archaea. The multivariate patterns of the latter groups were different from each other. In addition, the multivariate patterns for all the microbial groups were very different from those observed for the macrofaunal communities (Figure 5).

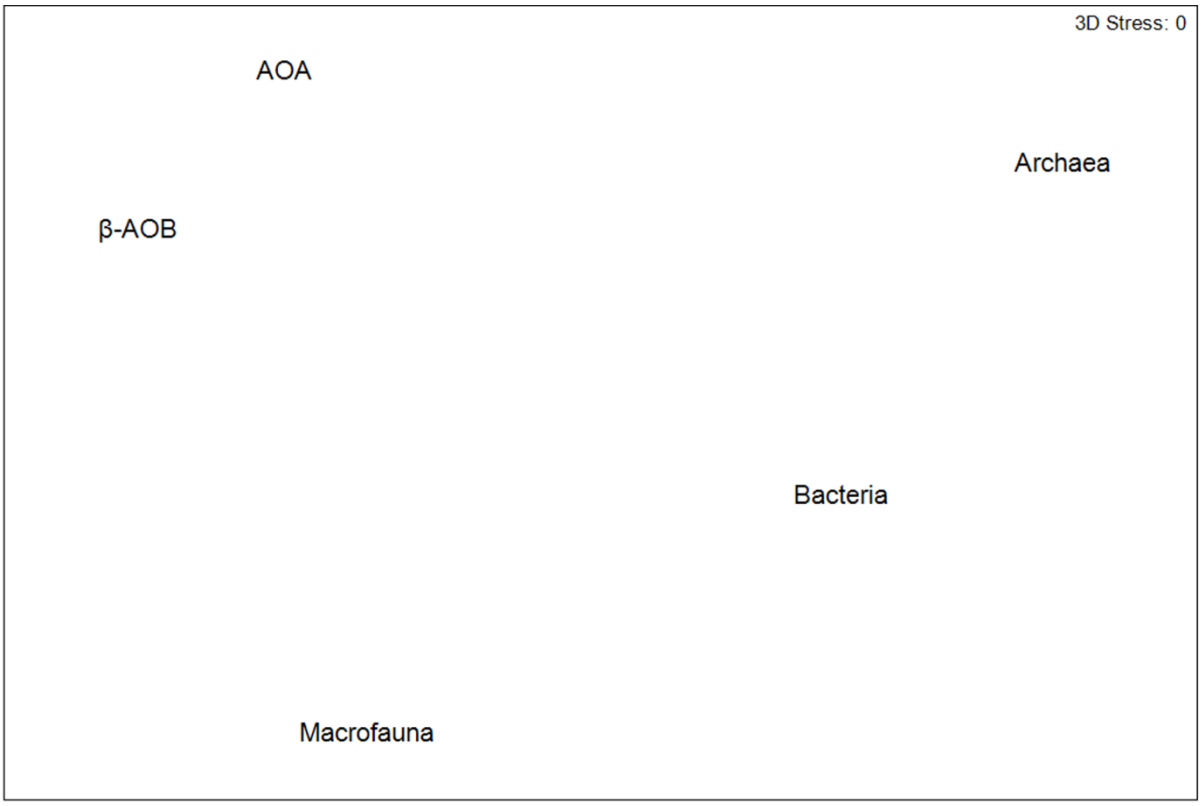


Figure 5: Second stage MDS for community composition of all investigated microbial groups and macrofauna. Data are square root transformed.

3.2. Linking Microbial Communities with Biotic and Abiotic Sediment Characteristics

Overall, DistLM results (Table 1) indicated that bacterial community composition (total and β-AOB) was better explained by biotic and abiotic sediment characteristics (≈ 25%) than archaeal community composition (total and AOA; ≈ 8%). Median grain size (MGS) contributed significantly to the variability of all studied communities. For AOA, this variable was the only significant one in the model while together with MGS, one or more proxies for the quality and quantity of organic matter (chl-*a* concentration, PAP ratio, C:N) significantly contributed to the models of the other groups as well. The macrofaunal bioturbation index, BPc, was only important for the total bacterial community composition.

Table 1: Distance-based linear model (DistLM) of microbial community composition and diversity indices against biotic (macrofauna) and abiotic factors

	Variable	*AIC, AIC_c*	*SS(trace)*	*Pseudo-F*	*P*	*Prop*	*Cumul*	*res.df*
Bacterial community composition	*chl-a*	494.31	15850.00	6.40	0.000	0.09	0.09	61
	MGS[a]	490.10	14196.00	6.22	0.000	0.08	0.18	60
	C:N ratio	487.75	9126.90	4.21	0.000	0.05	0.23	59
	BPc[b]	487.25	4969.60	2.35	0.012	0.03	0.26	58
Archaeal community composition	*chl-a*	507.32	9601.40	3.15	0.000	0.05	0.05	61
	MGS	506.79	7307.40	2.46	0.001	0.04	0.09	60
β-AOB community composition	*chl-a*	497.12	20553.00	6.99	0.000	0.10	0.10	60
	C:N ratio	493.93	14175.00	5.15	0.001	0.07	0.18	59
	MGS	492.76	8095.70	3.04	0.017	0.04	0.22	58
	PAP ratio[c]	491.79	7218.30	2.80	0.029	0.04	0.25	57
AOA community composition	*MGS*	494.56	14313.00	5.07	0.001	0.08	0.08	60
Bacterial OTU richness	*MGS*	209.85	211.35	7.80	0.007	0.11	0.11	61
	C:N ratio	203.91	195.91	8.06	0.007	0.10	0.22	60
	BPc	198.95	152.50	6.89	0.009	0.08	0.30	59
Archaeal OTU richness	*No significant variable*							
β-AOB OTU richness	*C:N ratio*	250.27	485.45	8.85	0.003	0.13	0.13	60
	PAP ratio	247.55	241.53	4.67	0.037	0.06	0.19	59
	chl-a	244.23	250.61	5.19	0.028	0.07	0.26	58
AOA OTU richness	*chl-a*	244.94	348.07	6.91	0.012	0.10	0.10	60
	BPc	241.10	271.39	5.82	0.017	0.08	0.18	59
Bacterial Shannon diversity	*MGS*	-123.95	0.94	6.92	0.011	0.10	0.10	61
	C:N ratio	-129.00	0.87	7.10	0.010	0.09	0.20	60
Archaeal Shannon diversity	*No significant variable*							
β-AOB Shannon diversity	*C:N ratio*	-28.18	10.65	17.32	0.001	0.22	0.22	60
	chl-a	-31.64	3.11	5.43	0.025	0.06	0.29	59
	Macrofauna density	-34.21	2.40	4.44	0.036	0.05	0.34	58
AOA Shannon diversity	*C:N ratio*	-1.05	4.77	5.01	0.026	0.08	0.08	60
	BPc	-4.22	4.57	5.13	0.026	0.07	0.15	59

Predictor variables subjected to a sequential step-wise selection procedure using the AIC and AIC_c criterions for multivariate (community composition) and univariate (richness and diversity) response variables, respectively. [a]*MGS=median grain size;* [b]*BPc= Bioturbation Potential of the Community;* [c]*PAP ratio=The ratio of phaeopigments to the sum of chl-a + phaeopigments (Boon and Duineveld, 1998)*

Evaluating the variables affecting richness and diversity yielded different results (Table 1): macrofaunal densities and/or bioturbation potential (BPc) contributed significantly to diversity aspects (richness and/or diversity) of some microbial groups. Only total archaeal richness and diversity were not affected by macrofauna or any other variables. Generally, the biotic and abiotic variables in the model explained the variation in richness and diversity of total bacteria and β-AOB (20-34%) better than was the case for total archaea and AOA (0-18%). While MGS was incorporated in all models for community composition, it was only part of the model for the total bacterial diversity indices (≈ 10%).

All other environmental variables in the model were related to quantity and quality of the organic matter. While MGS and C:N were important contributors to the model for total bacterial diversity indices (≈ 20%), C:N ratio together with chl-*a* concentration

were incorporated in the models for β-AOB and AOA richness and/or diversity. PAP ratio contributed significantly only to the β-AOB richness model explaining 6% of the variation.

3.3. Linking the N-cycle with Abiotic and Biotic Factors

DistLM models (Table 2) identified MGS (18% of variation explained), and depending on the model, β-AOB richness or AOA diversity (10 and 6% of the variation explained) as the variables significantly affecting nitrification rates.

Denitrification could not be explained by the diversity or richness of the total pool of bacteria and archaea; however, bioturbation potential (BPc) significantly affected denitrification rates (30%) together with the abiotic variables MGS, PAP ratio, and chl-*a* concentrations in the sediment, each contributing ≈ 5% to the models.

Both BPc (28%) and bacterial richness or diversity (≈ 5%) contributed significantly to the models for total N-mineralization rates. Abiotic variables significantly retained in the model were PAP ratio (6%) and chl-*a* concentration (15%). In total, 54% of the variation in N-mineralization could be explained by these factors.

Table 2: Distance-based linear model (DistLM) of N-cycle processes against biotic (micro- and macrofauna) and abiotic factors

	Variable	*AIC*	*SS(trace)*	*Pseudo-F*	*P*	*Prop*	*Cumul*	*res.df*
Nitrification[1]	*MGS*[a]	180.55	230.62	12.94	0.000	0.18	0.18	60
	AOA Shannon diversity	177.95	76.43	4.54	0.038	0.06	0.24	59
Nitrification[2]	*MGS*	180.55	230.62	12.94	0.000	0.18	0.18	60
	β-AOB OTU richness	174.70	127.06	7.96	0.007	0.10	0.27	59
Denitrification[1]	*BPc*[b]	244.47	1261.60	25.25	0.000	0.30	0.30	60
	MGS	241.24	242.26	5.19	0.022	0.06	0.35	59
	PAP ratio[c]	237.55	241.86	5.58	0.020	0.06	0.41	58
	chl-a	233.81	222.00	5.52	0.019	0.05	0.46	57
Denitrification[2]	*BPc*	244.47	1261.60	25.25	0.000	0.30	0.30	60
	MGS	241.24	242.26	5.19	0.023	0.06	0.35	59
	PAP ratio	237.55	241.86	5.58	0.021	0.06	0.41	58
	chl-a	233.81	222.00	5.52	0.018	0.05	0.46	57
N-mineralization[1]	*BPc*	70.04	70.95	23.67	0.000	0.28	0.28	60
	chl-a	57.75	37.02	15.29	0.000	0.15	0.43	59
	PAP ratio	53.13	14.46	6.53	0.013	0.06	0.49	58
	Bacterial Shannon diversity	45.97	13.87	7.24	0.009	0.05	0.54	56
N-mineralization[2]	*BPc*	70.04	70.95	23.67	0.000	0.28	0.28	60
	chl-a	57.75	37.02	15.29	0.000	0.15	0.43	59
	PAP ratio	53.13	14.45	6.53	0.015	0.06	0.49	58
	Bacterial OTU richness	46.20	13.47	7.01	0.009	0.05	0.54	56

DistLM analyses were run two times for every process separating microbial species richness and diversity in each model (processes run using [1]Shannon diversity or [2]OTU richness). Predictor variables subjected to a sequential step-wise selection procedure using the AIC criterion. [a]MGS=median grain size; [b]BPc= Bioturbation Potential of the Community; [c]PAP ratio=The ratio of phaeopigments to the sum of chl-a + phaeopigments (Boon and Duineveld, 1998)

4. Discussion

4.1. Effects of Abiotic Factors on Microbial Communities in the Sediment

While sediment grain size usually correlates to organic matter content, nutrient concentration and oxygen penetration depth (Bergamaschi *et al*., 1997; Magni *et al*., 2008; Braeckman *et al*., 2014c), most studies on microbial communities so far considered granulometric variables in isolation (Sapp *et al*., 2010). Sediments with coarser gradients are characterized by low amounts of organic matter especially at top layer and deep oxygen penetration. When sediments become finer, hydrodynamic forces are less strong, hence deposited organic matter can accumulate near the surface (Ehrenhauss and Huettel, 2004; Ehrenhauss *et al*., 2004) and result in oxygen stress during periods of intense mineralization (Provoost *et al*., 2013).

Our study indicated that MGS indeed significantly explained a part of the variation of total bacterial richness and diversity, and community composition of all investigated microbial groups (DistLM; Table 1). Consistent with earlier findings in the BPNS for bacteria (Franco *et al*., 2007) and the other studies (Mills *et al*., 2008; Böer *et al*., 2009), generally low richness for bacteria and β-AOB was observed in the surface layer of permeable sediments, which was related to strong hydrodynamic forces (advective currents through the sediment) (Franco *et al*., 2007; Mills *et al*., 2008; Böer *et al*., 2009). However, this was not the case for AOA and total archaea: richness and diversity of these two groups in permeable sediments were comparable with those in fine sediments in all sampling months (Tables S8 and S9). Permeable sediments even harbored generally higher values of richness and diversity than muddy sediments in total archaea (Figures 3 and 4). Such differences between archaeal (total or AOA) and bacterial (total or β-AOB) communities were also observed in terms of community composition as permeable sediments showed generally different community composition from the other sediment types in bacterial (total or β-AOB) communities (Table S5, Addendum 2).

Two explanations are possible: *(i)* archaeal communities (total or AOA) are more resistant against hydrodynamic forces by establishing more particle associated rather than free-living communities (Zhang *et al*., 2014). *(ii)* These forces do not directly affect microbial communities but do alter concentrations of labile organic matter through washing them out into deeper layers in permeable sediments (Ehrenhauss and Huettel, 2004; Ehrenhauss *et al*., 2004). The latter explanation corroborates

earlier studies (Abell *et al*., 2010; Zheng *et al*., 2013; Tait *et al*., 2014) and our findings (DistLM, Table 1) indicating there is a lower dependency on sedimentary abiotic factors (organic matter quantity and quality) in community composition and diversity indices of archaea (total or AOA) compared with bacteria (total or β-AOB). Richness and diversity of total archaea were not even related to any of the measured variables. This suggests that the sedimentation of organic matter and the subsequent changes in the biogeochemical environment have a stronger impact on β-AOB and total bacterial communities in our study area. In addition, this also explains why total bacterial and especially β-AOB community composition varied more seasonally than spatially (visualized by PCO, Figure 2) as proxies of organic matter quantity and quality in the upper cm of sediment show a larger variation temporally than the relatively stable granulometric variables. This was reflected in a clear separation of β-AOB community composition in June (phytoplankton bloom deposition) as chl-*a* and PAP ratio explained higher proportion of the total variation than MGS (14 to 4 %) in this group.

The Belgian coastal zone is characterized by high primary production (M'harzi *et al*., 1998; Rousseau *et al*., 2002; Rousseau *et al*., 2006) where 70-75% of the phytoplankton biomass production at the time of spring bloom is under the form of *Phaeocystis* colonies which is mainly mineralised by bacterial activity (Rousseau *et al*., 2006). As this reflected seasonal changes in bacterial community composition ([Rousseau *et al*., 2006]; see also Figure 2), the highest bacterial richness and diversity were observed in June (Tables S6 and S7; Figures 3 and 4). In addition, spatial differences in bacterial richness were also most prominent in June (Table S8, Addendum 2). However, as the obligate chemolithoautotrophic AOB (Mosier, 2011) do not directly rely on the availability of organic matter, the sedimentation of the phytoplankton bloom has no direct effect on this community. Therefore, although community composition and diversity indices of total bacteria and β-AOB are both related to organic matter quantity and quality, they showed different patterns (Figure 5). The richness values of metabolically active β-AOB peaked in September (Table S6, Addendum 2; Figure 3). Following the increase in temperature in summer time, the degradation of organic matter accelerates in September (Provoost *et al*., 2013). This process produces ammonia as a source of energy for nitrifying organisms. The highest richness values of active AOA were also observed in September (second stage MDS showed more similar patterns in AOA and β-AOB in comparison with

those for total bacteria and archaea; Figure 5). However, AOA exhibit a variety of metabolic pathways compared to AOB. They are capable of getting energy through different carbon-fixing pathways by autotrophic activities (Abell *et al*., 2010) as well as the ability of heterotrophic metabolism through oxidizing organic matter (Mosier, 2011; Mußmann *et al*., 2011; Qin *et al*., 2014). Furthermore, there are reports indicating high affinities of AOA to the substrate (ammonia and oxygen) concentration (Abell *et al*., 2010; Park *et al*., 2010). The reasoning above, together with the lower dependency of AOA and total archaea on sedimentary abiotic factors in our study area suggests a generally lower spatio-temporal variability in archaeal (total and AOA) than in bacterial (total or β-AOB) communities. Indeed, our results showed that changes in total archaeal community composition and diversity indices were very limited in space and time (no temporal variation in diversity indices). AOA's did differ seasonally and spatially but were more stable than β-AOB. This is in agreement with findings by Sapp *et al*. (2010) in the central part of the North Sea between total bacteria and archaea and earlier studies, which showed that AOA are ubiquitous in sediments, whereas β-AOB were not detected in all the investigated samples (Beman and Francis, 2006; Abell *et al*., 2010).

4.2. Macrofauna, Microbes and the Benthic N-cycle

Interactions between macrofaunal and microbial communities are important for biogeochemical processes in coastal benthic ecosystems but still our knowledge is very limited (Laverock *et al*., 2014). Most research has been based on lab incubations (Satoh *et al*., 2007; Laverock *et al*., 2010; Gilbertson *et al*., 2012; Stauffert *et al*., 2014) or has focused on the effects of single macrofaunal species in field studies (Papaspyrou *et al*., 2005 and 2006; Bertics and Ziebis, 2009; Laverock *et al*., 2014). Results from such small-scale studies have often revealed species-specific differences in these interactions. For example, for some macrofaunal species, microbial communities in burrow walls and surrounding sediments were more similar than communities in burrow walls and surface sediments (Lucas *et al*., 2003; Papaspyrou *et al*., 2005 and 2006) while for others burrow wall communities were more similar to those in the surface layer (Steward *et al*., 1996; Bertics and Ziebis, 2009; Laverock *et al*., 2010). As a consequence, the results of these studies cannot readily be translated to complex field situations. In the present study, we adopted an integrated field approach by simultaneously collecting information on

macrofauna, important microbial groups and biogeochemical processes over time and in contrasting subtidal sediment types. More specifically, we investigated the effect of the density and bioturbation potential of the whole macrofaunal communities on microbial community composition, diversity and N-cycling in these sediments. As the microbial communities were sampled from homogenized sediments from the upper cm layer, small scale distribution patterns will have been disrupted, and specific relationships between macrofauna and certain microbial community descriptors may as a result have gone undetected. Nevertheless, a striking and significant contribution of macrofauna to explaining the overall variation in composition, diversity and richness of different microbial communities was observed. Patterns in macrofaunal communities were very different from those for the microbial groups (Figure 5), suggesting that changes in the identity of the macrofaunal species were not reflected in the spatio-temporal patterns of the microbial communities. Richness of β-AOB and diversity of AOA was significantly linked to variation in nitrification rate (DistLM; Table 2). At the same time, a pronounced, significant contribution of macrofaunal abundance and functional diversity was observed explaining respectively the variation in diversity of the metabolically active β-AOB and both richness and diversity of the active AOA (Table 1). While the first finding suggests a biodiversity-ecosystem functioning relationship between ammonia oxidizers and nitrification rates in our study area, the second finding indicates that this relationship is modulated by the activities of the macrofauna.

Intermittent ventilation activities of certain macrofaunal species increase oxygen concentrations during the ventilation and excretion activity, followed by oxygen depletion during resting periods of the animals (Stief, 2013). As both oxygen and organic matter are required for the nitrification activity of β-AOB and AOA, we suggest that sediment heterogeneity created by the presence of macrofauna lies at the basis of the significant relationship between macrofauna and diversity aspects of the active nitrifying microbes. Indeed, the fauna in our study area does affect sediment oxygenation of the upper cm in fine sandy sediments (Braeckman *et al.,* 2010) and redistributes organic matter (Braeckman *et al*., 2010 and 2011). Strikingly, total archaeal communities were not affected by the macrofaunal communities (Table 1). The difference observed between the total archaeal community and AOA in our findings revealed that while overviews of total community in relation to macrofauna

may be useful, investigations of specific microbial groups are fundamental in establishing a generic mechanistic understanding (Gilbertson *et al*., 2012).

The functional index BPc significantly contributed to the models for total bacterial community composition and richness while both BPc and bacterial richness and diversity were significantly linked with N-mineralization (Tables 1 and 2). Macrofauna affect organic matter mineralization by degrading organic matter directly through ingestion (Sun *et al*., 1999; van Nugteren *et al*., 2009) and through stimulating microbial mineralization (Kristensen and Mikkelsen, 2006). Among the total pool of microbial communities (bacteria and archaea), only aspects of bacterial diversity were significantly linked with N-mineralization rates. Below the oxic layer in marine sediments, organic matter is mineralised mainly by bacteria (i.e. fermenting, denitrifying, sulphate-reducing) (Jørgensen, 1982), of which sulphate-reducing bacteria account for up to 50% of the entire organic matter degradation in coastal and shelf ecosystems (Jørgensen, 1982; Plugge *et al*., 2011). Annual maximum oxygen penetration depth was only a few mm in muddy and fine sediments, which are characterized by relatively high mineralization rates (Braeckman *et al*., 2014c), so that we can expect a more important role of bacteria compared with archaea in mineralization rates.

Assuming that the total bacterial and archaeal communities comprised denitrifiers as well, a significant relationship between these general communities and denitrification could have been detected (Table 2). This was not the case, which can partly be attributed to the fact that we did not target a functional gene for denitrification, and the fact that parts of denitrification occur at deeper sediment layers not included in this study. However, BPc was retained in models for denitrification (Table 2) reflecting an increase in the coupled nitrification-denitrification processes especially in fine sediment as macrofaunal activity increases (characterized by rich functional macrobenthic diversity [Braeckman *et al*., 2014c]). Denitrification can be increased by bioturbation and bio-irrigation for two reasons. A first reason is the increase of the surface for coupled nitrification-denitrification (Howe *et al*., 2004; Birchenough *et al*., 2012). A second reason is the increased fluxes of O_2 and NH_4^+ due to bio-irrigation, causing higher nitrification fuelling denitrification (Howe *et al*., 2004). As we did not find a relation between nitrification rates and macrofauna density and bioturbation, we believe that macrofauna activity adds complexity to the biogeochemical settings

of the sediment matrix, reflected in an increased surface for the coupled nitrification-denitrification processes.

In conclusion, this study provides evidence to support the link between microbial biodiversity and ecosystem functioning in marine sediments as well as to support the hypothesis that this relationship is modulated by macrofaunal density and functional diversity. Indirect effects of macrofaunal communities on N-cycle processes were found important as well as diversity aspects of microbial communities mediating these processes. As such, our study is major step forward in a general understanding on how marine ecosystem functioning is affected by interactions between organisms with very different body size.

Acknowledgements

We thank the crew of RV “Zeeleeuw” for assistance on board and help in sampling. Also, we are very grateful to Bart Beuselinck for the analysis of granulometric variables, to Sofie Jacob for help with sample processing, to the staff of the research group Marine Biology and the Laboratory of Microbiology, Ghent University for technical assistance. We thank the anonymous reviewers for their constructive comments that improved the quality of the manuscript.

Chapter 4

The Effect of Bio-irrigation By the Polychaete *Lanice conchilega* on Denitrifiers: Distribution, Diversity and Composition of *nosZ* Transcripts

In preparation as “Yazdani Foshtomi, M., Leliaert, F., Derycke, S., Willems, A., Vincx, M., Vanaverbeke, J., The effect of bio-irrigation by the tube-building polychaete *Lanice conchilega* on denitrifiers: distribution, diversity and composition of *nosZ* transcripts”.

ABSTRACT

The presence of large densities of the piston-pumping polychaete *Lanice conchilega* can have important consequences for the functioning of marine sediments. It is considered both an allogenic and an autogenic ecosystem engineer, affecting spatial and temporal biogeochemical gradients (O_2 concentrations, oxygen penetration depth and nutrient concentrations) and physical properties (grain size) of marine sediments, which could affect functional properties of sediment-inhabiting microbial communities. Here we investigated whether density-dependent effects of *L. conchilega* affected horizontal (m-scale) and vertical (cm-scale) patterns in the expression of the typical *nosZ* gene, which plays a major role in N_2O reduction in coastal ecosystems as the last step completing the denitrification pathway. We showed that both vertical and horizontal composition and richness of *nosZ* transcripts were indeed significantly affected when large densities of the bio-irrigator were present. This could be directly related to allogenic ecosystem engineering effects on the environment, reflected in increased oxygen penetration depth and oxygen concentrations in the upper cm of the sediment in high *Lanice* densities. A higher diversity (Shannon diversity and inverse Simpson) in *nosZ* transcripts observed in patches with high *Lanice* densities (3,185-3,440 ind. m^{-2}) suggests a downward transport of NO_3^- to deeper layers resulting from bio-irrigation as well. Hence, our results show the effect of *L. conchilega* bio-irrigation activity on denitrifying organisms in *Lanice* reefs.

Keywords: *Lanice conchilega*, bio-irrigation, *nosZ*, denitrification, gene expression

1. Introduction

Denitrification is a key process in the biogeochemical cycling of nitrogen. It is a primary loss mechanism for nitrogen in the nitrogen budget of coastal ecosystems (Jenkins and Zehr, 2008). It is a four-step respiratory process, in which nitrate is reduced sequentially to nitrite, nitric oxide, nitrous oxide, and nitrogen gas, and is mediated by a taxonomically diverse group of microorganisms, mainly bacteria (Canfield *et al*., 2005). Denitrification counteracts eutrophication by removing N_2, but it can contribute to global warming and ozone depletion due to the release of NO and N_2O (Knowles, 1982; Sanford *et al*., 2012; Kohn, 2015).

The distribution of denitrifying organisms and denitrification rates in both terrestrial and marine ecosystems is affected by different environmental factors including organic matter content, concentration gradients of dissolved inorganic nitrogen (DIN: NO_3^-, NO_2^- and NH_4^+) and the availability of oxygen (Deutsch *et al*., 2010; Huang *et al*., 2011). Stratification in the sediment results from the vertical distribution of these environmental factors and their effects on distribution of denitrifying organisms (Tiquia *et al*., 2006; Huang *et al*., 2011).

Denitrifiers are facultative anaerobes and denitrification is often coupled with nitrification across oxic/anoxic interfaces in the sediment (Zehr and Ward, 2002). With increasing sediment depth, oxygen concentrations decrease and denitrifying communities shift from facultative anaerobes to strict anaerobes (Tiquia *et al*., 2006). However, maximum denitrification rates do not necessarily occur at the lowest O_2 concentration in deep layers (Tiquia *et al*., 2006; Gao *et al*., 2010; Li *et al*., 2010) due to low/absent NO_3^- and NO_2^- concentrations. The NO_x^- concentration may therefore act together with O_2 to control denitrification rates and the diversity and structure of the denitrifying community (Tiquia *et al*., 2006; Gao *et al*., 2010; Zheng *et al*., 2011). In addition, the effects of species-specific adaptation and variations in substrate threshold among denitrifying organisms need to be considered as well. For example, while oxygen has an inhibitory effect on the denitrification enzymes of *Agrobacterium tumefaciens* (Kampschreur *et al*., 2012), *Pseudomonas stutzeri* is able to denitrify under aerobic conditions (Su *et al.*, 2001).

The capacity for denitrification can be deduced from functional genes involved in denitrification pathways, which reflect the distribution and function of denitrifying organisms in the environment (Jenkins and Zehr, 2008). However, the phylogeny of

the functional genes does not necessarily reflect 16S rRNA gene-based phylogeny and taxonomy (Jones *et al*., 2008). The genes encoding for nitrite reductase (*nirS* or *nirK*) and nitrous oxide reductase (*nosZ*) have been used as marker genes in studies improving the understanding of microbial communities involved in different steps of denitrification (e.g. Wei *et al*., 2015; Zhang *et al*., 2015). However, the detection of a gene in the environment (DNA-based methods) does not imply that the corresponding activity is present (Philippot and Hallin, 2005; Wang *et al*., 2014). Instead, the expression of genes (RNA-based methods) and the detection of enzymes (protein-based methods) allow investigating the direct link between the composition and density of the transcribed denitrification genes and rate of denitrification (Philippot and Hallin 2005).

Microbial communities in marine ecosystems can vary in structure from large (kilometers or meters) to small scales (centimeters) (Scala and Kerkhof, 2000; Bertics and Ziebis, 2009; Laverock *et al*., 2011). The small scale (cm) variation is a result of gradients in substrate availability and geochemical conditions associated with the activity of larger biotic communities (Scala and Kerkhof, 2000; Wallenstein *et al*., 2006; Bertics and Ziebis, 2009). Along the vertical gradient within the sediment, the boundary between oxic and anoxic conditions migrates on spatial scales of micrometers to centimeters and on time scales of seconds to hours (Joye and Hollibaugh, 1995). Denitrifiers have the ability to adapt to oscillations of oxygen concentrations during the rapid transition from oxic to anoxic conditions (Bergaust *et al*., 2008; Liu *et al.,* 2013a). Such oxygen oscillations and different oxygen regimes have effects on the structure of the denitrifying community (Wittorf *et al.,* 2016).

Functional diversity and abundance of larger organisms affect the biogeochemical cycles and variations in composition and diversity of microbial communities (Yazdani Foshtomi *et al*., 2015), creating small-scale heterogeneity in the sediment as microniches (Kristensen, 2000; Bertics and Ziebis, 2009). Building and irrigating of burrows by bioturbating organisms like worms, for example, can provide a unique environment for micro-organisms inhabiting the burrow wall (Laverock *et al*., 2011 and 2014) or enhance total microbial metabolism in adjacent surrounding sediments (Kristensen and Kostka, 2004). However, the impact of bioturbation depends on individual functional effects of burrowing organisms (Papaspyrou *et al*., 2006; Bertics and Ziebis, 2009; Laverock *et al*., 2014).

Lanice conchilega is a tube-building worm that glues sediment grains together to form tubes reaching 10-30 cm vertically into the sediment (Forster and Graf, 1995; Degraer *et al*., 2006) and extending 2-3 cm into the water (Heuers, 1998). It can be found in dense aggregations (Van Hoey *et al*., 2008), referred to as biogenic reefs (Rabaut *et al*., 2009). *L. conchilega* manifests both autogenic and allogenic ecosystem engineering properties via, respectively, its own physical structures and by transforming living/non-living materials from one physical state to another (Godet *et al*., 2008). The autogenic engineering effect provides new habitat for associated species by increasing bed stability (Rabaut *et al*., 2009) and trapping organic matter from the water column. It substantially affects the structure and abundance of associated communities and food-web properties (Callaway, 2006; De Smet *et al*., 2015 and 2016). The allogenic engineering capacity of *L. conchilega* is reflected in its piston-pumping activity (Forster and Graf, 1995) causing water and solute exchanges between tubes and the overlying water (bio-irrigation). This stimulates nutrient fluxes, mineralisation and denitrification processes in coastal sediments (as deduced from lab experiments) and seems to be density-dependent (Braeckman *et al.,* 2010). However, the effect of *L. conchilega* on the microbial communities mediating these processes remains unexplored.

In this study, we investigated how the expression of the *nosZ* gene, encoding the enzyme which catalyses the last step of denitrification (conversion of N_2O to N_2 gas; Canfield *et al.,* 2005), and the biogeochemical environment (oscillation in O_2 concentration, sediment grain size and chlorophyll *a*) are affected by *L. conchilega* bio-irrigation activity vertically at small (cm) scales along the sediment depth profiles and also at larger horizontal scales (m) at different natural *Lanice* densities within the reef. The observed patterns in *nosZ* transcripts were then statistically linked to environmental variables to objectively assess the link between faunal-mediated heterogeneity and expression of *nosZ* genes.

To our knowledge, the current study is novel as most of the previous studies were limited to the bioturbation and bioirrigation impact of larger organisms on the rate of denitrification (Gilbert *et al*., 1997; Howe *et al*., 2004; Webb and Eyre, 2004; Nizzoli *et al*., 2006; Bertics *et al*., 2012) and only a few studies have investigated this effect on the denitrifying functional genes (Fernandes *et al*., 2012; Laverock *et al*., 2014; Poulsen *et al*., 2014 but the latter is in freshwater sediments) and still the active community investigation was neglected. As such, the current study can improve our

understanding of the effects of bio-irrigation on diversity of *nosZ* transcripts in marine sediments at small spatial scales.

2. Materials and Methods

2.1. Study Site and Sampling

Sampling was done in October 2014 at the intertidal zone of the sandy seashore of Boulogne-sur-Mer, along the northern part of the English Channel (50° 44.01' N, 01° 35.15' E; Northern France; Figure 1). Sediments of the *L. conchilega* reef were collected by core (Plexiglas, 78.5 cm^2 surface area; height: 25 cm) from a limited area of the western reef zone (about 4000 m^2) located higher on the beach and exposed at every low water (Rabaut *et al*., 2008).

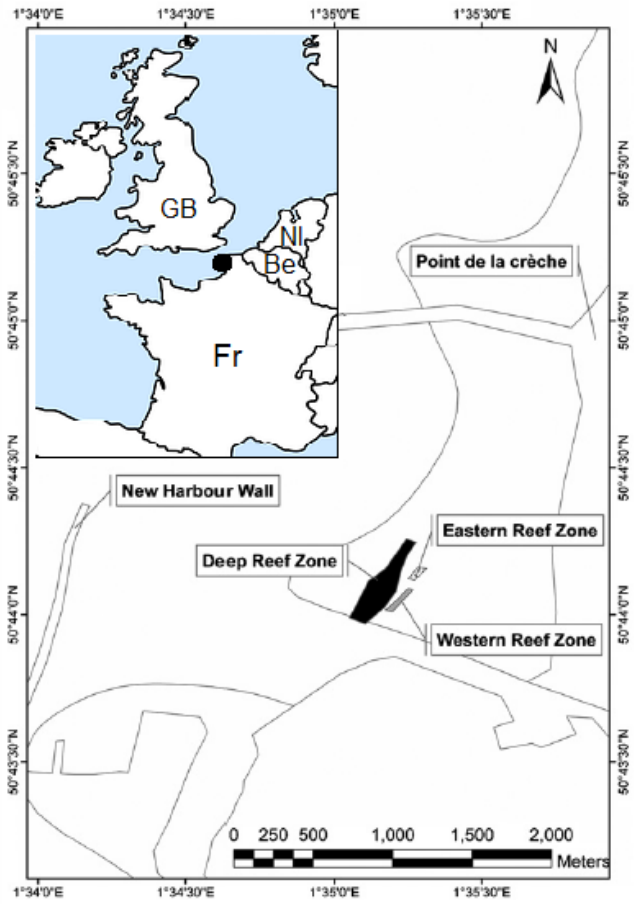


Figure 1: Sampling location (Western reef zone) at Boulogne-sur-Mer in France (modified after: Rabaut *et al*., 2008)

Three replicate cores were taken from three different areas within the reefs: *(i)* an area with an average density of *Lanice* individuals (based on counts of fringed tubes, Van Hoey *et al*., 2006) of 25-27 tubes per core surface (3,185-3,440 ind. m^{-2}) (hereafter referred to "high *Lanice* treatment") *(ii)* an area with lower *Lanice* densities

(5 tubes per core surface; 637 ind. m^{-2}) located on the edge of the patches ("low *Lanice* treatment") *(iii)* and sediments without *Lanice* between the reef patches ("control treatment").

The intact sediment cores (3 treatments × 3 replicates) were transported to the lab and submerged, uncapped without creating suspension of the sediment in tanks containing continuously aerated seawater at an *in situ* temperature-controlled room (17 °C in October). Every core was aerated separately by a stream of fine bubbles in the overlying water. In order to create water circulation inside the cores, teflon-coated magnets were inserted at appropriate distance from the sediment surface and rotated by a central magnet in the tanks at a speed below the resuspension limit.

Within two days after sampling, vertical profiles of sediment oxygen concentration were measured (three profiles per core) in the ambient sediment at 1 to 2 cm distance from tubes, using Unisense oxygen microsensors (type ox100) in vertical increments of 250 µm. Bio-irrigation activities of *Lanice* individuals were assessed as well, by logging changes of sedimentary O_2 concentrations every second for 30-35 min at 1.5 mm sediment depth, at 1 cm distance from the tube, using the same Unisense micro sensors.

At the end (three days after sampling), the upper sediment layers were sliced in 0.5 cm intervals (0-0.5, 0.5-1, 1-1.5 cm) to investigate microbial communities in the oxic and anoxic zones (based on the measured oxygen profiles). A fourth layer (2.5-3 cm) was collected as well because *Lanice* was reported to ventilate its tube up to 2.4 cm tube length (Forster and Graf, 1995). All sediment slices were homogenized before collecting subsamples for further analyses.

The sediment was subsampled by taking 3 to 5 ml using a cut-off syringe for the analysis of labile organic matter (chl-*a*), % clay and silt content and median grain size (MGS). Around 4 g of sediment (wet weight) was subsampled using sterilized tools for microbial analyses and stored in sterile 15-ml falcon tubes. Microbial and chl-*a* samples were immediately frozen at -80 °C, whereas the samples for grain size were dried at 60 °C before analysis.

Chl-*a* was determined by HPLC (Gilson, Middleton, Wisconsin, USA) analysis according to Wright and Jeffrey (1997) and sediment granulometry by laser diffraction (Malvern Instruments, Malvern, UK).

2.2. RNA Extraction and *nosZ* Sequencing

RNA was extracted from 4 g homogenised sediment (wet weight) from each depth layer (36 samples = 3 treatments × 3 replicates × 4 layers) using the RNA Power Soil Total RNA isolation kit (MO BIO Laboratories). Integrity and purity of RNA extractions were checked following Yazdani Foshtomi *et al.* (2015). The DNA-free RNA samples were reverse transcribed into cDNA using Omniscript Reverse Transcriptase Kit (Qiagen) according to the instructions of the manufacturer and using 10 µM of Random Hexamer primers (Applied Biosystems) and 5 µl RNA template per total volume of a reaction (20 µl).

RT-PCR amplification of *nosZ* gene fragments (267 bp) was performed in three technical replicates for each sample using specific primer sets (*nosZ*-2F: 5'-CGCRACGGCAASAAGGTSMSSGT-3' / *nosZ*-2R: 5'-CAKRTGCAKSGCRTGGCA GAA-3') (Henry *et al*., 2006) for the typical *nosZ* gene cluster (Sanford *et al*., 2012; Graf *et al*., 2014). The forward primer contained the 5' Illumina adaptor, forward primer pad and linker and the *nosZ*-2F primer. The reverse primer consisted of the reverse complement of the 3' Illumina adapter, 12-bp multiplex identifiers (MIDs), the reverse primer pad and linker and the *nosZ*-2R primer. MIDs in the reverse primer were used to identify the different samples (see Addendum 3, Table S1 for adaptor, MID, pad, linker and primer sequences for the forward and reverse data; Caporaso *et al*., 2012). A control (no template) was also included to ensure that no contamination occurred in the laboratory.

Amplifications were performed in volumes of 25 µl containing 5 µl of target cDNA, 5 µl of 5xKAPA HiFi Buffer (containing 2 mM $MgCl_2$ at 1x; Kapa Biosystems, Boston, Massachusetts, United States), 0.4 µg/µl BSA, 0.2 mM dNTPs, 0.4 µM of each primer, and 1.5 U KAPA HiFi HotStart DNA Polymerase (1 U/µl) (Kapa Biosystems). The 'touchdown' PCR condition was as follows: after an initial denaturation step at 95 $^{\circ}$C for 5 min, 20 cycles were performed consisting of three steps: denaturation (98 $^{\circ}$C, 20 sec), annealing (70 $^{\circ}$C, 1 min, decreasing 0.5 $^{\circ}$C cycle^{-1} to 60 $^{\circ}$C) and extension (72 $^{\circ}$C, 1 min) followed by 15 additional cycles in which the annealing temperature was 60 $^{\circ}$C (Bai *et al*., 2012). The final elongation step was performed at 72 $^{\circ}$C for 10 min.

PCR amplicons of the three technical replicates of each sample were combined, purified using E-Gel (Invitrogen, Life technologies) and measured with a Qubit fluorometer (Life Technologies). Samples were then pooled equimolarly and loaded on a Bioanalyzer 2100 (Agilent Technologies) to check the presence of the single peak. Purified pooled libraries were submitted to Genomics Core (Center for Human Genetics UZ - K.U. Leuven) for 150×2-cycle paired-end sequencing on an Illumina Miseq platform.

2.3. Sequence Analyses

Demultiplexing of the data was carried out by Genomics Core using the Illumina standard procedure by applying the bcl2fastq tool of Illumina which allows for 1 mismatch between the barcodes. Raw demultiplexed Illumina fastq files were then quality-filtered and merged using Pear v0.9.5 (Paired-End read mergeR; Zhang *et al*., 2014). Reads shorter than 100 bp or longer than 400 bp and low-quality reads (scores <25) were removed from the output. Any reads containing uncalled bases and singletons were also discarded. The final file was checked for quality control with Fastqc v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Marconi *et al*., 2014).

We eliminated potential chimeras using the USEARCH package (Edgar *et al*., 2011; Fish *et al*., 2013). Non-target reads (non-*nosZ* reads) were filtered out using FrameBot included in the Fungene pipeline (http://fungene.cme.msu.edu/). We retrieved a reference set of *nosZ* protein sequences of 163 different species with high scores (> 919) and 98% coverage with a hidden Markov model (HMM) from Fungene. FrameBot compared each member of these reference sequences to the query nucleotide sequence in both forward and reverse directions. In addition, FrameBot corrected insertion and deletion errors, and translated DNA sequences to frameshift-corrected protein sequences (Fish *et al*., 2013; Wang *et al*., 2013).

nosZ diversity was determined at the amino acid level. Therefore, a complete identity threshold (100%) of amino acid sequences was applied in USEARCH. These units (unique AA *nosZ* transcripts, hereafter referred to as "*nosZ*-UAT") are equivalent to the "OTU" (Operational Taxonomic Units) defined in previous studies (e.g. Palmer *et al*., 2012). However, because "OTU" is commonly used in a 16S rDNA taxonomic context, and our study is based on a functional gene, which does not necessarily correspond to 16S rDNA taxonomy (due to confounding effects of horizontal gene

transfer [HGT] and other phenomena such as gene duplication/divergence), we prefer not to use the term here (Canfield *et al*., 2005; Jones *et al*., 2008; Burke *et al*., 2011).

Prior to further analysis, *nosZ*-UATs with < 0.005% relative abundance in all samples, which together accounted for less than 2% of the total reads and mostly represented singletons and doubletons, were discarded (Bokulich *et al*., 2012; Kartzinel *et al*., 2015).

Maximum likelihood phylogeny of unique *nosZ* AA sequences was inferred using RAxML v8.2.6 on the CIPRES Science Gateway (Miller *et al*., 2010), under a JTT+I+G model as determined using the lowest AIC criterion in ProtTest version 2.4 (Abascal *et al*., 2005). Node confidence was determined using 200 bootstrap replicates. In order to construct a phylogeny with reference sequences, we performed protein BLAST searches against the NCBI non-redundant protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of 25 *nosZ*-UAT representatives selected randomly across the phylogenetic tree (Figure S2, Addendum 3). For each blastp search, the best 100 blast hits were retained. To reduce the size of the tree, identical sequences were removed as well as sequences differing in 1 or 2 AA positions. A final alignment of 208 *nosZ* sequences from the database was used to construct an ML tree using RAxML as described above.

2.4. Data Analysis

2.4.1. Environmental Data Analyses

We used permutational multivariate ANOVA (PERMANOVA; Primer v6.1.10., Primer-E Ltd., Plymouth, United Kingdom with the PERMANOVA + add-on package; Anderson *et al*., 2008) performing a two-way fixed factor model design ("treatment" and "depth" as factors) while "replicate" was nested as a random factor in "treatment" (Braeckman *et al*., 2011) in order to test significant differences in environmental factors (MGS, % silt+clay [mud] content and chl-*a* concentration). To test for the differences in maximum oxygen penetration depth (max OPD) and oxygen profiles (both depth and time profiles) between levels of *Lanice* density, PERMANOVA with a one-way fixed factor model design ("Treatment" as a factor) was performed. Prior to the PERMANOVA analyses for oxygen profiles, a multivariate data matrix was constructed in which each depth horizon (for depth profiles) and each second (for time profiles) was considered a "variable" (Widdicombe *et al*., 2013). Pairwise tests

were performed for significant terms using Monte Carlo p-values when the number of possible permutations was restricted (Anderson *et al*., 2008).

Euclidean distance was used to calculate the resemblance matrices in PERMANOVA for the univariate environmental data (Anderson *et al*., 2008) and also for the multivariate data matrix corresponding to oxygen depth and time profiles (Widdicombe *et al*., 2013). Homogeneity of multivariate dispersion ('variance') was tested with PERMDISP for any of the significant terms in PERMANOVA to verify whether dispersion effects affected the PERMANOVA tests.

For oxygen depth profiles, SIMPER analysis was used to determine which depth was responsible for the identified differences.

2.4.2. Microbial Community Analyses and Diversity Estimates

To correct for technical bias related to read number variations among the samples, the data was normalized (Dillies *et al*., 2012). All samples were normalized by taking the proportions of each *nosZ*-UAT, multiplying it with the minimum sample size (1022 reads) and rounding to the nearest integer (McMurdie and Holmes, 2014; Props *et al.,* 2016).

To calculate alpha diversity and to assess how the sampled community of *nosZ* genes reflects a "true" diversity per sample (Hughes *et al.,* 2001), we made random sub-samples of the non-normalized data matrix to the minimum number of reads (1022) in R version 3.3.1. This was repeated 1000 times and the average diversity (richness, Shannon-Wiener [log e] and inverse Simpson) of each sample was calculated from the estimated diversity of each trial.

Rarefaction curves were also calculated in R for the number of unique genes expressed (*nosZ*-UAT) vs. the total number of reads, which reflects the variation of the *nosZ* gene expression in a sampling effort in our samples.

Generalized UniFrac distances (α = 0.5) (Chen *et al*., 2012) were calculated on the composition of *nosZ* transcripts from normalized data with the GUniFrac package in R (Team, 2008). PERMANOVA was conducted on these UniFrac distances using the Adonis package in R. PERMDISP and pairwise difference tests were also performed in R.

Euclidean distance was used to calculate the resemblance matrices in PERMANOVA (Primer v6.1.10) for the diversity indices (estimated as average values).

PERMANOVA was performed as a two-way fixed factor model design ("treatment" and "depth" as factors while "replicate" was nested in "treatment") to test significant differences among factors in composition and diversity indices of *nosZ* transcripts.

Principal coordinates analysis (PCoA) plots were generated using the Ade4 package in R to visualize variation in composition of *nosZ* transcripts among treatments and depths.

In addition, we investigated whether differences among factors were caused by differences in non-abundant *nosZ*-UATs (< 1% relative abundance), by constructing a dataset with abundant *nosZ*-UATs (defined as *nosZ*-UATs with >1% relative abundance in at least one treatment-depth combination). This resulted in a second dataset with 21 abundant *nosZ*-UATs out of the total 502 *nosZ*-UATs and with 439624 reads. Statistical analyses on the dataset with the abundant *nosZ*-ATUs were performed as described above.

Multivariate data corresponding to *nosZ* transcripts were reduced to binary data (*nosZ*-UATs were counted as present or absent) in Excel (Fulthorpe *et al*., 2008). Jaccard similarity index and Venn diagrams were calculated and drawn based on the number of shared and unique *nosZ* transcripts among treatments per depth layer (Schloss and Handelsman, 2006; Fulthorpe *et al*., 2008).

2.4.3. Multiple Linear Regressions

Multiple linear regressions were performed to identify which variables contributed significantly to the variations observed in the average values of diversity indices of *nosZ* transcripts (richness, Shannon diversity and Inverse Simpson as response variables) at different depths and treatments. Multivariate data related to oxygen profiles, both depth and time profiles, was also included in the regression analyses but as two univariate variables: mean values of oxygen concentration per depth layer ("OX") and coefficient of oxygen variations over time ("CV"), respectively. To avoid multi-collinearity among independent variables, the regression analyses were performed after removing highly correlated variables based on: *(i)* the correlation coefficient (|r| ≥ 0.85) among numerical independent variables *(ii)* graphical exploratory techniques (boxplots) displaying the distribution of each numerical variable at different depths and treatments (categorical independent variables). No collinearity was observed among numerical variables while "depth" and "treatment" were collinear with chl-*a* and max OPD, respectively. Six explanatory variables were

ultimately entered into the models: chl-*a* concentration, MGS, % silt+clay [mud] content, CV, max OPD and OX. All assumptions (Zuur *et al*., 2010) were checked after model selection. An initial linear regression analysis showed violation of homogeneity of residuals in all models. We therefore applied linear regression with generalized least-squares (GLS) using restricted maximum-likelihood (REML) estimation. This incorporates variance–covariates to model the variance structure. To find the optimal random structure, the full linear regression model was compared to the GLS models of specific variance structures using lowest Akaike information criteria (AIC). This procedure resulted in the use of a variance structure that allowed for different variances per stratum for "max OPD" (varIdent function, R package nlme). The optimal fixed components in the final model were obtained by applying a backward selection using the likelihood ratio test obtained by ML estimation. A graphical model validation was applied to check for homogeneity and normality in the final models (Zuur *et al*., 2009).

3. Results

3.1. Sediment Environmental Factors

The granulometric variables, including MGS and mud content (%), were not significantly influenced by interactive and single effects of treatments (high, low and no (=control) *L. conchilega*) and depths (PERMANOVA, $p > 0.05$). Chl-*a* concentration was significantly affected by depth (PERMANOVA, pseudo-F = 12.455, p = 0.000). Highest chl-*a* concentrations were observed at the top layer (0-0.5 cm) (pairwise test, all $0.001 < p < 0.01$) followed by the second layer which was significantly higher than the deepest layer (2.5-3 cm) (pairwise test, p = 0.014; Table 1).

O_2 depth profiles (Figure 2) were significantly affected by the treatment effect (PERMANOVA, pseudo-F = 5.33, p = 0.006) (Table S2, Addendum 3). Pair-wise tests (Table S2, Addendum 3) revealed significant differences in vertical O_2 distribution between control and both *Lanice* treatments (p = 0.002 and p = 0.039 for high and low *Lanice* density, respectively). There were no significant differences between both *Lanice* treatments. SIMPER analyses showed that the difference in oxygen profiles between the control treatment and high *Lanice* treatment was attributed to 1-4 mm sediment depth whereas this difference was between 1-3.25 mm for the control and low *Lanice* treatments.

Table 1: Variations of sedimentary environmental factors in three *Lanice* treatments (high, low densities and control) and four depths. Capital letters stand for significant vertical differences (A>B>C) across treatments (p > 0.05). MGS: Median grain size

	Chlorophyll a (µg g^{-1})			***MGS (µm)***			***Mud content (%)***		
	High	**Low**	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**
Depth (cm)									
0-0.5	2.01±0.28^{A}	1.55±0.06^{A}	1.80±0.20^{A}	238.12±6.35^{A}	227.14±2.96^{A}	243.23±6.22^{A}	1.55±1.55^{A}	0.20±0.20^{A}	0.00±0.00^{A}
0.5-1	1.34.±0.19^{B}	0.79±0.23^{B}	1.50±0.06^{B}	250.97±4.14^{A}	228.81±4.96^{A}	245.96±5.62^{A}	2.17±2.17^{A}	0.28±0.28^{A}	0.15±0.15^{A}
1-1.5	0.65±0.15^{BC}	1.04±0.55^{BC}	1.29±0.12^{BC}	239.25±6.43^{A}	232.76±6.11^{A}	242.12±3.36^{A}	0.00±0.00^{A}	0.00±0.00^{A}	1.46±1.11^{A}
2.5-3	0.59±0.51^{C}	0.56±0.13^{C}	1.02±0.49^{C}	235.31±8.97^{A}	238.03±7.71^{A}	234.62±1.39^{A}	2.94±2.94^{A}	0.00±0.00^{A}	1.31±1.31^{A}

Oxygen concentrations over time (over a period of 30-35 min) at 1 cm distance from the tube and at 1.5 mm depth stayed high in the high *Lanice* treatment ranging between 93 and 235 µmol l^{-1} followed by the low *Lanice* treatment (60-203 µmol l^{-1}). The lowest values were in the control treatment (80-157 µmol l^{-1}) (Figure 3). However, O_2 time profiles were not significantly affected by the treatment effect (p > 0.05; Table S2, Addendum 3).

Maximum OPD was significantly affected by "treatment" (pseudo-F= 12.19; p = 0.000) and pair-wise tests showed a significantly higher max OPD in the high *Lanice* density treatment (5.17 ± 0.44mm; mean±se) compared to the low (3.69 ± 0.17mm; $p_{(MC)}$ = 0.008) and control (3.30 ± 0.12mm; $p_{(MC)}$=0.001) treatments (Table S2, Addendum 3; Figure 2).

PERMDISP analyses showed some heterogeneity of multivariate dispersion (F= 3.16, p = 0.028) in the analysis of maximum OPD but this was not the case for the analysis of the entire O_2 depth profiles (p > 0.05).

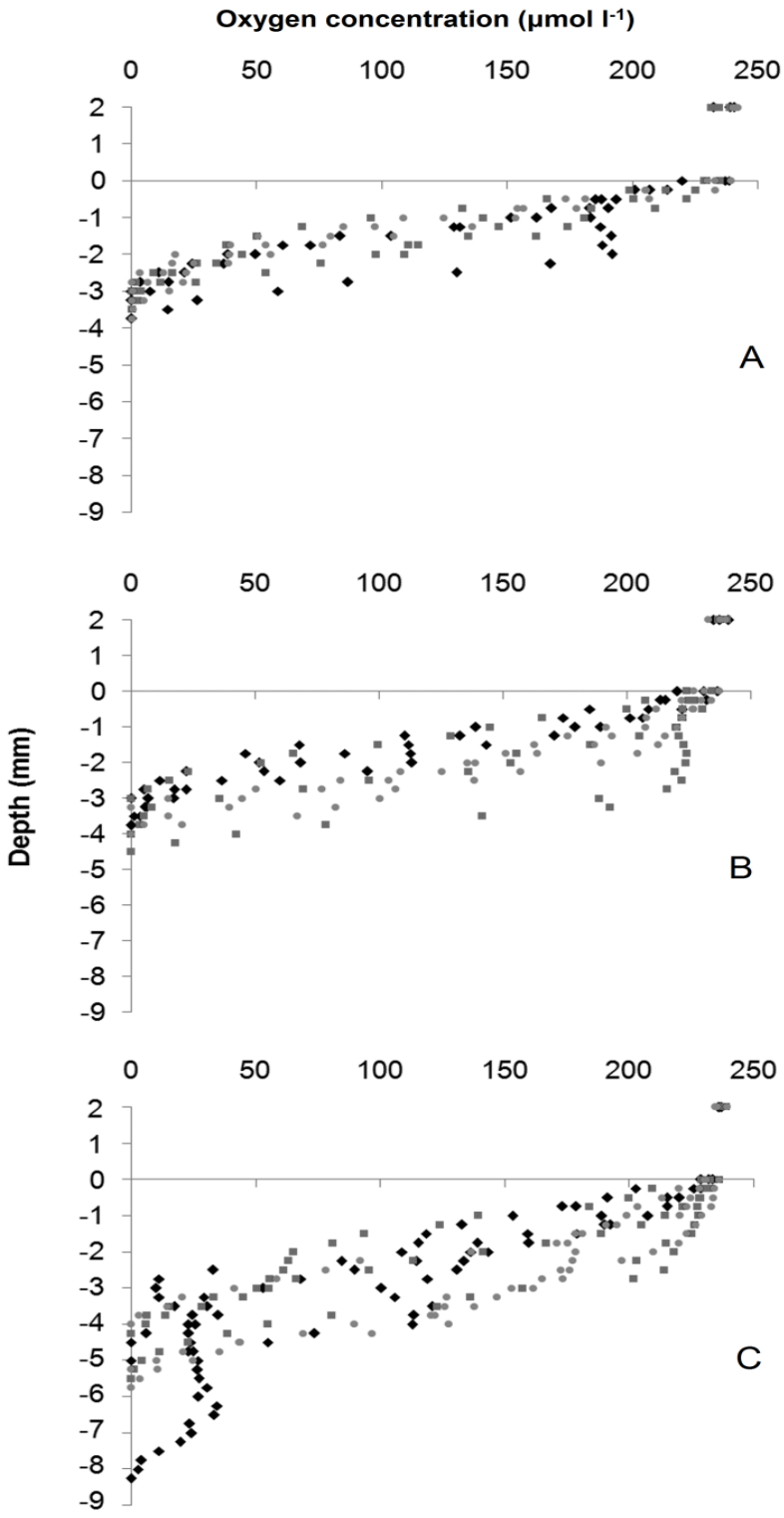


Figure 2: O_2 depth profiles in the overlying water and sediment in control (A), low (B) and high *Lanice* (C) treatments. Nine profiles were measured per treatment (shown with different symbols per core replicate). The zero depth in the profiles represents the sediment-water interface.

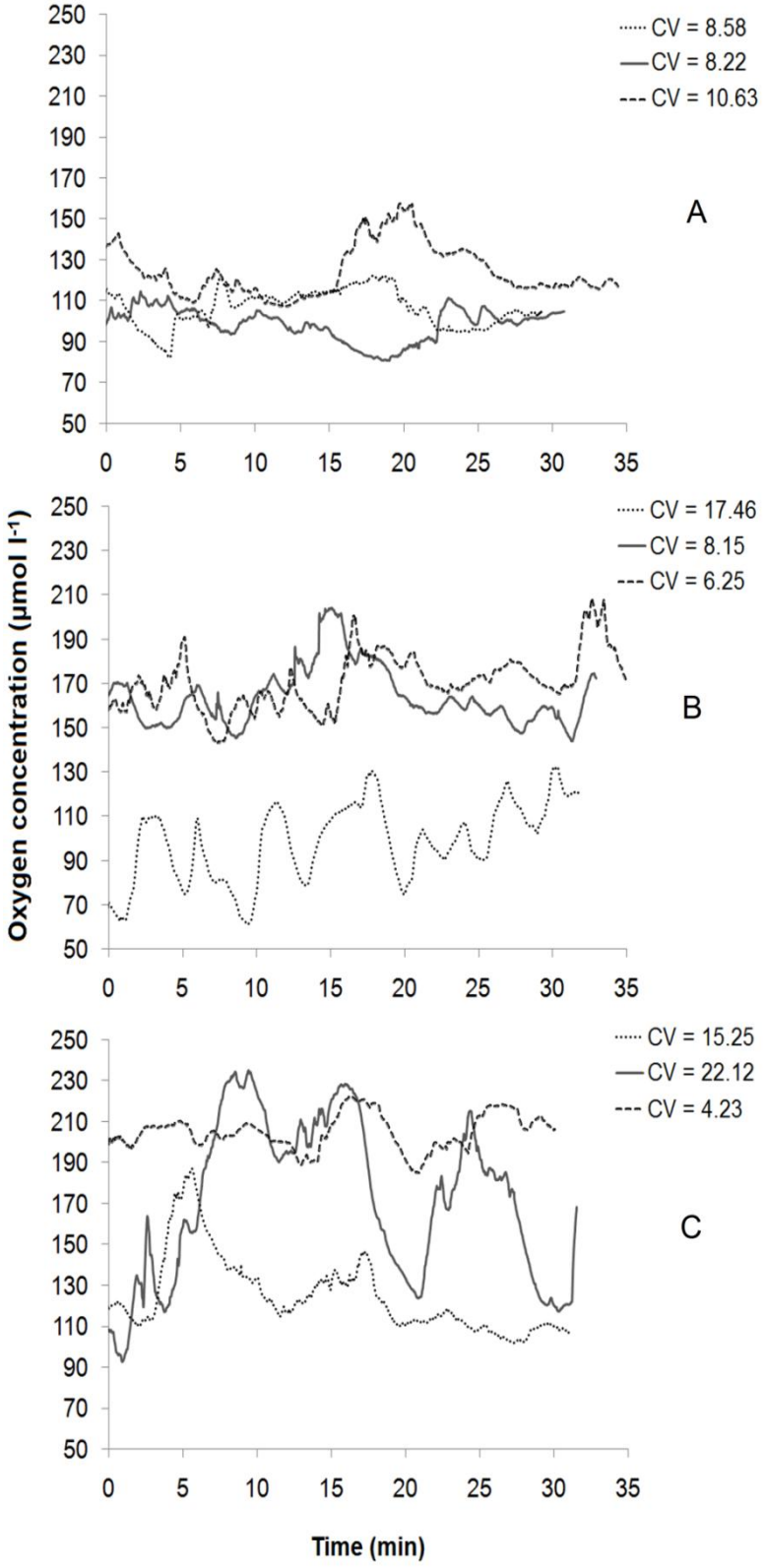


Figure 3: Time variations (in 30-35 min) of O_2 concentration as a measure for bio-irrigation activities of *Lanice* at 1.5 mm depth in control (A), low (B) and high *Lanice* (C) treatments. Three profiles were measured per treatment. “CV” indicates coefficient of oxygen variations over time.

3.2. Composition of *nosZ* Transcripts and Diversity Indices

In total, 545,742 *nosZ* reads were assigned to 502 unique amino acid sequences (referred to "*nosZ*-UAT"). The current sampling effort was sufficient in most samples to reflect the variation of *nosZ* transcripts (Figure S1 A, Addendum 3). Only samples of the Hd4 treatment did not reach an asymptotic rarefaction curve. Instead, read numbers of the high *Lanice* treatment decreased with increasing depth. This pattern was consistent across replicate samples, suggesting that the low read numbers are not a technical artifact but probably a reflection of the lower abundance of *nosZ*-UAT's in this treatment.

Only 21 of 502 *nosZ* transcripts were found to be abundant (> 1% relative abundance in at least one treatment-depth combination). Of these abundant *nosZ* transcripts, the two dominant *nosZ*-UAT 1 and 2 transcripts combined constituted 50-77% of the community where *nosZ*-UAT 1 alone accounted for 44-71% of the relative abundance in different samples (Figure 4).

Numbers of reads and total numbers of *nosZ*-UATs per sample as well as the numbers of abundant *nosZ*-UATs (> 1% relative abundance) in treatment-depth combinations are provided in Table S3 (Addendum 3).

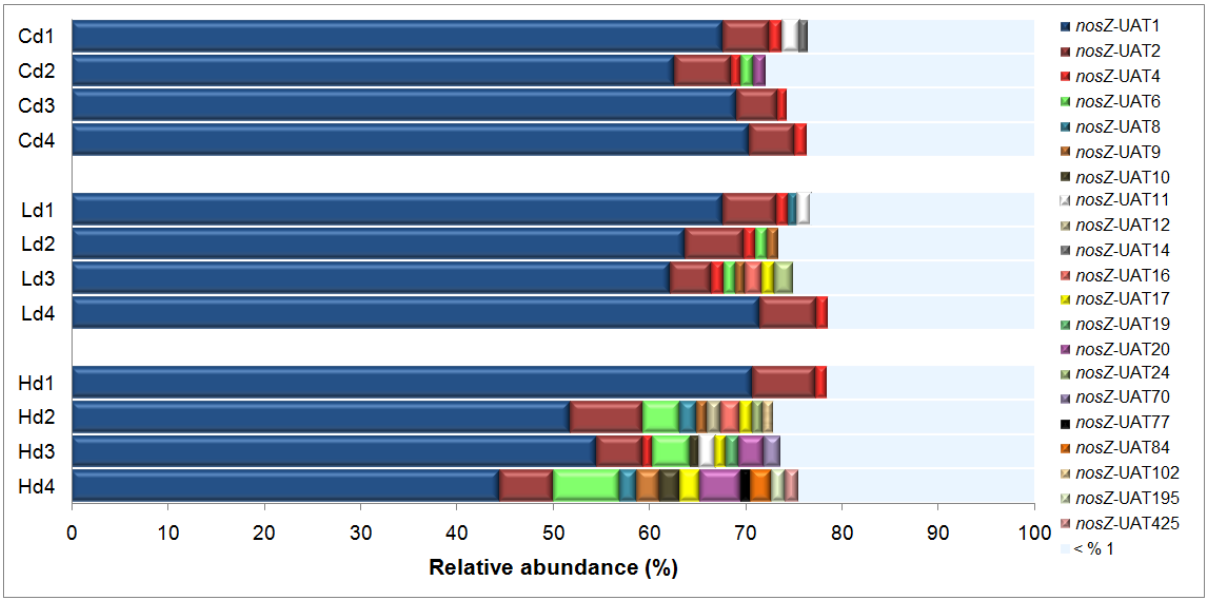


Figure 4: Distribution of abundant (relative abundance > 1%) and non-abundant (< 1%) *nosZ*-UAT at different depths and treatments. Abundant *nosZ*-UATs were defined as *nosZ*-UATs with > 1% relative abundance in at least one treatment-depth combination (values are averages of replicates). "H" indicates high *Lanice* treatment. "L": low *Lanice* treatment, "C": control treatment, "d1": 0-0.5 cm depth, "d2": 0.5-1 cm, "d3": 1-1.5 cm, "d4": 2.5-3 cm.

The results of the protein blast indicate that among the abundant *nosZ* sequences, *nosZ*-UAT 1, 2, 4 and 10 were most closely (> 97% AA identity) related to *nosZ* sequences from *Pseudomonas stutzeri*. The other abundant *nosZ* sequences, however, were less than 95% similar to protein sequences available in sequence databases.

The maximum likelihood tree of *nosZ* sequences retrieved from our samples showed considerable phylogenetic diversity (Figure S2, Addendum 3). No clear phylogenetic signal could be observed with respect to treatment, i.e. samples from the three treatments (control, low and high *Lanice*) contained sequences from across the phylogenetic tree (Figure S2, Addendum 3). Phylogenetic analysis of representative *nosZ*-UATs together with related sequences derived from pBlast searches showed that abundant as well as non-abundant *nosZ*-UATs are dispersed among Alpha-, Beta- and Gammaproteobacteria (Figure S3, Addendum 3).

PERMANOVA based on Generalized Unifrac distance showed that the composition of *nosZ* transcripts with and without including non-abundant *nosZ*-UAT was significantly affected by the single effect of treatment and depth, regardless of the dataset used (Table 2).

Table 2: Results from PERMANOVA analysis main tests for differences in composition of *nosZ* transcripts among treatments (high and low *Lanice* densities and control) and depths (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm). Analyses were carried out based on Generalized UniFrac distances (α = 0.5) on the data sets using all *nosZ*-UATs or only abundant *nosZ*-UATs with relative abundance > 1%. Single factor results are shown while the interaction treatment x depth is not significant.

	factor	df_{term}	*Pseudo-F*	*P value*
All nosZ-UATs	treatment	2	2.82	**0.026**
	depth	3	2.68	**0.012**
Abundant (> 1%) nosZ-UATs	treatment	2	3.35	**0.025**
	depth	3	2.59	**0.046**

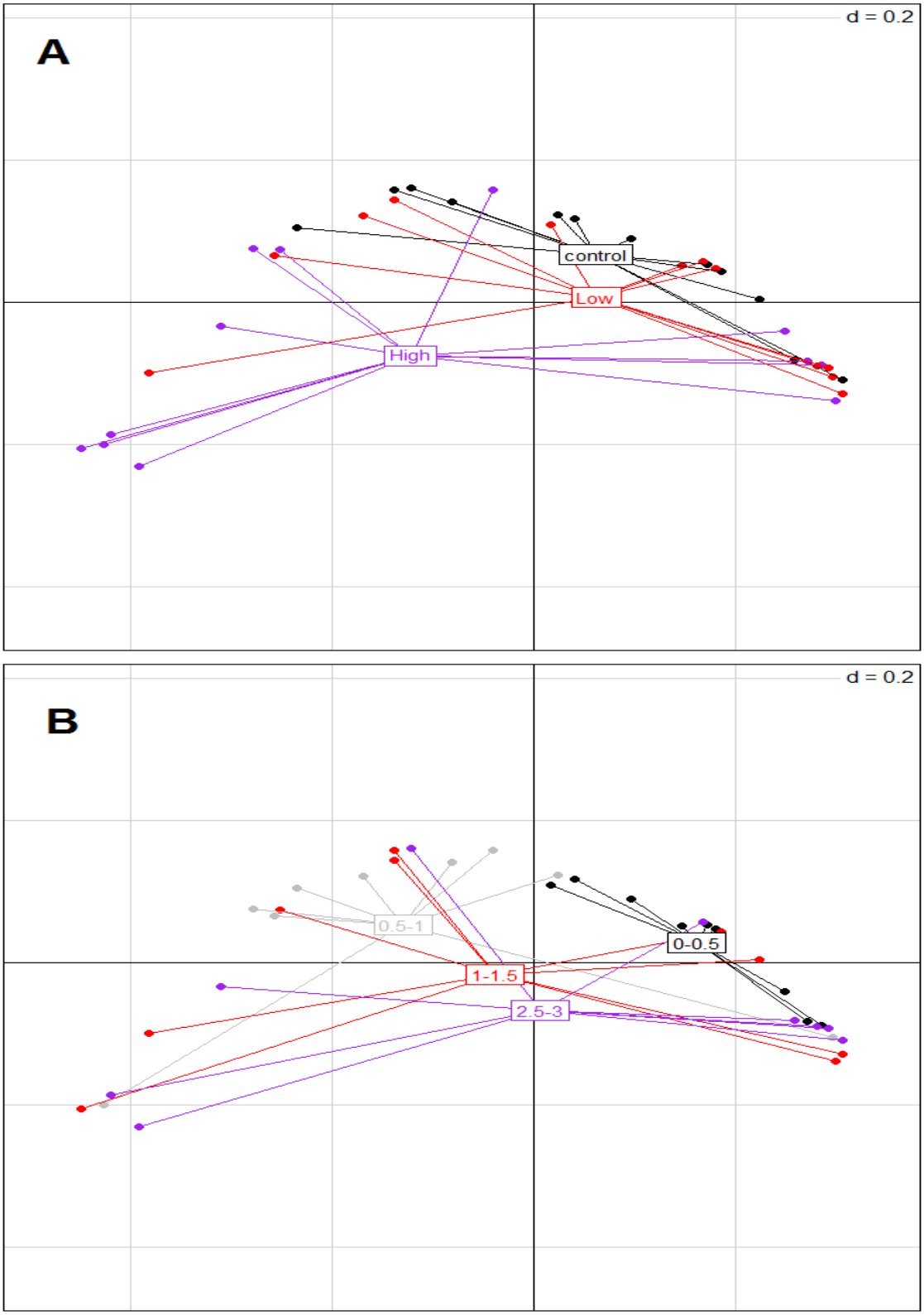


Figure 5: Principal coordinates analysis plot (PCoA) based on generalized UniFrac distances on the normalized data including all *nosZ*-UATs (A: per treatment; B: per depth). Each point represents a sample. Three treatments (High: high *Lanice* treatment, Low: low *Lanice* treatment, Control) and four depth layers (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm) are shown.

Pairwise tests (Table S4, Addendum 3) indicated that the composition of *nosZ* transcripts in the treatment with high density of *Lanice* was significantly different from the control treatment ($p = 0.036$). However, no difference in *nosZ* transcript composition was observed between the low and high *Lanice* treatments, and also between low *Lanice* and control treatments ($p>0.05$).

In the vertical depth distribution, pairwise tests revealed that the composition of *nosZ* transcripts at the top layer (0-0.5 cm) was significantly different from the other layers (all pairwise test $p<0.05$; Table S4, Addendum 3).

The highly significant PERMDISP results in both analyses with all *nosZ*-UATs (factor "treatment": $F = 10.07$, $p = 0.000$; factor "depth": $F = 8.95$, $p = 0.000$) and only abundant *nosZ*-UATs (factor "treatment": $F = 10.93$, $p = 0.000$; factor "depth": $F = 7.56$, $p = 0.000$) indicated a dispersion effect as well. Visualization by PCoA plot (Figure 5 and Figure S4, Addendum 3) showed that dispersion increased with increasing *Lanice* densities and with increasing depth in the sediment.

The Venn diagram and Jaccard similarity index (Figure 6) indicated the highest similarity in *nosZ* transcripts among treatments at the top layer (0-0.5 cm) with more than 91% similarity. With increasing depth, the number of shared *nosZ*-UATs was generally reduced between the high *Lanice* density and the two other treatments, reaching the lowest similarity (< 50%) at the deepest sediment layer (2.5-3 cm). In addition, Jaccard similarity index vertically at different depths per treatment indicated the low similarities in the high *Lanice* treatment with the lowest values (47%) between the top layer (0-0.5 cm) and 2.5-3 cm (Table 3).

PERMANOVA did not show any significant effect on Shannon diversity and inverse Simpson for the factors "depth", "treatment" or the interaction (all $p > 0.05$) although higher values were observed in the high *Lanice* treatment at highest depth (Table S5, Addendum 3; Figure 7). On the other hand, richness of *nosZ* transcripts was affected by the interaction effect "treatment x depth" (PERMANOVA, pseudo-$F = 5.45$, $p = 0.043$; Table S5, Addendum 3). Pairwise tests (Table S6, Addendum 3; Figure 7) did not reveal significant differences in gene richness among treatments at the top layer (0-0.5 cm) ($p_{(MC)} > 0.05$). In deeper sediment layers (2.5-3 cm), significantly lowest values were detected in the high *Lanice* treatment (pairwise tests, $p_{(MC)} = 0.015$ and $p_{(MC)} = 0.016$ compared with the control and low *Lanice* treatments). In addition, richness was significantly lower in the high *Lanice* compared to the control at the depth 0.5-1 cm (pairwise test, $p_{(MC)} = 0.026$).

We generally found a decreasing trend in transcript richness in the high *Lanice* treatment with increasing depth (Figure 7). These changes were significant between the top (0-0.5 cm) and deepest (2.5-3 cm) layer (pairwise test, $p_{(MC)}$ = 0.022; Table S6, Addendum 3).

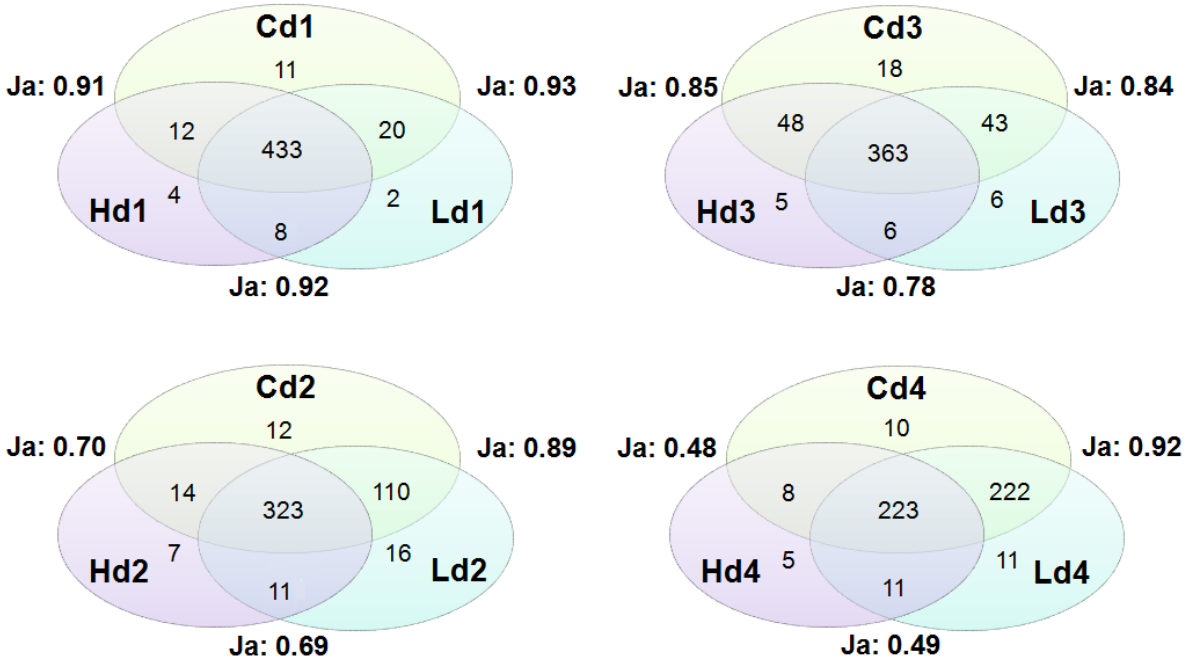


Figure 6: Venn diagrams of the numbers of unique and shared *nosZ*-UATs and Jaccard similarity index (Ja) among treatments (high *Lanice* treatment [H], low *Lanice* treatment [L], Control [C]) in each depth (0-0.5 [d1], 0.5-1 [d2], 1-1.5 [d3] and 2.5-3 cm [d4]).

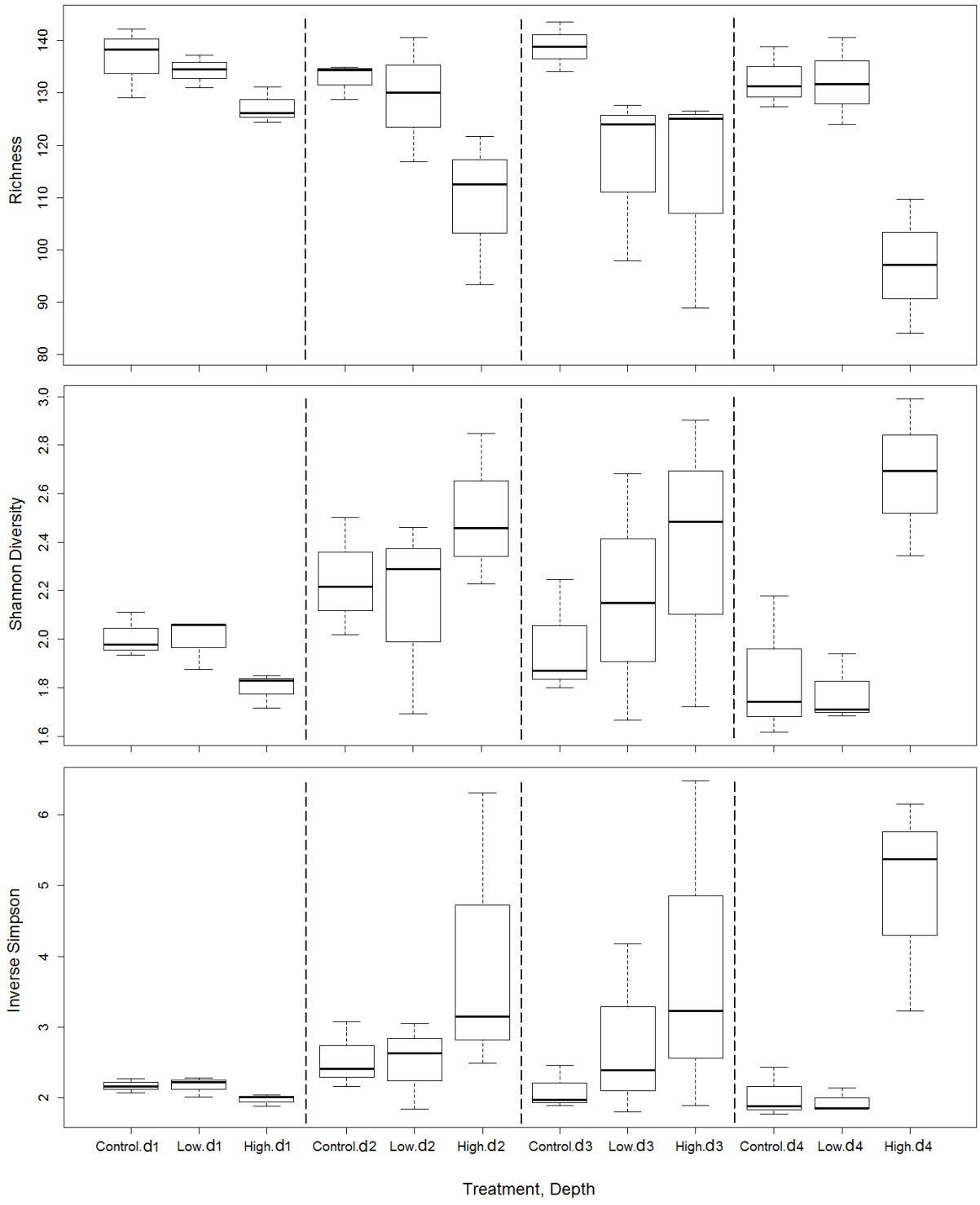


Figure 7: Vertical distribution of diversity indices (richness, Shannon diversity and inverse Simpson) of *nosZ* transcripts (obtained from 1000 sub-samples of the data matrix to the minimum number of reads) at three treatments (high *Lanice*, low *Lanice* and control treatments). “d1”, “d2”, “d3” and “d4” indicated four depth layers (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm, respectively). The solid horizontal line shows the median. Box is drawn between the quartiles (the middle 50% of the data). Dotted lines extend to the minimum and maximum values.

Table 3: Jaccard similarity index in each treatment between the top layer (0-0.5 cm) and other depth layers. Treatments: high *Lanice* treatment [H], low *Lanice* treatment [L], Control [C]. Depths: 0-0.5 [d1], 0.5-1 [d2], 1-1.5 [d3] and 2.5-3 cm [d4]

High Lanice	Jaccard	***Low Lanice***	Jaccard	***Control***	Jaccard
Hd1-Hd2	0.67	Ld1-Ld2	0.89	Cd1-Cd2	0.91
Hd1-Hd3	0.82	Ld1-Ld3	0.81	Cd1-Cd3	0.93
Hd1-Hd4	0.47	Ld1-Ld4	0.89	Cd1-Cd4	0.92

3.3. Statistical Modeling to Link Abiotic Sediment Characteristics with the Diversity Indices of *nosZ* Transcripts in *L. conchilega* Reefs

Maximum oxygen penetration depth (max OPD; collinear with "treatment") was an important variable incorporated in all diversity models. However, it was not significant in the model for the inverse Simpson index (p >0.05). We found significant negative relation ($\beta = -8.10 \times 10^{-3}$, $p < 0.01$) between max OPD and gene richness; and a positive relation between this variable and Shannon diversity ($\beta = 1.96 \times 10^{-4}$, $p < 0.01$) (Table 4). The average oxygen concentrations per depth layer (OX) and % mud content were further important explanatory variables in the model of Shannon diversity. Shannon diversity was negatively related with both OX ($\beta = -3.19 \times 10^{-3}$) and % mud content ($\beta = -0.06$). Apart from max OPD, chl-*a* concentration contributed significantly ($\beta = 7.24$, $p < 0.001$) to the model of richness.

Table 4: The average values of diversity indices of nosZ transcripts (richness [S], Shannon diversity [H'] and inverse Simpson [1/λ]) as a function of environmental variables: maximum oxygen penetration depth (max OPD), average of oxygen concentrations per depth layer (OX), % mud content (Mud) and chlorophyll *a* (Chl-*a*)

Model	formula
Richness	S = 154.40*** - 8.10 e^{-03} max OPD** + 7.24 Chl-*a* ***
Shannon diversity	H' = 1.45*** - 0.06 Mud* + 1.96e^{-04} max OPD**- 3.19e^{-03} OX *
Inverse Simpson	1/λ = 1.47*** + 2.1e^{-04} max OPD

Significance of terms (* P < 0.05, ** P < 0.01, *** P < 0.001)

4. Discussion

In the current study, we investigated the denitrifying community in *L. conchilega* aggregations at the intertidal zone by focusing on expression of the gene encoding nitrous-oxide reductase (*nosZ*), the enzyme catalysing the last step of denitrification. Microbial taxa possess divergent *nosZ* clusters (typical and atypical *nosZ*) with genes that are related yet evolutionarily distinct from each other. Here, we investigated typical *nosZ* genes, which more commonly occur in bacteria with a complete denitrification pathway (co-occurrence of denitrifying genes; Sanford *et al*., 2012; Graf *et al*., 2014) and play the major role in N_2O reduction in coastal ecosystems (Wittorf *et al.,* 2016). However, we should take into consideration that detecting the denitrifying genes in some specific bacterial taxa (Verbaendert *et al*., 2014) as well as denitrifying archaea (Sanford *et al*., 2012) may still fail using the current primers. In addition, the denitrification pathway may also be truncated leading to N_2O emission rather than N_2 when organisms lack the *nosZ* gene (Zumft, 1997; Wang *et al*., 2008) in some denitrifying organisms, which we may miss in our study. However, in recent work in the marine coastal sediments, denitrifiers with a complete pathway were identified as the dominant community (dominance of *nirS*-type community in co-occurrence with *nosZ* gene) (Wittorf *et al*., 2016).

In comparison with previous studies on functional genes for denitrification (*nirS*, *nirK* and *nosZ*) in marine sediments (Scala and Kerkhof, 1999; Falk *et al*., 2007; Zheng *et al*., 2011; Decleyre *et al*., 2015), our results show a diverse active *nosZ* community (502 *nosZ* transcripts) in the *Lanice* reef (area of *Lanice* aggregations plus zones without *Lanice* as a control). This is what can be expected in intertidal sediments where regular oxygen oscillations support a high genetic potential for denitrification (Wittorf *et al*., 2016).

4.1. *nosZ* Phylogenetic Analysis

The phylogenetic tree constructed for the *nosZ* transcripts showed that there were three major clusters belonging to the Alpha, Beta and Gamma classes of the Proteobacteria. A high percentage of the sequences (76%) belonging to *nosZ*-UATs 1, 2, 4 and 10 were closely related to the *nosZ* genes of *Pseudomonas stutzeri* (class Gammaproteobacteria, > 97% identity at the amino acid level). However, the highest phylogenetic diversity was found in the Alphaproteobacteria clade, which also included 13 of the 21 abundant *nosZ*-UATs. This is in agreement with previous

studies on the *nosZ* gene in shelf sediments (Hunter *et al*., 2006; Wittorf *et al*., 2016) and shallow coastal sediments (Mills *et al*., 2008; Gihring *et al*., 2009; Zheng *et al*., 2011) which indicate Alphaproteobacteria as the most diverse group involved in denitrification and *Pseudomonas stutzeri* as a common species in coastal sediments harboring all genes involved in a complete denitrification pathway (Lalucat *et al*., 2006). In addition, the high relative abundance of Gammaproteobacteria-related phylotypes in RNA-derived libraries suggests high levels of metabolic activity within this group (Mills *et al.,* 2008).

4.2. Horizontal and Vertical Patterns of *nosZ* Transcripts in *Lanice* Aggregations

The composition of microbial functional communities (including specific functional guilds like nitrifiers and denitrifiers) in marine sediments as well as total microbial communities differs at large horizontal scales (m, km) due to the wide range of environmental conditions such as changes in median grain size and organic matter concentration (Scala and Kerkhof, 2000; Braker *et al.,* 2001; Sapp *et al*., 2010; Yazdani Foshtomi *et al.,* 2015). At small vertical scales (cm), the community is rather uniform in spite of dramatic changes in redox gradients and differences seem to be mainly structurally related to the presence and absence of genes (Scala and Kerkhof, 2000; Braker *et al.,* 2001; Falk *et al.,* 2007). However, these patterns are not consistent, and other studies have shown no changes in microbial composition at metre scales despite differences in organic matter inputs (Decleyre *et al*., 2015) as well as significant differences in populations at small vertical scale across different redox conditions (Zheng *et al*., 2011). Our study revealed differences in the composition of *nosZ* transcripts at both horizontal (m) and vertical (cm) scales between high *Lanice* and control treatments, and between top oxic (0-0.5 cm) and deeper layers at different sediment depths (Table S4, Addendum 3). These differences in composition were mainly due to changes in the relative contributions of abundant *nosZ*-UAT to the community (Table S4, Addendum 3). The same pattern was also observed in the active *nosZ* community structure, mainly showing a decrease in the number of non-abundant and rare *nosZ*-UATs (data not shown) in the high *Lanice* treatment at a depth of 2.5-3 cm (Jaccard similarity index < 50%; Figure 6). Statistical modelling of diversity measures suggested that biogeochemical heterogeneity (O_2 concentrations and O_2 penetration depth) created by the activity of

high *Lanice* aggregations is the driving factor behind the observed differences, next to chl-*a* and mud content (%). This suggests a structuring effect of *L. conchilega* on the expression of *nosZ* genes in our study site. However, MGS and % mud were not affected by treatment nor depth, and chl-*a* was only affected by depth in the sediment. As such, there was a mainly allogenic effect of *L. conchilega* on the environment in our case study.

4.3. Allogenic Effect of *Lanice conchilega*

Lanice bio-irrigates its tube through piston-pumping activity for 1.5 min generally every 4 min, thereby transporting about 3 mmol O_2 m^{-2} d^{-1} into the sediment (Forster and Graf, 1995). Burrowing benthic animals (mainly polychaeta and Crustacean) also bioirrigate their burrows. However, the mechanism by which benthic animals propel the water current through their burrows or tubes varies considerably within and among taxonomic groups. The exact irrigation cycles therefore differ between species (Kristensen and Kostka, 2005). For example, the burrowing crustacean *Callianassa subterranea* bioirrigates its burrow less frequently for 2.6 min every 40 min (Forster and Graf, 1995). However, to determine the amount of oxygen transporting into the sediment, some other factors such as amplitude of the water flow in each ventilation event, the ventilating surface of burrow or tube and density of animals need to be considered (Forster and Graf, 1995; Christensen *et al*., 2000; Kristensen and Kostka, 2005). *Lanice* introduces oxygen-rich water in layers where oxygen is absent (Forster and Graf, 1995). This intermittent ventilation results in temporal changes in oxygen concentrations in the upper layers of the sediment (Forster and Graf, 1995), increased oxygen concentrations closer to the *Lanice* tubes and a deeper OPD (Forster and Graf, 1995; Braeckman *et al.,* 2010). Our results confirm that OPD is deeper in the presence of *Lanice*, and further show a density-dependent effect of *Lanice* bio-irrigation on the OPD: a significant increase in maximum penetration depth of oxygen in the high *Lanice* treatment (5.17 ± 0.44mm) was present compared with the low (3.69 ± 0.17mm) and control (3.30 ± 0.12mm) treatments (Figure 2; Table S2, Addendum 3). The bio-irrigating effects of *Lanice* also affected O_2 concentrations with a significantly higher oxygen content at the top sediment layer (mainly 1-4 mm; SIMPER) in high and low *Lanice* treatments compared to the control sediment (Figure 2; Table S2, Addendum 3). In addition, the periodical piston-pumping activity resulted in oxygen variations over time. We

recorded temporal changes in O_2 concentration (over a period of 30-35 min) up to a distance of 1 cm from the tube at 1.5 mm depth. Our results revealed that the oscillations were generally higher in both *Lanice* treatments compared to the control treatment (Figure 3), although there is no statistically significant difference. This could be caused by the fact that for some measurements, variations in oxygen concentration (the values of CV) were comparable with those in the control treatment. This can be explained by simultaneous piston pumping activity of adjacent animals in their tubes or by a high frequency of occurrence of this pumping activity in one animal resulting in merging individual pulses (Forster and Graf, 1995).

Although not directly measured, this bio-irrigation behaviour can have an effect on the NO_3^- concentrations in the sediment (Gilbert *et al*., 1997), which is used as a substrate for denitrification. This can be done by directly pumping NO_3^- (Gilbert *et al*., 1997) and/or by bringing O_2 to deeper layers (Forster and Graf, 1995) thereby increasing coupled nitrification-denitrification (Gilbert *et al*., 1997; Fernandes *et al*., 2012).

The different availability of NO_3^- between treatments in the low oxygen or anaerobic deeper layers probably can explain differences observed in diversity indices of *nosZ* transcripts between treatments at depth. Generally low richness and high diversity (Shannon and inverse Simpson) of *nosZ* transcripts was observed in the high *Lanice* treatment compared with the control treatment at depth and these differences were most prominent at the deepest sediment layer (2.5-3 cm) and between the high and low *Lanice* treatments as well. In a vertical sediment depth profile, nitrate coexists with oxygen in the top layer. In the deeper layers where oxygen is low or absent, the nitrate concentration is an important factor determining similarity or difference in anaerobic denitrifying communities (Liu *et al.,* 2003; Jayakumar *et al.,* 2009). As anoxic conditions continue at depth, nitrate concentrations decrease (Tiquia *et al.,* 2006) while denitrification progresses. This trend coincides with the decrease in diversity of denitrifying organisms and the rate of their activity with increasing sediment depth (Tiquia *et al.,* 2006; Middelburg and Levin, 2009; Jäntti and Hietanen, 2012). During time series incubations of oxygen minimum zone sediments, high diversity of denitrifiers (high Shannon diversity and evenness) has also been found associated with high nitrate concentrations. As denitrification proceeds, nitrate concentrations decrease, coinciding with a lower diversity in denitrifying organisms (Jayakumar *et al.,* 2009). Such trends in *nosZ* transcript diversity were also observed

in control and low *Lanice* treatments (although not significant): a decrease in *nosZ* transcript diversity with increasing depth (Figures 4 and 6) probably indicated low NO_3^- supply for denitrification at depth in control and low *Lanice* treatments. In contrast, in the high *Lanice* treatment, an increasing trend in *nosZ* transcript diversity (Shannon diversity and inverse Simpson) was observed with increasing depth (Figures 4 and 6). Also in agreement with previously mentioned studies, this can be attributed to the higher availability of NO_3^- in the high *Lanice* treatment at depth and more intensive irrigation activity in this treatment, compared to the low *Lanice* and control treatments. As mentioned earlier, the most prominent differences were at the deepest sediment layer (2.5-3 cm) between the high *Lanice* and the other two treatments. *Lanice conchilega* ventilates its tube in each pumping activity by a volume of water equivalent to 2.4 cm tube length (Forster and Graf, 1995). Therefore, the most suitable condition for denitrification (concomitant high availability of NO_3^- and little or no O_2 in the surrounding sediment) was met at 2.5-3 cm sediment depth in the high *Lanice* treatment, where high *nosZ* transcript diversity and lowest richness was observed. In the upper layers, high richness values resulted from the presence of a larger number of non-abundant and rare *nosZ* transcripts mainly belonging to the Alphaproteobacteria (data not shown). This highly diverse pool of transcripts may provide a background reservoir for suboptimal denitrification conditions increasing niche creation (Tilman *et al*., 1997; Bent and Forney, 2008; Jayakumar *et al*., 2009). In addition, it should be considered that observed differences between the high *Lanice* and the other two treatments at 2.5-3 cm (where the lowest Jaccard similarity index, less than 50%, was observed; Figure 6) are not due to adaptation of certain transcripts specific to this treatment. 93.5% (231 out of 247) and 94.7 % (234 out of 247) of *nosZ* transcripts in Hd4 were shared with Cd4 and Ld4, respectively (Figure 6). Therefore, observed differences between treatments are also due to reduction of non-abundant and rare *nosZ* transcripts in Hd4 compared with Cd4 and Ld4.

In the high *Lanice* treatment, therefore, the depth of possible denitrification is speculated to shift to the deeper layers compared to the other two treatments following an increase in transcript diversity at 2.5-3 cm. This is consistent with previous studies indicating that in bioturbated sediments denitrification occurs in deeper layers (Bertics *et al.,* 2012) and at higher rates (Gilbert *et al*., 1997; Webb and Eyre, 2004) compared to non-bioturbated or defaunated sediments.

In accordance with a previous study (Bertics *et al.,* 2012) indicating higher variations in denitrification rates in bioturbated sediment, our results on denitrifying transcript distribution showed higher variations in composition of *nosZ* transcripts in the *Lanice* treatments compared with the control; and in "depth" with increasing depth (Figure 5). This pattern can also be visualized in diversity indices (Figure 7). In bioturbated sediments, nitrogen cycle processes are most likely driven by the formation of microniches surrounding the burrow systems (Bertics *et al.,* 2012). As we sliced the cores and homogenized the sediment, the microniche formation and biogeochemical differences in the sediment were reflected as higher variations in transcript distribution in *Lanice* treatments, especially in the high *Lanice* treatment, and in "depth" with increasing depth.

4.4. Autogenic Effect of *Lanice conchilega*

Lanice conchilega is known to affect the environment and other species "autogenically" via its own physical structures (Godet *et al*., 2008) by affecting sediment characteristics (Rabaut *et al*., 2009) and trapping organic matter from the water column (De Smet *et al*., 2016).

Our results showed that measures of *nosZ*-UAT diversity (richness and Shannon diversity) were related to chl-*a* concentration and mud content (%) in the sediment (Table 4). However, MGS and % mud were not affected by treatment nor depth in consistence with the previous study in our sampling location in Boulogne-sur-Mer (De Smet *et al*., 2015), suggesting that the autogenic effect of the *Lanice* aggregations was not fully expressed at our study site. Hence, we believe that the autogenic effect of the aggregations was limited. In a separate site along the French side of the English Channel (the bay of the Mont Saint-Michel; BMSM) with higher median grain size (MGS in BMSM: 196-324 µm and in Boulogne: 185-261 µm), however, *Lanice* aggregations were shown to affect the grain size, decreasing median grain size to the finer sediments containing higher mud (De Smet *et al*., 2015). As such, autogenic effects of *L. conchilega* on the denitrifying communities cannot be excluded.

4.5. Aerobic Denitrification in Marine Sediments

The presence of *nosZ* transcripts in all treatments in the top oxic layer (Figure 7) points to the occurrence of aerobic denitrification (co-consuming O_2 and NO_3^- as electron acceptors) in the sediment of our study area, a process that has been

observed before in marine sediments (Gao *et al*., 2010). In addition, the presence of abundant *nosZ*-UATs in all depth layers indicates that denitrifiers may have the ability for co-respiration of NO_3^- and O_2 in the top oxic layer while switching to only NO_3^- respiration at deeper layers (Gao *et al*., 2010). The ability of co-respiration of nitrate and O_2 represents an adaptation of denitrifiers to the induced oxygen fluctuations and also to the rapid transition from oxic to anoxic conditions (Bergaust *et al*., 2008; Gao *et al*., 2010). The range of oxygen concentrations for aerobic denitrification differs from one organism to another (Robertson and Kuenen, 1990). Aerobic denitrification by *Pseudomonas stutzeri*, a common denitrifying species in marine sediments (Mills *et al*., 2008; Gihring *et al*., 2009; Zheng *et al*., 2011), was reported at higher rate in high oxygen conditions than observed for *Paracoccus pantotrophus* (Su *et al*., 2001). The high presence of *nosZ* types tolerant to oxygen in our samples (76% of the sequences were closely related to the *nosZ* genes of *Pseudomonas stutzeri*) may reflect a long term adaptation to oxygen oscillation in the intertidal area (Wallenstein *et al*., 2006). Microorganisms tolerate higher oxygen fluctuations in intertidal areas where they are exposed to increasing oxygen concentrations and penetration depths during low tide (Brotas *et al*., 1990; Bertics and Ziebis, 2009). Therefore, in the *Lanice* aggregations in the intertidal zone, oscillating conditions from the tidal regime as well as from *Lanice* activity affect microbial communities in sediments. However, as we submerged cores in the water in the lab, the short effects of tidal oscillating conditions were disrupted.

4.6. Importance of *Lanice conchilega* to Denitrifiers in Relation to Associated Communities in its Aggregations

Lanice aggregations have been shown to support a high diversity and abundance of macrofauna in comparison with the adjacent sediments without *Lanice* (De Smet *et al*., 2015). In our study, we investigated the effect of *L. conchilega* on denitrifying organisms in intact sediment cores from the reef. Differences observed in denitrifying organisms in the presence and absence of *Lanice* in its biogenic reefs might therefore be due not only to *Lanice* activity but also to activities of the associated community which is different from the control situation. Previous studies showed how total microbial and active functional communities and their activities were influenced by the bioturbation potential of the macrofaunal communities (Yazdani Foshtomi *et*

al., 2015). In our sampling site, the amphipods from the genus *Urothoe* and the polychaetes *Eumida sanguinea*, *Pygospio elegans*, *Heteromastus filiformis* and *Capitella sp.* were the dominant macrofaunal species after *L. conchilega* (De Smet *et al*., 2015). Some of these species, i.e. the surficial modifier *Urothoe* and the fixed tube builder *Pygospio elegans*, are small (max 1.5 cm; Degraer *et al*., 2006; Marine Species Identification Portal; Queirós *et al*., 2013) and cannot affect the ecosystem as strongly as the bio-irrigator *L. conchilega*, making also up 20-25% of total macrofaunal density in the sampling month (De Smet *et al*., 2015). Visual inspection did also not show abundance of other large organisms in our sampling cores while *Heteromastus filiformis* and *Capitella sp.* show limited movements in the sediment (Queirós *et al*., 2013). *Eumida sanguinea* is a biodiffuser (Queirós *et al*., 2013) but there is no record of the functional importance of this species for N-cycle processes. Considering the high ability of *L. conchilega* to build reef structures (Rabaut *et al*., 2007; Rabaut *et al*., 2009), the effects on the denitrifying community in our study are therefore considered to result mainly from the presence and activity of *L. conchilega*.

Acknowledgements

We are very grateful to Bart Beuselinck for the analysis of granulometric variables, to Dirk Van Gansbeke for analysis chlorophyll *a*. We are very grateful to Mehrshad Taheri and Renata Alves Mamede da Silva for sampling assistance. We also thank Nele De Meester from Marine Biology Department, Ghent University for her assistance and great thanks to Frederiek-Maarten Kerckhof and Ruben Props from Department of Biochemical and Microbial Technology, Ghent University for their useful suggestions in some statistical analyses. Financial support was given by the Ministry of Science, Research and Technology, Iran, scholarship nr 24-88200022 and by the Special Research Fund of Ghent University (BOF-UGent grant 01G01911). This paper also contributes to project G.0033.11 from the Flemish Fund for Scientific Research (FWO).

Chapter 5

General Discussion and Future Challenges

Chapter 5: General Discussion, Conclusions and Future Challenges

The overall aim of this PhD research was to investigate the effect of macrofauna (at the community and species level) on benthic microorganism-mediated N-cycle processes (nitrification and denitrification) and microbial communities (total bacterial and archaeal communities and specific functional groups, nitrifiers and denitrifiers). This was achieved by means of lab-field experiments and application of various molecular techniques (DGGE and NGS) on total microbial communities (DNA-based research) and active (RNA-based investigations) nitrifying and denitrifying communities.

In this chapter (**chapter 5**), I will discuss my own findings, derived from the experiments on the interactions between macrofaunal communities and microorganisms. I will focus on how structural and functional diversity of large benthic organisms affect benthic ecosystem processes in shallow coastal ecosystems.

In figure 1 (see below) the factors that affect and control the N-cycle processes in bioturbated sediments in coastal areas are presented as the framework in which this PhD study was performed. We examined how macrofauna affects the local environment, thereby influencing the microbial communities and their activity. In addition, we took into account the structuring effect of “global” environmental factors as well. “Global” environmental variables are considered as the environmental variables not or only to a limited extent impacted by larger benthic animals. They include water temperature, chl-*a* concentrations in water and sediment, grain size, % mud, % organic carbon and nitrogen in the sediment. Local environmental variables include the sedimentary parameters (O_2 concentration and dissolved inorganic nitrogen fluxes [DIN; NO_3^-, NO_2^- and NH_4^+]) that are influenced by macrofaunal activities at the local scales which ultimately affect the distribution and composition of microbial communities in the sediment. Macrofaunal communities were characterized by structural characteristics (density and biomass) and functional traits (mobility and sediment reworking, summarized as BPc; and bio-irrigation).

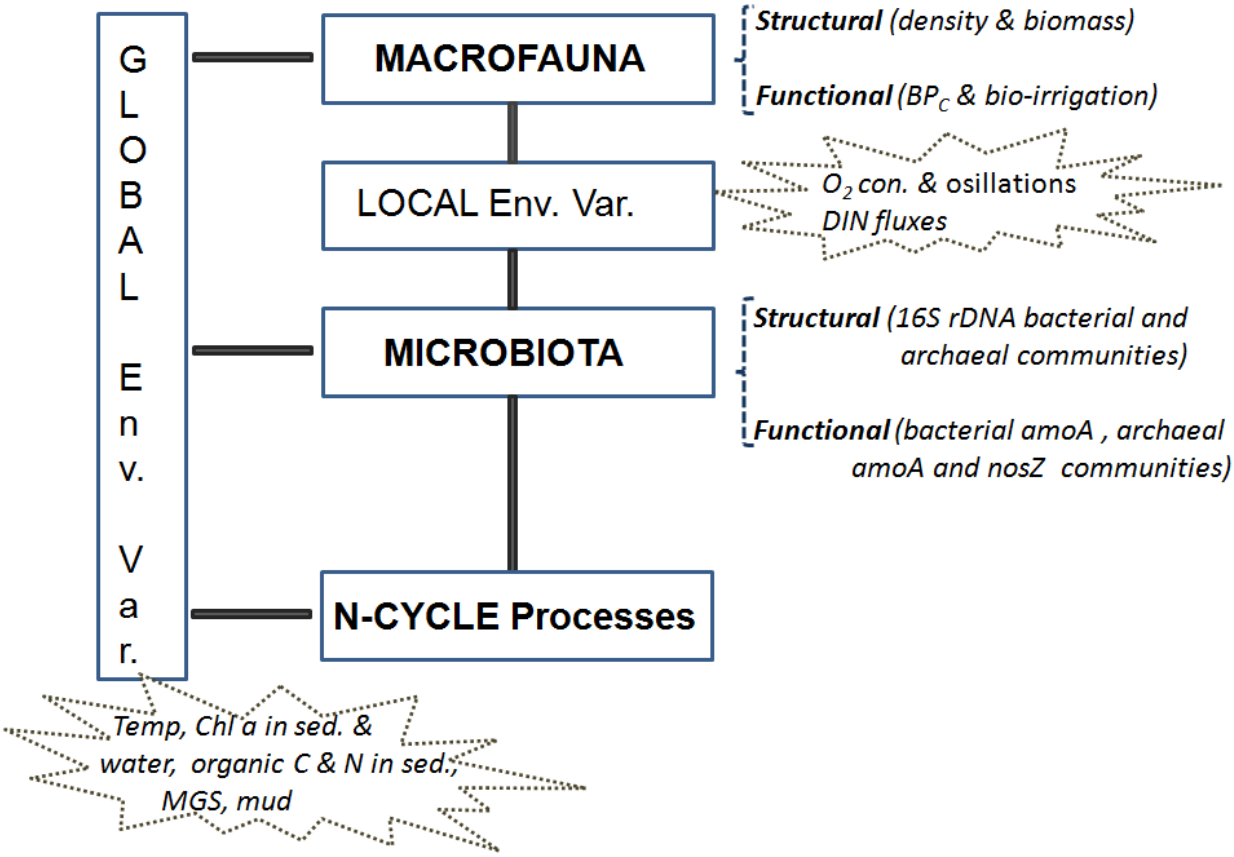


Figure 1: Schematic presentation of the investigated interaction between macrofauna, microbiota and the environment. "LOCAL Env. Var" and "GLOBAL Env. Var" indicate local and global environmental variables. Global environmental variables are considered as the environmental parameters generally not impacted by macrofauna. Measured "Local environmental variables" include: O_2 concentration and oscillation in the sediment and DIN (NO_3^-, NO_2^- and NH_4^+) fluxes across the sediment-water interface. Measured "Global environmental variables" include: water temperature, chlorophyll *a* concentration in water and sediment, median grain size (MGS), mud and organic carbon and nitrogen content in the sediment.

5.1. Biodiversity, Ecosystem Functioning, Coastal Management

Coastal zones are among the most productive areas in the world with high ecological and economic value. They offer a wide variety of valuable habitats and ecosystem services that have always attracted humans and human activities (Camarsa *et al*., 2012). Excessive human use of the coastal zone puts extensive pressure on coastal ecosystems leading to biodiversity loss and habitat destruction (Hooper *et al*., 2005; Solan, 2009; Camarsa *et al*., 2012). Growing marine biodiversity loss and/or decline in locally abundant species changes the internal functioning of ecosystems, which is reflected in the variation of the ocean's ability to provide food, maintain water quality, regulate perturbations and other essential ecosystem services to humans (Hooper *et*

al., 2005; Naeem *et al*., 2009; Pinto *et al*., 2014). Marine protected areas will not be able to overcome the current trends of loss of marine biodiversity (Mora and Sale, 2011). It is essential, therefore, to implement marine Ecosystem-Based Management (EBM) - the conservation and management of marine systems - by enhancing the protection of coastal resources whilst increasing the efficiency of their uses (McLeod *et al*., 2005; Tallis *et al*., 2010). Such EBM needs to be based on solid scientific evidence, including a detailed understanding of the link between biodiversity and ecosystem functioning.

A number of studies have attempted to link biodiversity to ecosystem functioning (Naeem *et al*., 2009; Pinto *et al*., 2014; Tilman *et al*., 2014 and references therein). Hooper *et al*. (2005) state that ecosystem functioning depends on biodiversity in terms of the *functional* characteristics of organisms present in the ecosystem and the distribution and abundance of those organisms over space and time.

In the marine environment, variations in benthic faunal composition caused by human or natural perturbations can lead to changes in dominant functional traits, with strong implications for the biogeochemical functioning of the sea-floor (Kristensen *et al*., 2014; Pinto *et al*., 2014 and references therein). The shallow estuary of OdenseFjord in Denmark experienced dramatic shifts in benthic fauna community structure and functional traits over years. During the expansion period of burrow-dwelling polychaetes, *Arenicola marina* and *Marenzelleria viridis*, and a decline in the population of *Nereis diversicolor*, elevated concentrations of NH_4^+ and NO_3^- (DIN) in the overlying water produced by the benthic system stimulated phytoplankton concentration. This altered benthic-pelagic coupling may subsequently affect the benthic biogeochemical system (Kristensen *et al*., 2014). In fact, any shift in species dominance, for example, is tightly coupled to interactions among the functional traits of the involved species and ultimately affects sediment biogeochemistry (Kristensen *et al*., 2014). In our study sites in the Belgian Part of the North Sea, fine sandy sediments are characterized by macrofaunal communities with high abundance, biomass and richness and high values for the bioturbation potential (BPc). However, the very high BPc in these sediments were mainly made up by the BPc of biodiffusers (*Echinocardium cordatum*, *Sagartia troglodytes* and *Abra alba*; Table S5, Addendum 1), which in total proportion in the community were not different from other sediment types, but reached highest biomass in these sediments. Increased abundance and biomass of surficial modifiers (mainly *Owenia fusiformis*) also

contributed to a higher BPc in this sediment type. As changes in community composition due to human activities more frequently takes place in the relative abundance of species rather than changes in absolute species richness (Wittebolle, 2009; Wohlgemuth *et al*., 2016), it seems as if any natural or anthropogenic activities leading to the biodiversity loss in BPNS would significantly influence bioturbation potential and subsequently benthic ecosystem functioning in this area. However, the question on how the functional traits of macroorganisms, through the interactions with microorganisms, affect microbial-mediated ecosystem functioning is still not fully understood. Such links have recently received significant attention and coastal and marine managers not long before started to recognize the importance of this issue for environmental management (Kristensen *et al*., 2005).

Braeckman *et al*. (2014a) provided a comprehensive overview of the potential use of ecosystem engineers (e.g. the bio-irrigating *Lanice conchilega*) in marine Ecosystem-Based Management, with a focus on North Sea management approaches. The European Marine Strategy Framework Directive (MSFD; Rice *et al*., 2010 and 2012) is considered as one of the important instruments for the management of the North Sea. Within this study, our findings especially contribute to increasing knowledge relevant for Descriptor 6 of the MSFD which refers to *Sea-Floor Integrity,* stating that "*Sea-floor integrity is at a level that ensures that the structure and functions of the ecosystems are safeguarded and benthic ecosystems, in particular, are not adversely affected"* (Rice *et al*., 2010 and 2012)*.* Within this thesis, sea-floor functioning is considered as the link between macrofauna and their role for ecosystem functioning, through the interaction with microbiota. The establishment of such link strengthens the call to include benthic microbial indicators as well as macrofaunal indicators and to construct methods integrating these indicators for assessment of sea-floor integrity in policy affairs. The present microbial indicators are mainly classified to the pollution and ecosystem health indicators in air, wastewater and marine ecosystems (e.g. Ashbolt *et al*., 2001; Stewart *et al*., 2008; Witt, 2012; Sumampouw and Risjani, 2014). For example, the use of faecal coliforms as bacterial indicator for marine pollution is accepted worldwide (Engelbrecht and Tredoux, 2004) or the use of microbial assemblages was suggested as indicators of heavy metal pollution in salt marsh sediments (Córdova-Kreylos *et al*., 2006). Although microbial assemblages provide a major contribution to global biodiversity and play a crucial role in the functioning of marine ecosystems, are largely ignored by the MSFD. However, there is a growing

consensus to consider prokaryotic assemblage in the MSFD approach and in implementing some MSFD Descriptors with microbial indicators (Caruso *et al*., 2015). Therefore, knowledge improvements and methodological challenges appear to be needed in this context in order to improve our understanding of the role of microbes in ecosystem functioning (Cochrane *et al*., 2010; Schroeder, 2010).

A recent study (Caruso *et al*., 2015) reviewed several contexts for which the monitoring of prokaryotic assemblage would implement the assessment of quality of marine environments. This extensive literature survey resulted in the identification of some suitable microbial indicators of marine ecosystem status. However, the evaluation of the strengths and weaknesses of these prokaryotic indicators will benefit of further validation. In addition, these microbial indicators did not take into account microbial functional guilds while the analysis of microbial communities based on functional genes gives information about dynamics of processes occurring. Thus, we recommend the inclusion of abundance and diversity of the microbial functional genes to develop microbial indicators in future implementations of the MSFD. Considering Descriptors 6 (Sea-floor integrity), in coastal systems due to strong heterogeneity of sediment characteristics, rapid and patchy physicochemical changes and gradients, it is hard to be predicted the environmental status. Microbial indicators can represent useful tools when incorporated in multimetric indexes merging physical, chemical and other biological parameters (Caruso *et al*., 2015).

Development of these indicators defines future directions of research in EBM and public policy. However, no single specific indicator may suite or logistically feasible to meet the needs of all countries and in all regional seas (Rice *et al*., 2012; Nelson, 2013).

5.2. Microorganisms and their Function

Microorganisms are vital in mediating the earth's biogeochemical cycles (Graham *et al*., 2016). However, the relationship between microbial community structure and particular ecosystem processes might not be reflected in total community diversity but rather in the diversity of narrow functional guilds (Graham *et al*., 2016). For example, although the total bacteria investigated comprised denitrifiers, we found no significant relationship between diversity aspects of the total bacterial community and the estimated denitrification rates in our spatio-temporal study on the BPNS (**Chapter 3**). Our results showed the link of microbial biodiversity and ecosystem functioning,

particularly between diversity aspects of *amoA* gene carrying communities of active nitrifiers (AOA and β-AOB) and nitrification rates in the late summer. However, among different sediment types in September, β-AOB richness and diversity followed the same pattern as nitrification rates, with the highest values noted in fine sediments while AOA diversity indices did not show such spatial differences among sediment types (**Chapter 3**). Apart from the smaller cell volumes (10 to 100 times) in most AOA (Hatzenpichler, 2012), which is thought to be responsible for lower rates of ammonia oxidation in AOA compared to AOB (Hatzenpichler, 2012; Veuger *et al*., 2013), biochemical adaptations of AOA are thought to contribute to their wide distribution compared to AOB (Hatzenpichler, 2012). Our results in the BPNS revealed that the bacterial (total and β-AOB) community generally showed more spatio-temporal variation than the archaeal (total and AOA) community as sedimentation of organic matter and the subsequent changes in the environment had a stronger impact on bacteria (total and β-AOB) community composition and diversity indices. In addition, fine sandy sediments in our study display a high macrofaunal abundance, biomass and functional diversity (high BPc) (**Chapter 2**), which transfer water and solutes into the sediment. There are reports indicating the ability of AOA to live in low ammonia and oxygen concentration due to high affinities for the substrate (Abell *et al*., 2010; Park *et al*., 2010; Hatzenpichler, 2012). Furthermore, AOA exhibit a variety of metabolic pathways compared to AOB. They are able to obtain energy through different autotrophic carbon-fixing pathways (Hatzenpichler, 2012) as well as by heterotrophic metabolism through oxidizing organic matter (Mosier, 2011; Qin *et al*., 2014). In our study area, both AOA and β-AOB seem to play roles in sedimentary nitrification in fine sediments in September (**Chapter 3**, Table 2) but differentiation of their individual contributions was not investigated.

Correlations between nitrifying and denitrifying communities and ecosystem processes have been observed within numerous studies (e.g. Smith *et al*., 2007; Wankel *et al*., 2011). This information is mainly based on diversity and/or abundance data of a particular microbial community (e.g. Wankel *et al*., 2011). However, the uncoupled relation has also been reported (Zheng *et al*., 2014; Hou *et al*., 2013). For example, in a study in the intertidal sediments of the Yangtze Estuary, where bacterial *amoA* genes were more abundant than archaeal *amoA* genes, potential nitrification rates were not correlated with *amoA* gene abundance of AOB, but with AOA's (Zheng *et al.,* 2014). Such lack of correlation between functional gene

abundance and the associated activity was also reported in other studies (Wuchter *et al*., 2006; Caffrey *et al*., 2007; Bernhard *et al.*, 2010; Hou *et al*., 2013). Considering the fact that biogeochemical processes are mediated by microorganisms, this lack of correlation between abundance of microorganisms and the rate of their functions can be due to methodological artefacts (Bernhard *et al.*, 2010) in microbial techniques, targeting DNA rather than RNA as a template in cultivation-independent studies of microbial population (Spring *et al*., 2000; Philippot and Hallin, 2005), the effect of other controlling factors on the ultimate expression of a gene and activities or limitations in accuracy of the method applied in measuring the rate of microbial processes. Still other factors might be also involved.

Here, we explain briefly these different possible causes for the lack of correlation between microorganisms and their functionality, and how our research strategy avoided some of these issues. In general, methodological considerations are discussed below.

5.2.1. Methodological Considerations

Methodological artefacts (Bernhard *et al.*, 2010) in microbial techniques may include PCR bias (Acinas *et al*., 2005; Aird *et al*., 2011) and underestimation of microbial diversity due to the limitation of the primer pair (Yu *et al*., 2008; Liu *et al*., 2013b). For example, it seems the currently widely used primers for denitrification pathway often fail to detect Gram-positive denitrifiers (Verbaendert *et al*., 2014). In our study in **Chapter 4**, we also aimed to investigate bacterial and archaeal *amoA* genes involved in nitrification but the bacterial *amoA* gene failed to amplify. The optimal primer melting temperature for custom MiSeq primers is 65°C (Personal communication with the Genomics Core). However, it was less for the custom primers for this library which may have contributed to the failed amplification (β-AOB primer sets: amoA-1F (forward primer), 5'-GGGGHTTYTACTGGTGGT-3' and amoAr-new (reverse primer), 5'-CCCCTCBGSAAAVCCTTCTTC-3').

A weak point of many cultivation-independent population studies is the use of DNA as a template. It has been argued that genomic DNA of dead cells (extracellular DNA) can be very stable, surviving for long periods of time in the environment (Spring *et al*., 2000). Extracellular DNA concentrations in aquatic sediments are 3 to 4 orders of magnitude greater than those in the water column and account for a large fraction of the total DNA in the sediment, compared with intracellular DNA concentrations

(Corinaldesi *et al*., 2005). Therefore, we removed extracellular DNA from sediment samples in our research on total bacterial and archaeal communities (**Chapter 3**), which allowed for studying living cells (dormant and active cells) in the sediment. Considering dormant cells account for a large fraction of living cells in marine sediments (Luna *et al*., 2002), the use of extracted RNA as PCR template (see **Chapters 3** and **4**) gives a better overview of only the active microbial population by avoiding the background of dead (extracellular DNA) and inactive (dormant) cells (Spring *et al*., 2000). Philippot and Hallin (2005), in a review study on three denitrifying functional genes, revealed that the expression of genes (RNA-based methods) and the detection of enzymes (protein-based methods) allows for investigating the direct link between microbial biodiversity and their activity. Our study confirmed these findings and even showed that functional gene transcripts allows for a deeper or better understanding of benthic processes, as we could show that the links between the microbial organisms and the particular ecosystem processes are modulated by the macrofauna. To our knowledge, this is a novel study on the mechanisms that control the N-cycle in shallow coastal sediments integrating macrofauna, microbial functional gene transcripts and processes. Therefore, our approach offers a good way forward to conduct such integrative research on the mechanisms controlling ecosystem functioning in the productive coastal areas.

Although RNA-based methods allow for the direct link between active microorganisms and their activities compared with DNA-based methods, the half-lives of mRNAs in the cell are short; in the hour range in eukaryotes and in a few minutes in prokaryotes (Rauhut and Klug, 1999). In **Chapter 4**, we assessed the active microbial community and revealed a direct link between the effect of bio-irrigation activity of *L. conchilega* and the diversity and distribution of denitrifying organisms. We consider that the short half-lives of a few minutes in mRNAs of microorganisms (Rauhut and Klug, 1999) probably do not hamper investigating this link because the duration of pumping activity and resting time in *L. conchilega* is also in minute range (1.5 min active and 2.5 min quiescent period). In addition, degradation of transcripts in prokaryotic cells can even take longer (Geets *et al*., 2006). For example, a half-life of 13 minutes was reported in a study monitoring the kinetics of *nirS* expression in *Pseudomonas stutzeri* (Hartig and Zumft, 1999) or the total degradation of *amo* and *hao* transcripts was reported within 8 h of the depletion of ammonia in *Nitrosomonas europaea* cells (Geets *et al*., 2006).

The mRNA/DNA ratio can be also interesting as it gives us more information of the number of active cells in the total living cells in an environment (Yu *et al*., 2014).

It has to be noted that the transcription of DNA into mRNA does not under all circumstances result in the expression of a functional protein (Spring *et al*., 2000) and subsequently an activity. There can be substantial post-transcriptional and protein-level modification and/or environmental factors may ultimately control enzyme activity (Philippot and Hallin, 2005; Jones *et al*., 2011; Smith *et al*., 2015). Therefore, proteins may be better candidate than mRNA to relate microbial community and function. However, the stability and half-life of enzymes is also important (Philippot and Hallin, 2005). In addition, it seems that environmental variables can interact with the genetic potential in controlling the final rate of processes (Caffrey *et al*., 2007; Graham *et al*., 2016). Smith *et al*. (2015) reported a spatial correlation between transcript abundances of *nirS* and *nrfA* and their associated processes (respectively denitrification and DNRA) along estuarine sediments, but temporal relations were not observed. In a different study showing the effect of environmental factors on the ultimate control of activity in microbiota (Jones *et al*., 2011), denitrification rates in 14 closely related *Bacillus* soil isolates at pH 6 and 7 were found to be substantially more variable than genotypic variations. Hence, this may reflect the need of applying activity-correlated analyses in microbial studies (Bernhard *et al*., 2010; Hatzenpichler, 2012).

The approaches applied to assess the microbial diversity also require careful consideration. High Throughput Sequencing (HTS) methods were shown a promising tool for detecting the microorganisms which failed to be detected by other culture-independent techniques such as Denaturating Gradient Gel Electrophoresis (DGGE) (Samarajeewa *et al*., 2015). We applied both these techniques in **Chapters 3** and **4.** However, we cannot make a comparison between them based on our data as we applied them for different groups of organisms, respectively, nitrifying and denitrifying organisms while denitrifiers are known as diverse organisms (Canfield *et al*., 2005). DGGE analysis has been a commonly used technique to characterize microbial community composition and diversity (Frossard *et al*., 2012; Graue *et al*., 2012; Tang *et al*., 2013; Tiodjio *et al*., 2014; von Scheibner *et al*., 2014). However, it has also some methodological limitations such as co-migration of fragments (Buchholz-Cleven *et al*., 1997) and/or detection of a certain sequence in different well-separated bands (Bowman *et al*., 2003). Therefore, identification of the species behind specific bands

yet requires laborious procedure of cutting, cloning and sequencing of the bands. Instead, HTS has the advantages of short analysis time and deep genome sequencing (e.g. Chao *et al*., 2015; Kircher, 2011; Reuter *et al*., 2015). Nevertheless, some recent reports indicate that in comparison with HTS, DGGE-cloning is not obsolete approach (Kraková *et al*., 2016). In addition, despite advantages of HTS techniques, they also introduce errors. Overall error rate of Illumina platform, which was applied in **Chapter 4,** is the lowest of all commonly used platforms (such as 454 pyrosequencing and Ion Torrent) (Schirmer *et al*., 2015; Laehnemann *et al*., 2016). However, we still tried to reduce the errors by removing low-quality reads (scores <25) from the output as well as using FrameBot included in the Fungene pipeline (http://fungene.cme.msu.edu/) to correct for insertion and deletion errors (Fish *et al*., 2013).

Another methodological option would have been the simultaneous study of all genes present on the genome (metagenomics) rather than investigation of a single gene (Thomas *et al*., 2012). In **Chapter 4**, we investigated effects of *L. conchilage* on the expression of the typical *nosZ* gene, which based on previous studies more commonly occurs in bacteria with a complete denitrification pathway (co-occurance of the genes involved in dinitrification pathway; Sanford *et al*., 2012; Graf *et al*., 2014). Metatranscriptomics can provide a direct genetic analysis of transcripts of all denitrifying genes in a community and thus would give a much broader assessment of the effects of *Lanice* bio-irrigation on the denitrifying community in the study area. However, differentiation of denitrifiers with the truncated pathway (N_2O as the end product, Zumft, 1997; Wang *et al*., 2008) from denitrifiers with the complete pathway and also detection of non-denitrifying organisms only capable of N_2O reduction (Sanford *et al*., 2012) is not possible with this approach.

One of the crucial steps in sequence data analyses is the data normalization to correct for technical bias related to read number variations among the samples (Dillies *et al*., 2012). There is currently no consensus on the most appropriate method. For example, rarefying data based on the sample with the lowest read count was not supported by McMurdie and Homes (2014) because rarefied counts represent only a small fraction of the original data, increasing the error (see other reasons in McMurdie and Homes, 2014). In **Chapter 4**, because of the difference by an order of magnitude in our samples' read numbers, the samples for the data composition were analyzed on non-rarefied data (containing all *nosZ*-UATs) following

the methods used by McMurdie and Holmes, (2014) and Props *et al*., (2016) (see **Chapter 4**, data analyses). However, because rarefaction is still used in data normalization (e.g. Derycke *et al*., 2016), we made a comparison between rarefied and non-rarefied data. Data was rarefied in two replicates based on the lowest number of reads (1022). The main test results of PERMANOVA were found consistent in rarefied and non-rarefied data indicating the significant single effect of treatment and depth (Table 1; Figure 2).

Table 1: Results from PERMANOVA analysis main tests for differences in composition of *nosZ* transcripts based on Generalized UniFrac distances (α= 0.5) among treatments (high and low *Lanice* densities and control) and depths (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm). A comparison between using non-rarefied (containing all *nosZ*-UATs) but normalized dataset (described in chapter 4) and the rarefied dataset. The dataset was rarefied randomly in two replicates based on the lowest number of reads (1022). The interaction term (treatment x depth) was not significant in all three tests and therefore the results of single factors are shown.

PERMANOVA-main test	*factor*	df_{term}	*Pseudo-F*	*P value*
***All nosZ-UATs* (non-rarefied but normalized dataset)**	treatment	2	2.82	**0.026**
	depth	3	2.68	**0.012**
Rarefied dataset (replicate 1)	treatment	2	2.34	**0.039**
	depth	3	2.57	**0.021**
Rarefied dataset (replicate 2)	treatment	2	2.53	**0.037**
	depth	3	2.22	**0.041**

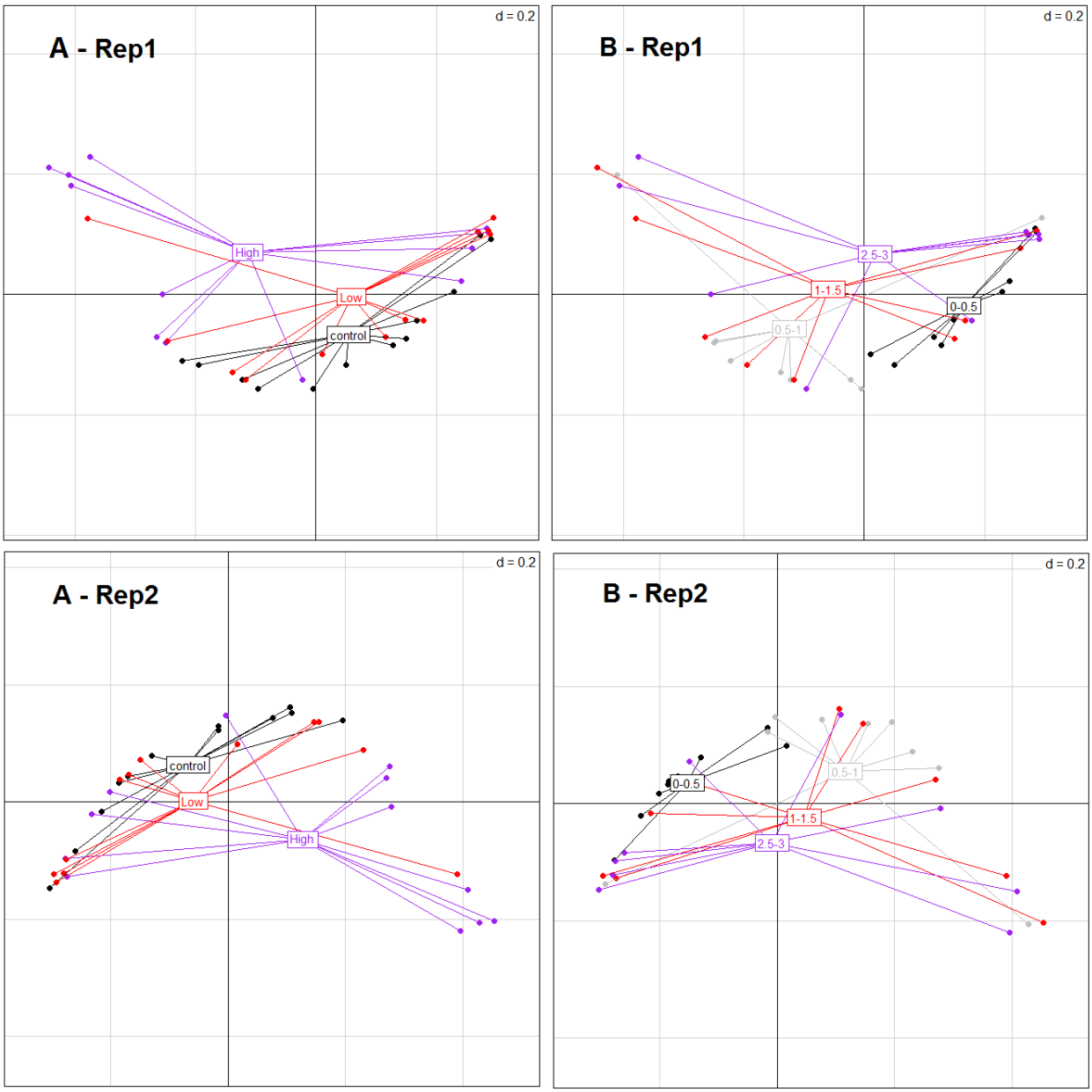


Figure 2: Principal coordinates analysis plot (PCoA) based on generalized UniFrac distances on the rarefied data (A: per treatment; B: per depth). The dataset was rarefied randomly in two replicates (Rep1 and Rep2: replicates 1 and 2) based on the lowest number of reads (1022). Each point represents a sample. Three treatments (High: high *Lanice* treatment, Low: low *Lanice* treatment, Control) and four depth layers (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm) are shown.

Apart from microbial methodological considerations, limitations in accuracy of the method applied in measuring the rates of microbial processes need to be considered. Several methods are in use to estimate N-cycle process rates (nitrification and denitrification) in the environment (Hou *et al*., 2013). These methods include generally (1) isotope-labeling methods; (2) adding a high concentration of substrate

and measuring the quantity of products afterwards; (3) blockage of the process using inhibitors and measuring the accumulated substances (inhibition techniques) (Fütterer, 2006; Wankel *et al*., 2011). However, these methods have limitations and disadvantages which reduce the accuracy in the process rate measurements (Jones *et al*., 1984; Fütterer, 2006). In **Chapters 2** and **3**, the rates of processes (denitrification, nitrification and N-mineralization) were estimated using an alternative technique, "Mass Budget Modeling" based on fluxes and modeling (Braeckman *et al*., 2010). This modeling (**Chapter 2**, see Text S1) was done by constructing an integrated mass budget of oxygen, nitrate and ammonium over the entire sediment column as a function of the source and sink processes (Soetaert *et al*., 2001). To contrast with the techniques of adding labeled substrates or adding excessive substrates, our estimations may be closer to the real rates in nature, particularly as we used intact sediment cores.

5.3. The Link Between Macrofaunal Activities, Microbial Biodiversity and Ecosystem Functioning in Subtidal and Intertidal Marine Sediments

Coastal sediments are typically inhabited by rich macrofaunal communities, which can exert an important control on sedimentary biogeochemical cycling (Aller, 1988; Meysman *et al*., 2006). Our results revealed that apart from the effect of global environmental variables (water and sediment chl-*a* concentration, water temperature, organic C:N ratio in the sediment and grain size in space and time) on the link of microbial biodiversity and N-cycle processes in intertidal and subtidal sediments, this link was modulated by macrofauna through their effects on local environmental variables (DIN fluxes, O_2 concentration and oscillations). Macrofauna affect the environment mainly through their allogenic effects by bioturbation (particle mixing) and bio-irrigation (solute transfer) (Solan *et al.,* 2004): *(i)* transferring water and solutes from the overlying water (Kristensen, 1988; Kristensen and Kostka, 2004) into the deeper sediment layers; *(ii)* extending the surface area available for diffusive solute exchange across the sediment-water interface (Howe *et al.,* 2004, Nizzoli *et al.,* 2007); *(iii)* creating microniches within sediments and the burrow wall (Bertics and Ziebis, 2009).

Our results further revealed the effect of macrofauna at the community and single species level (e.g. bio-irrigating polychaete, *L. conchilega*) on total microbial (bacteria

and archaea) communities and functional gene expression (*amoA*-carrying bacterial and archaeal nitrifiers and *nosZ*-carrying denitrifiers).

In the single species level such as *Lanice conchilega*, *Lanice* introduces oxygen-rich water in layers where oxygen is absent by bio-irrigating its tube (Forster and Graf, 1995) and also affects O_2 concentration over short-time intervals through periodical piston-pumping activity and intermittent ventilation (Forster and Graf, 1995; our study). We also found a density-dependent effect of *Lanice* bio-irrigation on the oxygen penetration depth within the *Lanice* reef. Increasing oxygen content and bringing oxygen deeper in the sediment by macrofauna enhance nitrification rates in the sediment which fuels denitrification (Howe *et al*., 2004). In addition, macrofaunal activity increases the surface for coupled nitrification-denitrification in the burrow wall (Howe *et al*., 2004; Birchenough *et al*., 2012) and probably in the sediment along the tube in tube building polychaetes (Kristensen and Kostka, 2005) such as *L. conchilega*. Under conditions of O_2 oscillation (such as the result of *Lanice* intermittent ventilation activity), nitrifying and denitrifying bacteria react to oxygen and nitrate in the environment by coordinating their respective activities (Gao *et al*., 2010) and such conditions support higher abundance of denitrifying genes (*nirS* and *nosZ*) compared to oxic or anoxic conditions alone (Wittorf *et al*., 2016). The bio-irrigating behaviour of macrofauna (e.g. *L. conchilega*) can also affect directly other solute exchanges such as NO_3^- (Gilbert *et al*., 1997), which is used as a substrate for denitrification.

Lanice conchilega manifests both autogenic and allogenic ecosystem engineering characteristics (Godet *et al*., 2008). However, the effect of macrofauna on nitrogen cycling processes exclusively via autogenic effects (i.e. through their own physical structures) has not been reported yet (Braeckman *et al*., 2014a). Although our results showed measures of *nosZ* diversity indices were related to chl-*a* concentration and % mud in the sediment (**Chapter 4**), this was not affected by granulometric differences and differences in chl-*a* concentration resulted from autogenic activities of *Lanice* in our study site.

Previous studies of the effects of macrofauna on biogeochemical processes involved mainly manipulative experiments (e.g. introducing of organisms to homogenised and/or defaunated sediments) in laboratory contexts (Braeckman *et al*., 2010; Laverock *et al*., 2010; Gilbertson *et al*., 2012; Stauffert *et al*., 2014) and the effect of single species of large burrowing macrofauna on the biogeochemical processes

(Bertics and Ziebis, 2009; Bertics *et al*., 2012; Laverock *et al*., 2014). In addition, our knowledge on interactions between macrofaunal and microbial communities is still very limited (Laverock *et al*., 2014). The impact of macrofaunal reworking activities on microbial communities was mainly assessed at the level of total bacterial community (Papaspyrou *et al*., 2005 and 2006; Bertics and Ziebis, 2009; Laverock *et al*., 2010; Sapp *et al*., 2010) while less attention was paid to the archaeal community (Sapp *et al*., 2010; Stauffert *et al*., 2014) and specific functional gene groups, such as nitrifiers and denitrifiers (Gilbertson *et al*., 2012; Laverock *et al*., 2014). The investigation of metabolically active microorganisms at RNA or protein level has also been rarely performed (technically challenging). In addition, an integrated study investigating simultaneously macrofauna, microbes and N-cycling processes (i.a. as done in this thesis, **Chapter 3**) was missing so far. We used the bioturbation potential of a community (BPc) as well as density and biomass to investigate the impact of macrofaunal communities. BPc is an index based on the classification of invertebrate taxa into discrete functional groups (Solan *et al.,* 2004; Birchenough *et al.,* 2012; Queirós *et al.,* 2013). It is rather the functional biodiversity than taxonomic biodiversity that matters for benthic ecosystem functioning (Emmerson and Raffaelli 2000; Leno *et al*., 2006; Cardinale *et al*., 2012). The functional biodiversity can be estimated by calculating the contribution of each species using e.g. bioturbation potential of each species (BPi), which is at the basis of the bioturbation potential of a community (BPc) (Solan *et al.,* 2004; Queirós *et al.,* 2013). Therefore, this index reflects the capacity of macrofaunal communities to mix sediments (Solan *et al.,* 2004; Queirós *et al*., 2013) and is related to the apparent redox potential discontinuity depth (aRPD) (Birchenough *et al.,* 2011), which is known as biogenic mixing depth as well (Solan *et al*., 2004; Birchenough *et al.,* 2011). Our results (**Chapters 2 and 3**) showed that macrofaunal activities (BPc) and/or abundance indeed were related to diversity indices of metabolically active β-AOB and AOA and also to the rates of nitrification in space and time. The models also indicated the significant importance of macrofaunal activities (BPc) to modulate the link between N-mineralization and total bacterial community affecting both bacteria and their mineralization activities. In addition, BPc was retained in our models for denitrification reflecting an increase in denitrification rates by maybe providing directly the nitrate from the overlying water (Nizzoli *et al.,* 2007) and/or an increase in the coupled nitrification-denitrification processes especially in fine sands characterized by a rich BPc (**Chapters 2** and **3**).

However, BPc also has some drawbacks. BPc cannot predict all bioturbation attributes: while this index successfully predicts bioturbation distance (average distance travelled by sediment particles), it is not related to the other attributes such as bioturbation depth, activity and biodiffusive transport (Queirós *et al.,* 2015). Ventilation of the burrow (bio-irrigation) is a mechanism which is also not accounted for in BPc. Bio-irrigating animals such as *L. conchilega* have a low score for M (mobility) and R (sediment reworking mode). However, bio-irrigation activity was shown to have indeed an important effect on benthic biogeochemical processes (Braeckman *et al*., 2010; Woodin *et al*., 2016). In **Chapter 4**, we also found density-dependent effects of the bio-irrigation activity of *L. conchilega* on the sediment heterogeneity and the expression of the *nosZ* gene, involved in the final step of the denitrification process.

Interaction between species is also not accounted for in BPc as BPc is a summation of single species effects, biomass, and abundance (Solan *et al*., 2004). However, despite the mentioned drawbacks of BPc, this index appears to explain the variability in measured fluxes and microbial processes much better than macrobenthic density or biomass alone (see **Chapter 2**; Van Colen *et al*., 2012). For future investigations, however, we suggest to develop better descriptions of bioturbation potential or extend the BPc index with a measure for bio-irrigation in the European Marine Strategy Framework Directive (MSFD).

5.4. Conclusions

This PhD increases the knowledge of factors controlling the microbial-mediated N-cycle processes in shallow coastal sediments and provides an important step in filling the existing gap how the functional traits of macroorganisms, through the interactions with microorganisms, affect microbial-mediated ecosystem functioning in coastal benthic ecosystems. We showed that the links between microorganisms and benthic biogeochemical N-cycle processes are affected by the density and/or activity of the macrofaunal communities causing heterogeneity in sedimentary environmental variables over local spatial and temporal scales as well as by changes in global environmental variables. We investigated this link in both intertidal and subtidal sediments (muddy, fine sandy and permeable sands) of shallow coastal areas and were able to relate the integrated effect of total macrofaunal activity and also bio-irrigation by a single macrofaunal species on sediment heterogeneity with functional

aspects of microbial communities. We showed that functional gene transcriptions (*amoA*-carrying bacterial and archaeal nitrifiers and *nosZ*-carrying denitrifiers) reveals better insights in the macrofauna – microbe – particular process interactions compared to the total microbial community (16S rDNA gene) investigation. Our results also indicated the effects of macrofauna on this link at both horizontal large scales (km and m) and small (cm) vertical scales.

Lanice conchilega in high densities affects denitrifying organisms causing an increase in the relative abundance of *nosZ* transcripts at anoxic depth layers in the sediment, which may stimulate the rates of denitrification at depth. They do this via their allogenic effects by increasing the oxygen content at the top sediment layer, increasing oxygen penetration depth and stimulating oscillation in oxygen concentrations, which all lead to increasing coupled nitrification-denitrification at depth. Bio-irrigation behaviour can further affect the exchanges of other solutes across the sediment-water interface, including NO_3^- which is a substrate for denitrification.

In our study area, the Belgian Part of the North Sea, the links between microbial biodiversity and ecosystem functioning in sediments in space and time are influenced by the annual spring phytoplankton bloom (water and sediment chl-*a* concentration), water temperature, sediment organic C:N ratio and grain size as well as larger fauna density and/or functional diversity. The importance of macrofauna depends on the sediment type. Fine sandy sediments are characterized by highest macrofauna densities, biomass and bioturbation potential (BPc). While nitrification and denitrification rates in permeable and muddy sediments were low, in the fine sandy sediments denitrification rates were high throughout the year. During summer, nitrification rates in fine sandy sediments were also higher than in muddy and permeable sediments. This was concomitant with the highest richness of metabolically active β-AOB and AOA in September. In fine sediments, AOA and β-AOB seem both to play roles in sedimentary nitrification in September but differentiation of their individual contributions was not investigated. Generally, our results revealed that bacteria (total and β-AOB) showed more spatio-temporal variation than archaea (total and AOA) as sedimentation of organic matter and the subsequent changes in the environment had a stronger impact on their community composition and diversity indices.

5.5. Future Challenges

Previous studies showed that the capacity of the macrofaunal communities to mix sediments is reflected in the location of the apparent redox potential discontinuity depth (aRPD) (Birchenough *et al*., 2011). However, there is also a need to investigate how these organisms affect the redox condition and the elements used as electron acceptors (NO_3^-, Mn^{4+}, Fe^{3+}, and SO_4^{2-}) for different functional microbial groups in the anoxic sediment at deeper layers in the sediment. This actually leads us to a comprehensive study of anaerobic microbial processes in bioturbated sediments. However, this is challenging due to complex recycling of redox elements (Fernandes *et al*., 2015) and investigation of different microbial respiration pathways. This PhD research was a good start linking macrofauna, metabolically active microorganisms and processes. However, to expand this link investigating different microbial respiration pathways requires additional studies.

As the oceans gradually become warmer and more acidified, an increasing number of studies test the effects of these changes on marine organisms (Wernberg *et al*., 2012; Kroeker *et al*., 2013). There is also evidence that the benthic biogeochemical cycling is affected by ocean acidification (Braeckman *et al.,* 2014b). However, the same effect was not reported in different coastal sediments (Gazeau *et al.,* 2014). Ocean acidification might have limited impacts on N-cycle processes in Arctic coastal sediments (Gazeau *et al.,* 2014). Hence, there is a large need to investigate the effect of these global changes on the microbial biodiversity and biogeochemical process link on the one hand and to do a comprehensive study integrating microbial communities and their activity with macroorganisms, on the other hand. This impact can be studied using a laboratory experiment with the null hypothesis that a decrease of 0.3 in pH after 14 days may affect the microbial community as a decrease in benthic nitrification rates after such period of time was observed before (Braeckman *et al.,* 2014b).

Considering the importance of coastal management, in this PhD thesis, we showed the importance of functional biodiversity of macrofauna and their role for ecosystem functioning, through the interaction with microbiota. But, we also clearly indicated the key role of bio-irrigation on the microbiota-ecosystem functioning link, which strengthens the call for a further development of an index, based in BPc, but integrating bio-irrigation. This can be considered especially important for the development of indicators relevant for Descriptor 6 of the MSFD, which refers to *Sea-*

Floor Integrity (safeguarding of structure and functions of the ecosystems), for the management of the North Sea. The establishment of the macrofauna – microbiota - process link also strengthens the call to include benthic microbial indicators.

Addenda

Addendum 1. Appendices to Chapter 2

Variable Importance of Macrofaunal Functional Biodiversity for Biogeochemical Cycling in Temperate Coastal Sediments

Table S1: Overview of performed experiments and *in situ* temperature

Experiment	*Date*	*In situ temperature (°C)*
St. 130, 145, 700, 120, 780, 230, 710	20/02/2011	6
St. 130, 145, 700, 120, 780, 230, 710	17/03/2011	6
St. 130, 145, 700, 120, 780, 230, 710	18/04/2011	12
St. 130, 145, 700, 120, 780, 230, 710	23/05/2011	16
St. 330, 215, ZG02	06/06/2011	16
St. 130, 145, 700, 120, 780, 230, 710	21/06/2011	16
St. 330, 215, ZG02	02/08/2011	18
St. 130, 145, 700, 120, 780, 230, 710	17/08/2011	18
St. 130, 145, 700, 120, 780, 230, 710	19/09/2011	16
St. 130, 780, 330	17/10/2011	16

Supplementary Text S1: Mass Budget Modelling

Oxygen is either directly consumed to oxidize organic carbon (oxic mineralization - OxicMin), or indirectly through the re-oxidation of reduced substances formed by anoxic mineralization (AnoxicMin). Part of the reduced substances remain buried in the sediment (pSolidDepo) and are not re-oxidized. We assume that one mole of oxygen is consumed for each mole of carbon originally mineralized (respiratory quotient = 1). Ammonium results from the mineralization of organic nitrogen (Nmineralization), whilst it is consumed by nitrification as ammonia, which requires two moles of oxygen for each mole of ammonia. Denitrification consumes 0.8 moles of NO_3 for one mole of carbon denitrified. Oxygen, nitrate, and ammonium are further exchanged through the sediment-water interface (O_2influx, NO_xinflux, NH_xinflux), while the lower boundary of the sediment is assumed to be a no flux boundary.

The resulting balances are summarized below.

$$\frac{dO_2}{dt} = O_2influx - OxicMin - AnoxicMin * (1 - pSolidDepo) - Nitrification * 2$$
$$\frac{dNH_x}{dt} = NH_x\, influx + Nmineralization - Nitrification$$
$$\frac{dNO_x}{dt} = NO_x influx + Nitrification - Denitrification * 0.8$$

The fluxes across the sediment-water interface (O_2inFlux, NH_xinFlux, NO_xinFlux) were estimated during the incubation experiments, while the rate of change of oxygen, nitrate and ammonium fluxes was assumed to be zero (geochemical steady state reached one week after introduction of animals). With six remaining unknowns (OxicMin, AnoxicMin, Nmineralization, Nitrification, Denitrification) and only three equations, the mass balance model is not closed. We therefore make the assumption that the burial of anoxic substances can be ignored (pSolidDepo = 0). This allows combining the oxic and anoxic mineralization into one quantity (OxicAnoxicMin). The extra equation to balance the model then imposes a relationship between nitrogen and carbon mineralization, using the average N:C ratio as measured in the surface sediment of each station and each month. The mass balances then become:

$$0 = O_2influx - OxicAnoxicMin - Nitrification * 2$$
$$0 = NH_x\, influx + Nmineralization - Nitrification$$
$$0 = NO_x\, influx + Nitrification - Denitrification * 0.8$$

Where:

$$Nmineralisation = (OxicAnoxicMin + Denitrification) * N{:}C\ ratio$$

With three equations and three unknowns this makes the model evenly determined. These three equations can be solved for the three unmeasured quantities (OxicAnoxicMin, Nitrification, Denitrification). The mass balance modeling was performed using package limSolve (Soetaert and others 2009) available in the open source software R (R development team, 2008).

Table S2: NO_x and NH_x concentrations (µmol l^{-1}) in the field and in the lab for each station over time

Station	*Sampling Date*	*Field NOx*		*Lab NOx*		*Field NHx*		*Lab NHx*	
		mean	*se*	*mean*	*se*	*mean*	*se*	*Mean*	se
120	21/02/2011	16.12	0.27	230.98	0.74	3.14	1.31	4.21	0.51
120	17/03/2011	0.81	0.42	226.23	1.89	0.08	0.08	3.60	1.39
120	18/04/2011	19.10	0.43	247.61	0.81	0.42	0.41	2.77	0.07
120	23/05/2011	0.93	0.30	215.52	0.52	4.36	0.55	4.70	0.45
120	21/06/2011	0.02	0.02	222.00	0.82	0.00	0.00	0.00	0.00
120	17/08/2011	1.69	0.24	226.89	0.39	0.31	0.25	0.00	0.00
120	19/09/2011	2.26	1.00	203.74	0.47	2.36	1.57	0.00	0.00
130	21/02/2011	23.93	0.29	231.85	0.79	7.36	4.81	4.17	0.36
130	17/03/2011	19.74	0.83	209.55	0.43	1.80	0.93	2.02	0.17
130	18/04/2011	58.69	1.45	243.93	1.54	2.23	0.31	0.79	0.24
130	23/05/2011	2.31	0.10	217.56	0.60	4.26	0.64	0.07	0.06
130	21/06/2011	1.67	0.17	222.98	0.86	0.21	0.11	0.23	0.09
130	17/08/2011	1.71	0.51	224.61	2.46	1.02	0.76	2.86	0.33
130	19/09/2011	3.81	0.51	234.42	0.50	8.29	1.77	0.00	0.00
130	17/10/2011	3.24	1.05	202.03	2.21	2.71	0.62	6.61	0.34
145	21/02/2011	59.24	0.58	230.83	0.66	8.07	1.18	2.32	0.36
145	17/03/2011	28.25	0.72	210.91	1.07	1.83	1.33	3.02	0.25
145	18/04/2011	64.76	5.53	245.74	4.19	8.93	1.27	0.32	0.07
145	23/05/2011	4.40	0.13	218.59	0.75	9.95	0.54	2.17	0.30
145	21/06/2011	0.19	0.08	226.10	0.25	0.26	0.23	0.99	0.16
145	17/08/2011	27.24	2.83	228.52	0.50	5.57	0.84	1.00	0.26
145	19/09/2011	5.48	0.17	209.03	2.72	5.02	0.17	0.00	0.00
215	02/08/2011	2.64	0.06	2.69	0.27	2.21	0.25	3.54	0.41
230	21/02/2011	19.12	0.59	227.86	1.16	6.98	5.70	0.79	0.29

230	17/03/2011	23.16	0.95	223.93	3.27	3.10	2.75	3.45	0.66
230	18/04/2011	46.20	1.13	246.70	0.84	1.66	0.40	3.42	0.38
230	23/05/2011	0.74	0.22	218.19	0.68	4.31	0.28	0.00	0.00
230	21/06/2011	1.07	0.05	224.71	0.56	0.17	0.17	3.15	0.37
230	17/08/2011	2.48	0.00	233.17	1.92	0.17	0.17	0.04	0.04
230	19/09/2011	0.07	0.10	230.52	3.18	0.00	0.00	0.30	0.09
330	02/08/2011	2.24	0.13	2.52	0.30	3.21	0.33	1.73	0.27
330	17/10/2011	3.10	1.02	210.35	6.26	0.00	0.00	1.52	0.17
700	21/02/2011	70.38	0.11	229.09	0.69	8.10	2.57	1.85	0.10
700	17/03/2011	32.63	0.47	208.33	1.03	0.40	0.40	1.76	0.39
700	18/04/2011	56.31	5.33	239.48	1.42	7.26	5.00	13.64	3.95
700	23/05/2011	18.07	0.57	214.42	0.62	6.00	0.37	5.09	0.43
700	21/06/2011	24.12	0.11	222.53	0.56	3.50	0.29	0.09	0.09
700	17/08/2011	21.26	0.80	158.63	31.44	5.62	0.87	1.66	0.39
700	19/09/2011	41.00	12.50	227.34	4.26	10.05	6.38	0.87	0.19
710	21/02/2011	42.19	1.04	227.89	0.51	6.98	1.22	1.12	0.31
710	17/03/2011	3.60	0.37	210.37	2.30	0.06	0.06	6.71	4.26
710	18/04/2011	n.d.	n.d.	236.23	3.29	n.d.	n.d.	0.22	0.15
710	23/05/2011	13.67	0.59	215.91	0.43	4.88	0.45	5.46	2.45
710	21/06/2011	12.10	0.06	222.74	0.55	0.88	0.09	0.00	0.00
710	17/08/2011	13.81	1.04	221.90	4.86	3.98	0.39	2.24	0.61
710	19/09/2011	19.74	2.49	223.89	1.20	7.26	3.08	0.00	0.00
780	21/02/2011	32.10	0.63	229.54	1.16	4.62	1.72	4.56	0.36
780	17/03/2011	5.00	0.74	211.13	1.18	0.05	0.05	3.69	0.65
780	18/04/2011	35.55	5.72	237.29	1.79	3.42	2.42	3.02	2.48
780	23/05/2011	12.24	1.02	216.70	0.38	6.93	0.91	0.98	0.25
780	21/06/2011	7.33	0.03	226.20	0.37	1.48	0.70	2.48	0.26
780	17/08/2011	7.55	0.24	227.25	0.58	3.57	0.07	0.00	0.00

780	19/09/2011	13.90	2.68	213.32	0.93	8.86	4.12	0.00	0.00
780	17/10/2011	12.52	0.11	201.80	0.40	1.17	0.14	6.40	0.32
ZG02	02/08/2011	0.29	0.29	2.75	0.49	1.17	0.48	1.48	0.35

Table S3: Water depth and surface sediment (0-2 cm) parameters averaged over all months (± sd)

Station	*Water depth (m)*	*Median grain size (µm)*	*% Silt (< 63µm)*	*% org. N*	*% org. C*	*Permeability (m^2)*	*Sediment type*
120	12	219 ± 27	14 ± 8	0.04 ± 0.01	0.40 ± 0.21	1.48 ± 2.00 $x10^{-12}$	Fine sandy
130	11	28 ± 14	78 ± 10	0.30 ± 0.17	1.91 ± 0.41	4.20 ± 1.90 $x10^{-15}$	Muddy
145	9	95 ± 72	50 ± 31	0.12 ± 0.10	1.10 ± 0.81	5.02 ± 1.45 $x10^{-13}$	Muddy
215	27	292 ± 24	0 ± 0	0.03 ± 0.02	0.15 ± 0.04	10.94 ± 2.81 $x10^{-12}$	Permeable
230	14	248 ± 17	3 ± 14	0.02 ± 0.04	0.23 ± 0.38	7.05 ± 1.67 $x10^{-12}$	Permeable
330	25	376 ± 8	0 ± 0	0.02 ± 0.01	0.11 ± 0.02	15.91 ± 1.23 $x10^{-12}$	Permeable
700	12	35 ± 22	73 ± 14	0.21 ± 0.05	1.77 ± 0.54	8.40 ± 14.00 $x10^{-15}$	Muddy
710	12	199 ± 68	13 ± 22	0.04 ± 0.05	0.25 ± 0.35	4.39 ± 3.13 $x10^{-12}$	Permeable
780	22	128 ± 18	27 ± 7	0.07 ± 0.02	0.52 ± 0.24	1.26 ± 3.25 $x10^{-13}$	Fine sandy
ZG02	15	464 ± 12	0 ± 0	0.09 ± 0.02	0.10 ± 0.01	26.84 ± 3.55 $x10^{-12}$	Permeable

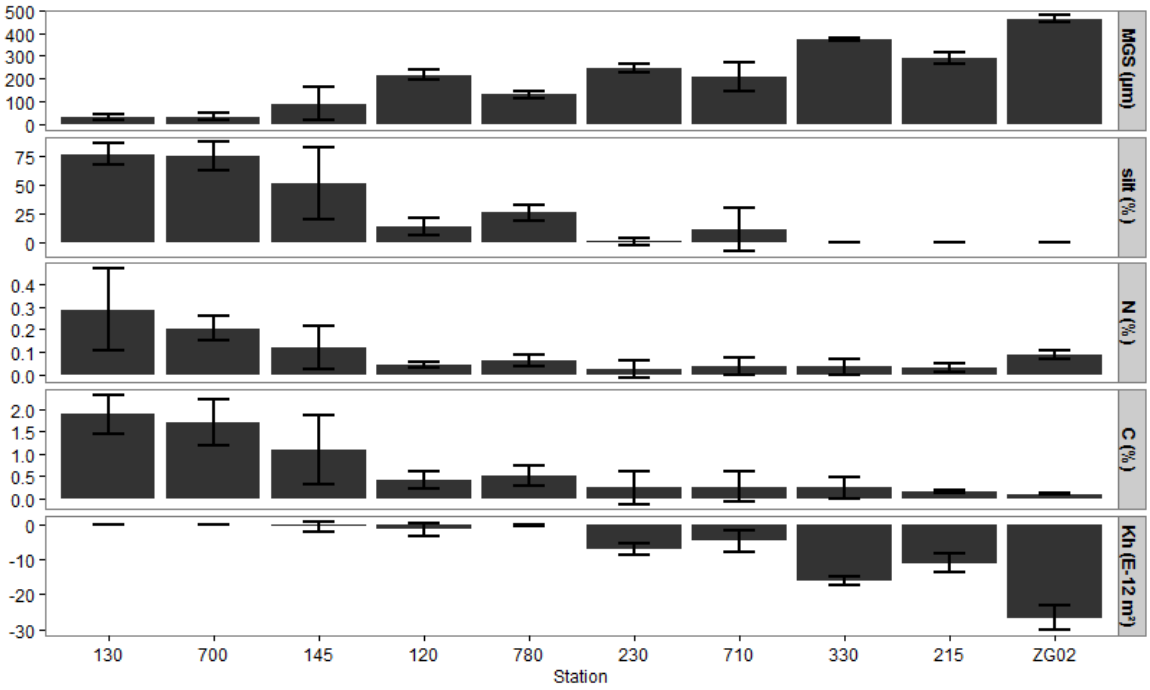


Figure S1: Average median grain size (MGS, µm), silt fraction (%), organic nitrogen (%N) and organic carbon (%C) content and permeability (Kh, $x10^{-12}$ m²) of the sediment at each station. Error bars denote standard deviation.

Table S4: Results of SIMPER analysis, indicating the macrobenthic species that contribute most to the community composition of each station

Station 120					
Av. similarity: 26.93					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	Functional type
Aphelochaeta marioni	794.28	7.12	26.44	26.44	S
Notomastus latericeus	388.05	6.79	25.2	51.64	UC
Capitella minima	491.12	4.62	17.15	68.79	UC
Scoloplos armiger	224.34	3.01	11.16	79.95	B
Eteone longa	97.01	1.14	4.22	84.17	B
Owenia fusiformis	66.7	0.7	2.61	86.78	S
Abra alba	109.14	0.67	2.51	89.28	B
Capitella capitata	127.33	0.66	2.44	91.72	UC
Station 130					

Av. similarity: 31.92					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Macoma balthica	394.11	29.1	91.19	91.19	S
Station 145					
Av. similarity: 27.61					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Macoma balthica	87.54	16.6	60.12	60.12	S
Owenia fusiformis	167.12	6.71	24.3	84.42	S
Aphelochaeta marioni	39.79	2.39	8.65	93.07	S
Station 230					
Av. similarity: 21.34					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Magelona johnstoni	668.47	13.68	64.12	64.12	S
*Capitella sp_*	101.86	2.63	12.3	76.42	UC
Scoloplos armiger	108.23	1.73	8.12	84.55	B
Ensis directus	101.86	1.4	6.55	91.1	S
Station 700					
Av. similarity: 27.94					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Macoma balthica	232.19	14.57	52.13	52.13	S
Aphelochaeta marioni	434.41	12.58	45	97.13	S
Station 710					
Av. similarity: 27.29					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Magelona johnstoni	156.71	21.72	79.57	79.57	S
Nephtys cirrosa	39.18	2.87	10.52	90.08	B
Station 780					
Av. similarity: 38.17					

Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Owenia fusiformis	4260.17	28.76	75.33	75.33	S
Abra alba	960.26	6.28	16.45	91.78	B
Station 215					
Av. similarity: 18.80					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Magelona johnstoni	223.38	11.97	63.64	63.64	S
Spiophanes bombyx	94.06	5.13	27.27	90.91	UC/DC
Station 330					
Av. similarity: 13.17					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Nephtys cirrosa	31.19	7.12	54.02	54.02	B
Ophelia limacina	26.45	2.67	20.29	74.31	B
Spiophanes bombyx	35.27	2.06	15.62	89.92	UC/DC
*Nephtys sp_*	11.76	0.87	6.63	96.55	B
Station ZG02					
Av. similarity: 50.00					
Species	Av.Abund	Av.Sim	Contrib%	Cum.%	
Nephtys cirrosa	35.27	50	100	100	B

This selection is based on the average abundance (Av. Abund) and average similarity (Av. Sim), which lead to the percentage of contribution to this similarity (Contrib%). Also the cumulative contribution (Cum.%) is given. Functional types: S – Surficial modifier, B – Biodiffuser, UC – Upward conveyor, UC/DC – Upward-Downward conveyor

Table S5: Contribution of species BPp to Total BPc per Sample and per Sediment Type. Mi and Ri are the reworking and mobility traits, and Fti is the corresponding sediment reworking functional types.

Sediment type	*Month*	*Station*	*Replicate*	*Genus*	*Species*	*Biomass ($g\ m^{-2}$)*	*Density ($ind.m^{-2}$)*	*Ri*	*Mi*	*Fti*	*BPi*	*BPp (m^{-2})*	*BPc (m^{-2})*	*%BPp_sample*	*%BPp_sedtype*
FINE SANDY	February	780	B	*Echinocardium*	*cordatum*	2911.17	127.33	4	3	B	57.37	7304.21	9042.64	0.81	0.04
	August	780	A	*Echinocardium*	*cordatum*	1686.52	127.33	4	3	B	43.66	5559.49	5937.61	0.94	0.03
	February	780	A	*Echinocardium*	*cordatum*	1682.32	127.33	4	3	B	43.61	5552.56	6892.69	0.81	0.03
	August	780	B	*Abra*	*alba*	535.45	3310.52	2	2	S	1.61	5324.33	10157.85	0.52	0.03
	May	780	C	*Sagartia*	*troglodytes*	479.24	381.98	4	3	B	13.44	5133.04	8585.61	0.60	0.03
	October	780	C	*Abra*	*alba*	477.35	2673.88	2	2	S	1.69	4518.00	9308.38	0.49	0.03
MUDDY	February	700	A	*Macoma*	*balthica*	247.16	636.64	2	2	S	2.49	1586.32	1586.32	1.00	0.06
	August	700	A	*Macoma*	*balthica*	154.19	636.64	2	2	S	1.97	1252.95	2138.45	0.59	0.05
	February	700	B	*Macoma*	*balthica*	166.88	509.31	2	2	S	2.29	1165.87	1165.87	1.00	0.04
	May	130	C	*Macoma*	*balthica*	32.73	2546.55	2	2	S	0.45	1154.46	1229.89	0.94	0.04
	August	145	C	*Sagartia*	*troglodytes*	58.60	127.33	4	3	B	8.14	1036.29	1235.68	0.84	0.04
	September	145	B	*Sagartia*	*troglodytes*	45.81	127.33	4	3	B	7.20	916.25	971.46	0.94	0.03
	August	130	A	*Macoma*	*balthica*	126.42	381.98	2	2	S	2.30	878.79	905.46	0.97	0.03
	June	130	C	*Macoma*	*balthica*	115.02	381.98	2	2	S	2.19	838.23	887.74	0.94	0.03
	October	130	C	*Macoma*	*balthica*	103.59	381.98	2	2	S	2.08	795.50	795.50	1.00	0.03
	April	700	C	*Macoma*	*balthica*	233.66	127.33	2	2	S	5.42	689.77	1415.30	0.49	0.03
	June	145	B	*Macoma*	*balthica*	110.05	254.66	2	2	S	2.63	669.47	844.09	0.79	0.03
PERMEABLE	March	710	A	*Echinocardium*	*cordatum*	97.73	127.33	4	3	B	10.51	1338.32	1338.32	1.00	0.07
	March	230	A	*Ensis*	*directus*	147.95	636.64	2	2	S	1.93	1227.32	1358.38	0.90	0.06
	March	230	B	*Ensis*	*directus*	129.07	509.31	2	2	S	2.01	1025.32	1199.45	0.85	0.05
	March	230	C	*Ensis*	*directus*	108.82	381.98	2	2	S	2.13	815.31	972.06	0.84	0.04
	May	230	B	*Glycera*	*alba*	29.18	127.33	4	3	B	5.74	731.34	823.88	0.89	0.04
	April	710	C	*Echinocardium*	*cordatum*	28.15	127.33	4	3	B	5.64	718.29	732.70	0.98	0.04
	February	230	C	*Ensis*	*directus*	82.04	381.98	2	2	S	1.85	707.92	758.60	0.93	0.03
	September	710	C	*Macoma*	*balthica*	199.62	127.33	2	2	S	5.01	637.55	766.69	0.83	0.03
	April	230	C	*Tellina*	*fabula*	98.24	254.66	2	2	S	2.48	632.53	724.79	0.87	0.03
	September	230	C	*Magelona*	*johnstoni*	9.80	2419.23	2	2	S	0.25	615.63	1102.90	0.56	0.03

Only species BPp contributing > 3% to total BPc per sediment type are shown.

Table S6: Generalized Least Squares Models of Oxygen Consumption (SCOC), Ammonium (NH_x) and Alkalinity (A_T) Effluxes and Nitrification and Denitrification Estimates per Sediment Type as a Function of Environmental Variables Temperature (Temp), Median Grain Size (MGS), Chlorophyll *a* Concentration in the Water Column (chl-*a*), Macrobenthic Density (Dens) and Bioturbation Potential of the Community (BP_C) and Their Centered Quadratic Terms

Sediment type	*Model*	*Model formula*	*AIC*
MUDDY	SCOC	SCOC = -30.19** + 3.75Temp*** + 0.28 Temp²** - 2.11 chla*** + 0.08chla²*	452.18
	NHx	NHx = -7.57 + 0.002**Dens**** + 0.04 MGS * + 4.84 e^{-04}MGS²**	378.23
	Nitrification	Nitrification = 0.27 – 0.002 **Dens**** - 0.06 MGS** - 5.5 e^{-04} MGS²*** + 0.86 Temp*** + 0.11 Temp²*** -0.37 chla***	377.53
	Denitrification	Denitrification = -0.46 + 0.35 chla***	396.65
	Alkalinity	Alkalinity = -54.47* + 0.01 **Dens**** + 0.29MGS* + 0.003MGS²**	577.99
FINE SANDY	SCOC	SCOC = -15.07 + 0.004**BPc***** + 2.55 Temp ***	334.30
	NHx	NHx = -1.08 + 9.74 e^{-04} **BPc***** +0.36chla*	238.00
	Nitrification	No significant model	
	Denitrification	Denitrification = -22.07** + 0.05MGS* + 1.42Temp*** + 9.43 e^{-04}**BPc***	278.68
	Alkalinity	Alkalinity = -0.76 + 0.003 **BPc****	352.23
PERMEABLE	SCOC	SCOC = -21.64*** + 0.01**BPc*** + 8 e^{-07} BPc²* + 1.09Temp*** + 0.17 Temp²*** -0.51 chla*	415.68
	NHx	NHx = 3.07*** + 0.12 chla*** - 0.01 MGS*** + 3.59 e^{-05} MGS²***	177.82
	Nitrification	Nitrification = -3.60** + 9 e^{-04}**BPc*** + 0.005MGS** -0.16 chla** + 0.17 Temp* + 0.03 Temp²**	249.31
	Denitrification	Denitrification = 0.05 + 0.005 **BPc** ***	317.56
	Alkalinity	Alkalinity = -1.48 + 0.64 chla ***	452.65

*Significance of terms (*p<0.05, **p<0.01, ***p<0.001) and the AIC as determined with restricted maximum likelihood of the best models are given.*

Addendum 2. Appendices to Chapter 3

The Link between Microbial Diversity and Nitrogen Cycling in Marine Sediments is Modulated by Macrofaunal Bioturbation

Supplementary Material and Methods (Text S1)

PCR Amplification of Total Archaeal and Bacterial 16S rDNA

The V3 region of the bacterial 16S rDNA gene was amplified for DGGE using the universal bacterial primers F357 and R518 (Table S1). Amplifications were performed in volumes of 50µl containing 2 µl of target DNA (>20 ng/µl), 6 µl of 10xPCR buffer (containing 15 mM $MgCl_2$; GeneAmp, Applied Biosystems, Life Technologies, Carlsbad, California, USA), 0.4 µg/µl BSA, 0.2 mM dNTPs, 0.5 µM of both primers, and 1.25 U AmpliTaq DNA polymerase (1 U/µl) (Applied Biosystems). The 'touchdown' PCR conditions were as follows: after an initial denaturation step at 94 °C for 5 min, 20 cycles were performed consisting of three steps: denaturation (94 °C, 1 min), annealing (65 °C, 1 min, decreasing 0.5 °C $cycle^{-1}$ to 55 °C) and extension (72 °C, 1 min) followed by 10 additional cycles in which the annealing temperature was 55 °C. The final elongation step was performed at 72 °C for 30 min to prevent the formation of artificial double bands in subsequent DGGE analysis (Janse *et al*., 2004).

A nested PCR design was used for 16S rDNA amplification of total archaea due to very low yield from direct PCR in some samples (Benlloch *et al*., 2002). In the first step of PCR, the mixture with a volume of 50 µl consisted of 5.55 µl of 10xTop Taq PCR buffer (containing 15 mM $MgCl_2$; Qiagen, Hilden, Germany), 0.4 µg/µl of BSA, 0.2 mM of each dNTP, 0.5 µM of primers A8F and A1492R (Table S1) amplifying approximately 1400 bp of the 16S rDNA genes of archaea, 2.5 U of Top Taq DNA Polymerase (Qiagen) and 5 µl DNA template (>20 ng/µl). The PCR amplification consisted of 5 min at 94 °C, 30 cycles of 1 min denaturation at 94 °C, 90 s annealing at 51 °C, 2 min extension at 72 °C, and a final step at 72 °C for 6 min. PCR products were checked on agarose gel, purified and diluted to 26 ng/µl. In the second step, a 'touchdown' PCR was performed

for DGGE with primers A344F and A915R to amplify 571 bp of the 16S rDNA fragments in archaea. To prevent complete melting of amplicons during DGGE, a 40-bp-long GC clamp was attached to the 5' end of primer A344F (Table S1). PCR mixtures with a volume of 25 µl consisted 2.5 µl of 10xCoralLoad, 2.5 µl of 10xTop Taq PCR buffer (containing 15 mM $MgCl_2$), 0.005 µg/µl of BSA, 0.2 mM of each dNTP, 0.5 µM of primers A344F and A915R, 0.625 U of Top Taq DNA Polymerase and 1 µl DNA template from the first step. The 'touchdown' PCR amplification started with an initial denaturing step at 94 °C for 5 min, followed by 10 cycles with 94 °C for 1 min, 71 °C for 1 min decreasing every cycle by 0.5 °C, and 72 °C for 3 min, followed by another 10 cycles at 94 °C for 1 min, annealing temperature at 61 °C for 1 min, and 72 °C for 3 min. The final elongation step was performed at 72 °C for 30 min.

cDNA and RT-PCR of Archaeal and Bacterial *amoA* Gene

Integrity and purity of RNA extractions were checked on a 1% agarose gel and by determining the ratio of absorbance at 260 nm and 280 nm and at 260 and 230 nm, which was ≈ 2.0 in all samples. The RTS DNase kit (MO BIO Laboratories, Carlsbad, California, USA) was used for the removal of genomic DNA and PCR amplifications with universal primer sets for archaea or bacteria (Table S2) were performed to verify the complete removal of DNA.

The DNA-free RNA samples were reverse transcribed into cDNA using Omniscript and Sensiscript Reverse Transcriptase Kits (Qiagen) respectively for samples containing ≥ or < 50 ng RNA per reaction according to the instructions of the manufacturer and using 10 µM of Random Hexamer primers (Applied Biosystems) and 2 µl RNA template per total volume of a reaction (20 µl).

RT-PCR amplification of *amoA* gene fragments for DGGE was performed using AOA and β-AOB specific primer sets (Table S1). RT-PCR mixtures with a volume of 25 µl were the same for AOA and β-AOB regarding the amount of template (5 µl of cDNA) and concentration of all used reagents except for primers and Top Taq DNA Polymerase containing 2.5 µl of 10xCoralLoad, 2.5 µl of 10xTop Taq PCR buffer (containing 15 mM $MgCl_2$), 0.005 µg/µl of BSA, 0.2 mM of each dNTP, 0.3 and 0.4 µM of each primer of AOA and β-AOB, respectively, 1.25 and 1.5 U of Top Taq DNA

Polymerase for AOA and β-AOB PCR mixtures. The 'touchdown' PCR for AOA and β-AOB started with a denaturing step at 94 °C for 5 min. Every cycle consisted of three steps, each for 1 min: 94 °C, annealing temperature and 72 °C. The initial annealing temperature (10 °C higher than annealing temperature, T_{an}) was decreased by 0.5 °C per cycle until a touchdown of T_{an}. 15 additional cycles were carried out at T_{an} (52.5 °C and 54.5 °C for AOA's and β-AOB's primers, respectively) to amplify archaeal and bacterial *amoA* genes. Final primer extension was performed at 72 °C for 30 min.

PCRs were performed in a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany). All PCR and RT-PCR products were inspected on 1% agarose gels, purified using the Qiaquick PCR purification kit (Qiagen) following the manufacturer's protocol and measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Denaturing Gradient Gel Electrophoresis (DGGE) of Bacteria, Archaea, AOA and β-AOB

DGGE analysis of PCR and RT-PCR amplicons was performed using the DCode Universal Mutation Detection System device (Bio-Rad, Hercules, California, USA). To enable fingerprint comparisons across gels, eight prokaryotic DNA samples from previous studies were pooled to generate DGGE standards for gels associated with total bacteria and archaea. Four separate prokaryotic DNA samples were also cloned to use as standards for AOA and β-AOB DGGE gels. Bands in the DGGE standards covered the entire gradient in the gels. 400ng of bacterial and 250ng of archaeal PCR products as well as 350ng of AOA and β-AOB RT-PCR products were loaded depending on the fragment size onto 8% (total bacteria and AOA) and 6% (total archaea and β-AOB) (w/v) polyacrylamide gels (30% [w/v] Bis-Acrylamide solution 37.5:1; National Diagnostics, Charlotte, North Carolina, USA). Optimal electrophoretic separation was obtained using 35-70% (bacteria), 35-65% (archaea), 35-50% (β-AOB) and 30-50% (AOA) denaturing gradients (100% denaturant contains 7 M urea and 40% formamide, Sigma-Aldrich, St. Louis, Missouri, USA), running for 16 h at 75 V in 1xTAE (Tris-acetate-EDTA) buffer at a constant temperature of 60 °C. The gels were stained with SYBR gold (Molecular Probes, Invitrogen, Life Technologies) for 30 min followed

by visualization and digital capturing of the profiles via the Molecular Imager Gel Doc XR System (Bio-Rad). Digital images were normalized and processed with BioNumerics (version 5.10, Applied Maths, Sint-Martens-Latem, Belgium).

Data Analysis

The DistLM routine analyses were performed after removing highly correlated independent variables (Draftsmans plot, lrl ≥ 0.90). Fourteen independent variables were entered into the models: chl-*a* concentration, PAP ratio, C:N ratio, MGS (rather than % silt), density of macrofauna, BPc (rather than biomass), diversity and richness of total bacteria, archaea, β-AOB and AOA. Prior to analyses, chl-*a* was ln transformed, whereas density of macrofauna, BPc and PAP ratio were square root transformed to remove right-skewness in the raw data. Predictor variables were then subjected to a sequential step-wise selection procedure using the Akaike's information selection criterion (AIC) for multivariate response variables and the AIC_c criterion in the analysis of univariate response (Anderson *et al*., 2008). To calculate resemblance in Permanova and DistLM, Bray-Curtis similarity and Euclidean distance were used for multivariate (square root transformed) and univariate datasets, respectively.

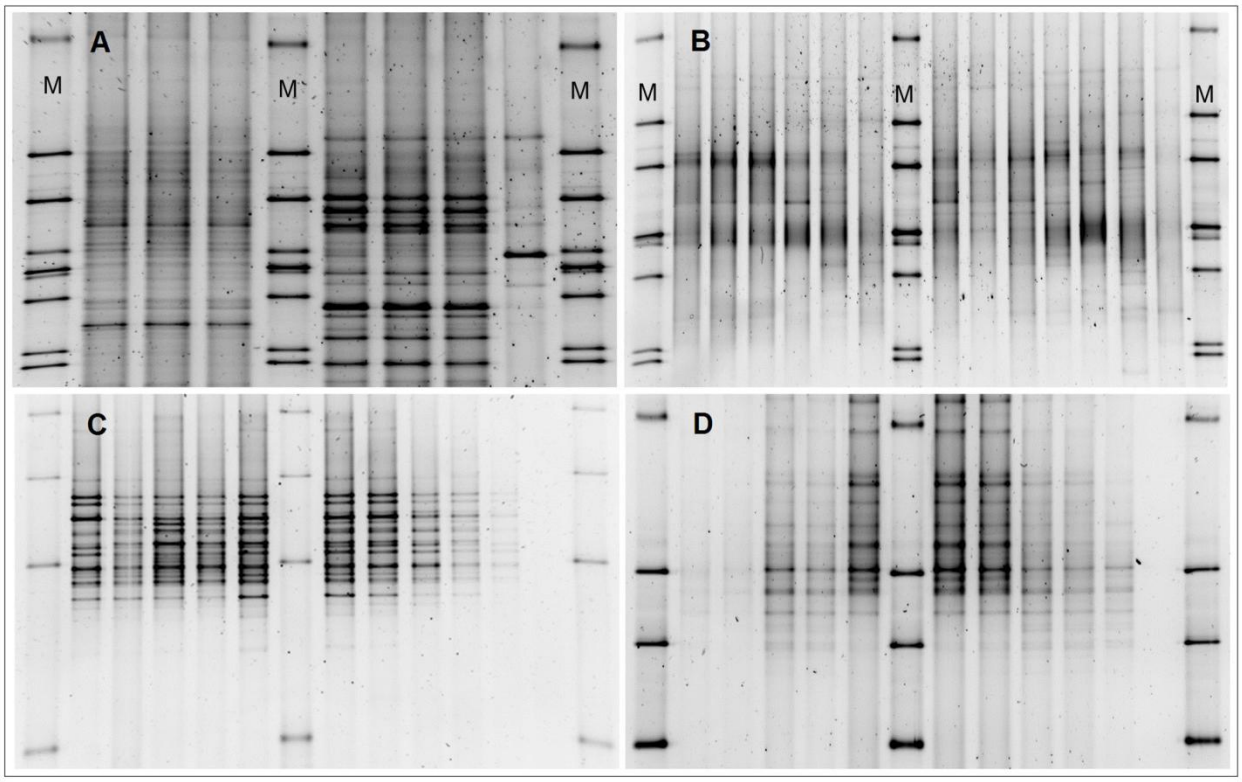


Figure S1. Example of DGGE profiles. Molecular markers: lanes M; Bacterial profile (A): lanes 2,3,4 replicates of St. 700 June; lanes 6,7,8, replicates of St. 230 June; lane 9 a replicate of St. 780 September. Archaeal profile (B): lanes 2,3,4 replicates of St. 700 June; lanes 5,6,7 replicates of St.710 June; lanes 9,10,11 replicates of St. 780 June; lanes 12,13,14 replicates of St. 230 June ; lane 15 a replicate of St. 145 September. β-AOB and AOA profiles (C and D, respectively): lane 2 a replicate of St.120 September; lanes 3,4,5 replicates of St.780 September; lanes 6,8,9 replicates of St.145 September; lanes 10,11,12 replicates of St. 710 September; lane 13 a replicate of St. 710 June.

Table S1. Spatio-temporal variations in surface sediment (0-1 cm) characteristics, N-cycle processes and macrofaunal characteristics (mean ± se, n=3); data from (Braeckman *et al*., 2014c).

		Muddy			***Fine Sandy***		***Permeable***	
Stations		**130**	**145**	**700**	**120**	**780**	**230**	**710**
Water depth (m)		11	9	12	12	22	14	12
abiotic factors								
MGS[a] (µm)	Apr	31.44±1.69	23.75±2.03	26.14±3.93	218.01±4.20	147.64±1.88	232.81±5.85	211.86±3.67
	Jun	31.56±1.23	164.38±2.94	26.95±0.33	192.25±30.84	129.78±4.70	259.00±7.71	168.39±73.06
	Sep	24.11±2.20	147.83±28.20	29.61±3.50	226.60±1.11	121.67±5.34	242.96±3.63	222.21±6.46
% Silt (<63µm)	Apr	70.36±2.09	83.16±2.94	83.21±4.12	12.92±3.14	19.84±0.63	3.38±3.38	0.00±0.00
	Jun	68.51±1.94	23.85±2.16	80.32±0.86	20.82±8.55	28.02±1.66	0.00±0.00	27.50±27.50
	Sep	80.44±3.90	21.16±12.92	76.75±3.30	12.81±1.69	29.03±3.67	0.00±0.00	0.00±0.00
% OrgN	Apr	0.21±0.01	0.26±0.02	0.25±0.04	0.07±0.01	0.05±0.00	0.01±0.00	0.02±0.00
	Jun	0.27±0.04	0.07±0.01	0.24±0.01	0.04±0.00	0.08±0.00	0.02±0.00	0.02±0.00
	Sep	0.31±0.01	0.06±0.02	0.20±0.02	0.05±0.00	0.09±0.01	0.02±0.00	0.02±0.00
% OrgC	Apr	1.39±0.01	1.84±0.12	1.74±0.17	0.35±0.09	0.37±0.00	0.09±0.00	0.07±0.00
	Jun	2.04±0.30	0.48±0.25	1.88±0.07	0.35±0.02	0.23±0.23	0.00±0.00	0.00±0.00
	Sep	2.31±0.04	0.48±0.21	1.56±0.15	0.36±0.04	0.44±0.22	0.13±0.00	0.12±0.01
Permeability ($\times 10^{-12}$ m²)	Apr	0.00±0.00	0.00±0.00	0.00±0.00	1.20±1.03	0.09±0.00	5.03±2.23	5.74±0.09
	Jun	0.00±0.00	0.01±0.00	0.01±0.00	2.57±1.13	0.08±0.01	7.44±0.11	0.03±0.03
	Sep	0.00±0.00	1.47±1.41	0.00±0.00	1.01±0.81	0.04±0.01	7.75±0.22	5.79±0.15
C:N ratio	Apr	6.59±0.39	7.14±0.43	7.21±0.77	4.89±0.46	6.81±0.69	5.19±0.03	4.00±0.94
	Jun	7.44±0.06	6.08±3.11	7.75±0.08	7.62±0.26	3.31±3.31	0.00±0.00	0.00±0.00
	Sep	7.36±0.16	7.91±0.54	7.70±0.06	7.56±0.26	4.88±2.46	6.68±0.28	6.30±0.58
PAP ratio[b]	Apr	0.37±0.27	0.07±0.01	0.09±0.00	0.29±0.04	0.13±0.03	0.21±0.04	0.26±0.02
	Jun	0.18±0.02	0.28±0.06	0.15±0.10	0.48±0.05	0.32±0.03	0.10±0.10	0.08±0.08
	Sep	0.16±0.01	0.19±0.01	0.15±0.02	0.39±0.04	0.40±0.04	0.00±0.00	0.00±0.00
Chlorophyll a (µg g^{-1})	Apr	0.02±0.01	0.02±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Jun	25.79±6.09	3.53±2.43	67.90±50.68	0.63±0.07	5.12±0.72	0.17±0.01	0.26±0.09
	Sep	18.56±0.71	1.84±0.76	17.01±3.28	0.52±0.10	1.91±0.37	0.23±0.05	0.13±0.01
Processes								
Nitrification (mmol $Nm^{-2}d^{-1}$)	Apr	-6.16±1.52	-2.34±0.88	-5.85±0.89	-2.34±1.79	2.17±0.93	0.27±0.31	0.92±0.43
	Jun	-12.80±3.03	-0.36±0.46	-3.58±1.58	-2.48±0.63	-3.18±0.76	0.88±0.37	-0.22±0.20
	Sep	0.10±1.20	3.39±0.73	2.33±1.05	6.32±1.16	6.50±0.43	-0.11±0.23	3.40±0.27
Denitrification (mmol $Nm^{-2}d^{-1}$)	Apr	-5.55±1.28	3.21±2.69	-14.14±10.93	8.86±2.27	3.16±0.91	3.51±1.60	2.96±2.32
	Jun	-10.14±3.15	7.53±1.62	-0.32±1.47	17.17±3.84	13.48±2.74	4.59±0.85	-0.73±0.65
	Sep	4.66±1.78	4.44±1.53	3.90±1.34	10.04±2.16	9.49±1.08	0.91±0.52	3.83±0.35
N-mineralization (mmol $Nm^{-2}d^{-1}$)	Apr	1.47±0.18	3.16±1.53	1.26±0.66	1.92±0.16	2.29±0.83	0.44±0.24	0.93±0.43
	Jun	2.68±0.81	1.56±0.15	1.57±0.64	4.27±0.97	6.31±1.16	0.70±0.26	0.00±0.00
	Sep	4.30±0.31	1.93±0.24	3.67±0.02	5.35±1.57	4.52±1.35	0.59±0.14	1.93±0.25
Macrofauna								
Biomass (g WW m^{-2})	Apr	3.52±3.47	0.00±0.00	142.45±76.81	150.65±77.03	283.02±140.49	34.72±32.33	9.98±9.13
	Jun	61.78±33.59	73.45±29.36	28.64±28.53	327.36±157.00	392.35±124.03	6.40±2.35	1.33±1.06
	Sep	24.02±12.44	81.44±14.87	6.14±6.14	182.81±70.27	328.21±172.24	7.74±6.15	67.62±66.36
Density (ind. m^{-2})	Apr	84.88±42.44	42.44±42.44	1443.05±658.89	3310.52±1155.34	3756.17±1568.51	381.98±194.50	339.54±84.88
	Jun	551.75±185.00	551.75±112.29	254.65±127.33	2758.77±153.03	4159.37±1672.04	2037.24±530.11	339.54±278.31
	Sep	127.33±0.00	169.77±42.44	42.44±42.44	2122.13±1181.55	5347.76±3014.02	1145.95±763.97	297.10±42.44
BPc[c] (m^{-2})	Apr	53.47±46.46	0.00±0.00	909.78±430.05	2431.40±580.83	2406.82±910.11	313.92±213.60	317.48±209.26
	Jun	531.98±259.66	508.61±179.24	216.64±202.13	4053.77±1553.22	4325.42±1445.86	466.14±40.81	161.04±144.30
	Sep	187.21±83.72	619.06±176.21	64.60±64.60	2273.09±494.086	3351.80±1687.64	430.95±336.15	371.66±198.31

[a]MGS=median grain size; [b]PAP ratio=The ratio of phaeopigments to the sum of chl-a + phaeopigments (Boon and Duineveld, 1998); [c]BPc= Bioturbation Potential of the Community

Table S2. Primers used for detection of total archaea, bacteria and ammonia oxidizers (AOA and β-AOB)

Target gene	*Primer pair*	*Sequences (5'-3')*	*Fragment (bp)*	*Reference*
16S rDNA Archaea (1_{st} round)	A8f A1492r	CGGTTGATCCTGCCGGA GGCTACCTTGTTACGACTT	1468	Casamayor *et al*. (2000) Casamayor *et al*. (2000)
16S rDNA Archaea (2_{nd} round)	A344f [a] A915r	ACGGGGTGCAGCAGGCGCGA GTGCTCCCCCGCCAATTCCT	571	Perreault *et al*. (2007) Raskin *et al*. (1994)
16S rDNA Bacteria	F357[a] R518	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	161	Muyzer *et al*. (1993) Muyzer *et al*. (1993)
amoA β-AOB	amoA-1F[a] amoAr-new	GGGGHTTYTACTGGTGGT CCCCTCBGSAAAVCCTTCTTC	490	Hornek *et al*. (2006) Hornek *et al*. (2006)
amoA AOA	Arch-amoA-for Arch-amoA-rev[a]	CTGAYTGGGCYTGGACATC TTCTTCTTTGTTGCCCAGTA	256	Wuchter *et al*. (2006) Wuchter *et al*. (2006)

[a] To prevent complete melting of amplicons during DGGE, a 40-bp-long GC clamp was attached to the 5' end of one primer. (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3') attached to Arch-amoA-rev and F357 primers (Muyzer et al., 1993); (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') attached to amoA-1F primer (Hornek et al., 2006); (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG-3') attached to A344f primer (Perreault et al., 2007).

Table S3. Results of PERMANOVA for microbial communities

	factor	df_{term}	df_{error}	*Pseudo-F*	*P(perm)*
Bacterial community composition	MoxSt	12	42	3.21	0.000
Archaeal community composition	MoxSt	12	42	2.06	0.000
β-AOB community composition	MoxSt	12	42	3.03	0.000
AOA community composition	MoxSt	12	42	2.79	0.000
Bacterial OTU richness	MoxSt	12	42	2.01	0.048
Archaeal OTU richness	St	6	42	2.98	0.023
β-AOB OTU richness	St	6	42	10.05	0.001
	Mo	2		13.25	0.001
AOA OTU richness	MoxSt	12	42	2.46	0.018
Bacterial Shannon diversity	St	6	42	4.16	0.002
	Mo	2		83.63	0.001
Archaeal Shannon diversity	St	6	42	3.11	0.022
β-AOB Shannon diversity	MoxSt	12	42	2.18	0.038
AOA Shannon diversity	MoxSt	12	42	2.56	0.024

Single factor results are only given where the interaction Month x Station (MoxSt) was not significant.

Table S4. Pairwise test results from PERMANOVA analysis for temporal differences of microbial community composition

Community composition	**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
	130		145		700		120		780		230		710	
Bacteria	t	P	t	P	t	P	t	P	t	P	t	P	t	P
April-June	2.55	**0.017**	1.97	**0.041**	1.40	0.110	2.23	**0.024**	2.07	**0.033**	3.21	**0.012**	3.70	**0.004**
April-Sept	1.97	**0.046**	1.38	0.173	1.64	0.088	3.19	**0.005**	1.45	0.145	4.14	**0.003**	3.32	**0.005**
June-Sept	2.23	**0.032**	1.60	0.100	2.25	**0.023**	3.51	**0.005**	1.76	0.083	9.31	**0.000**	3.75	**0.006**
Archaea														
April-June	1.92	**0.046**	1.58	0.094	2.87	**0.010**	1.20	0.256	1.59	0.103	1.34	0.171	1.45	0.130
April-Sept	1.10	0.054	1.10	0.348	2.37	**0.018**	1.28	0.207	1.36	0.163	0.97	0.46	1.81	0.050
June-Sept	2.20	**0.029**	1.01	0.344	1.63	0.093	1.18	0.281	1.79	0.062	0.84	0.604	1.60	0.086
β-AOB														
April-June	3.83	**0.004**	4.95	**0.004**	5.03	**0.001**	2.31	**0.029**	6.46	**0.000**	1.13	0.317	1.91	0.113
April-Sept	2.27	**0.025**	2.77	**0.022**	3.17	**0.011**	1.14	0.318	2.14	**0.034**	1.44	0.172	1.22	0.258
June-Sept	7.16	**0.000**	8.97	**0.000**	12.11	**0.000**	4.00	**0.003**	5.84	**0.000**	2.28	**0.028**	4.33	**0.002**
AOA														
April-June	4.41	**0.003**	1.62	0.149	1.54	0.131	1.83	0.122	1.17	0.281	0.88	0.442	2.93	**0.009**
April-Sept	2.10	**0.036**	1.72	0.138	1.55	0.138	1.75	0.071	1.29	0.233	1.79	0.116	1.30	0.227
June-Sept	2.86	**0.009**	4.83	**0.003**	3.73	**0.004**	5.96	**0.001**	2.07	**0.043**	1.28	0.239	10.74	**0.000**

P-values obtained from Monte-Carlo test, P (MC).

Table S5. Pairwise test results from PERMANOVA analysis for spatial differences of microbial community composition

Community composition		Muddy stations						Fine sandy stations				Permeable stations			
		130		145		700		120		780		230		710	
Bacteria		t	P	t	P	t	P	t	P	t	P	t	P	t	P
	130														
	145	1.47	0.148												
April	700	1.04	0.385	0.86	0.534										
	120	1.81	0.068	1.04	0.391	1.10	0.345								
	780	1.61	0.106	1.72	0.064	1.14	0.309	1.37	0.184						
	230	2.45	**0.017**	1.92	**0.047**	1.55	0.118	1.55	0.124	2.02	**0.034**				
	710	2.94	**0.009**	2.66	**0.014**	1.93	0.057	2.28	**0.028**	2.22	**0.029**	1.88	0.065		
	130														
	145	1.71	0.103												
June	700	2.26	**0.030**	1.90	0.068										
	120	2.80	**0.010**	1.26	0.233	2.97	**0.012**								
	780	3.07	**0.008**	2.08	**0.035**	3.22	**0.007**	2.82	**0.010**						
	230	5.56	**0.002**	2.37	**0.025**	5.91	**0.001**	2.76	**0.012**	3.07	**0.009**				
	710	4.62	**0.003**	2.26	**0.027**	4.91	**0.002**	2.85	**0.011**	2.70	**0.013**	2.86	**0.011**		
	130														
	145	0.92	0.452												
Sept	700	1.42	0.166	1.33	0.207										
	120	2.73	**0.017**	1.75	0.114	2.44	**0.022**								
	780	0.69	0.609	0.67	0.655	1.50	0.134	2.01	0.055						
	230	2.88	**0.012**	1.74	0.127	2.62	**0.021**	1.25	0.237	2.05	0.055				
	710	2.43	**0.024**	1.57	0.143	2.13	**0.038**	2.33	**0.021**	1.86	0.069	1.62	0.123		
Archaea															
	130														
	145	1.50	0.131												
April	700	1.89	0.060	1.57	0.117										
	120	1.06	0.374	1.25	0.225	1.70	0.070								
	780	1.56	0.118	1.27	0.240	1.37	0.170	1.25	0.229						
	230	1.33	0.206	1.32	0.201	1.51	0.123	1.21	0.255	0.94	0.500				
	710	1.82	0.061	1.54	0.112	1.78	0.060	1.49	0.115	1.19	0.271	1.31	0.191		
	130														
	145	1.55	0.112												
June	700	2.37	**0.015**	1.91	0.073										
	120	1.03	0.391	1.47	0.147	1.97	**0.041**								
	780	2.43	**0.018**	2.20	**0.031**	2.03	**0.041**	1.86	**0.047**						
	230	1.84	**0.044**	1.99	**0.043**	1.76	0.066	1.21	0.254	1.51	0.122				
	710	1.82	**0.046**	1.38	0.172	1.36	0.198	1.41	0.159	1.25	0.232	1.08	0.359		
	130														
	145	1.25	0.248												
Sept	700	2.32	**0.018**	1.00	0.427										
	120	1.53	0.113	0.70	0.741	0.95	0.477								
	780	1.73	0.062	0.99	0.438	1.40	0.146	0.96	0.470						
	230	1.67	0.081	0.77	0.684	0.87	0.544	0.61	0.830	1.06	0.367				
	710	2.10	**0.030**	1.34	0.176	1.78	0.061	1.55	0.103	1.53	0.105	1.40	0.147		

P-values obtained from Monte-Carlo test, P (MC).

Table S5 (continued):

Community composition		**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
		130		145		700		120		780		230		710	
β-AOB		t	P	t	P	t	P	t	P	t	P	t	P	t	P
	130														
	145	1.68	0.113												
April	700	0.59	0.758	2.15	**0.048**										
	120	1.40	0.176	1.12	0.336	1.62	0.108								
	780	1.71	0.091	2.69	**0.027**	1.89	0.064	1.51	0.147						
	230	1.05	0.381	1.22	0.280	1.21	0.261	0.73	0.622	1.54	0.115				
	710	1.70	0.074	1.26	0.255	1.91	0.054	0.48	0.789	1.77	0.074	0.81	0.568		
	130														
	145	1.50	0.152												
June	700	2.54	**0.022**	5.64	**0.001**										
	120	4.42	**0.002**	5.12	**0.002**	7.44	**0.001**								
	780	1.67	0.095	1.07	0.368	4.94	**0.001**	4.48	**0.004**						
	230	1.30	0.241	1.25	0.261	1.36	0.222	1.29	0.241	1.21	0.276				
	710	13.4	**0.000**	33.89	**0.000**	39.49	**0.000**	16.90	**0.000**	24.26	**0.000**	1.96	0.112		
	130														
	145	2.94	**0.010**												
Sept	700	1.58	0.103	4.64	**0.001**										
	120	1.95	0.054	1.25	0.250	2.44	**0.022**								
	780	2.23	**0.033**	2.11	**0.043**	2.51	**0.021**	1.06	0.358						
	230	2.50	**0.022**	1.64	0.105	3.47	**0.007**	1.46	0.155	2.18	**0.030**				
	710	1.81	0.070	1.09	0.340	2.22	**0.032**	0.74	0.630	1.15	0.303	1.06	0.364		
AOA															
	130														
	145	1.31	0.270												
April	700	1.41	0.191	0.85	0.547										
	120	2.31	**0.026**	1.13	0.343	1.22	0.265								
	780	1.65	0.091	0.97	0.453	1.05	0.376	1.14	0.313						
	230	2.26	**0.043**	1.15	0.317	0.99	0.404	0.96	0.423	1.32	0.197				
	710	1.66	0.096	0.96	0.462	1.12	0.321	1.40	0.164	0.79	0.631	1.37	0.201		
	130														
	145	3.56	**0.013**												
June	700	1.87	0.072	2.60	**0.021**										
	120	15.43	**0.000**	8.06	**0.000**	6.27	**0.000**								
	780	2.12	**0.045**	1.34	0.217	1.99	**0.046**	2.65	**0.016**						
	230	1.93	0.071	1.10	0.346	1.67	0.099	2.00	0.115	0.88	0.509				
	710	15.43	**0.000**	8.06	**0.000**	6.27	**0.001**	2.00	0.110	2.65	**0.015**	2.00	0.120		
	130														
	145	1.82	0.091												
Sept	700	1.67	0.097	5.75	**0.001**										
	120	1.90	0.062	3.22	**0.012**	3.22	**0.009**								
	780	1.48	0.148	1.61	0.141	3.10	**0.010**	1.66	0.109						
	230	1.21	0.274	2.33	**0.031**	2.55	**0.022**	1.60	0.123	1.50	0.173				
	710	2.05	**0.047**	3.02	**0.013**	4.07	**0.004**	1.71	0.112	0.99	0.391	1.69	0.099		

P-values obtained from Monte-Carlo test, P (MC).

Table S6. Pairwise test results from PERMANOVA analysis for temporal differences of microbial OTU richness

OTU richness	**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
	130		145		700		120		780		230		710	
Bacteria	t	P	t	P	t	P	t	P	t	P	t	P	t	P
April-June	15.59	**0.000**	0.73	0.515	4.08	**0.014**	0.38	0.724	2.78	0.052	2.52	0.065	4.69	**0.009**
April-Sept	1.58	0.233	1.58	0.190	2.53	0.067	6.04	**0.005**	3.89	**0.018**	2.56	0.058	1.92	0.126
June-Sept	11.02	**0.000**	2.07	0.103	10.54	**0.000**	9.17	**0.001**	5.08	**0.007**	4.36	**0.011**	4.58	**0.009**
AOA														
April-June	1.06	0.330	0.60	0.602	1.51	0.187	1.98	0.126	0.23	0.835	0.51	0.618	3.00	**0.030**
April-Sept	1.12	0.302	1.44	0.267	4.05	**0.011**	2.81	**0.047**	2.92	**0.038**	1.11	0.328	3.26	**0.033**
June-Sept	0.94	0.396	2.80	**0.044**	9.90	**0.002**	4.07	**0.014**	2.59	0.054	0.62	0.601	11.53	**0.002**
Archaea	t	P												
April-June	1.51	0.136												
April-Sept	1.50	0.136												
June-Sept	0.00	1.000												
β-AOB														
April-June	0.10	0.940												
April-Sept	4.08	**0.001**												
June-Sept	5.33	**0.001**												

P-values for bacteria and AOA obtained from Monte-Carlo test, P (MC) while those for archaea and β-AOB obtained from permutation, P (Perm).

Table S7. Pairwise test results from PERMANOVA analysis for temporal differences of microbial Shannon diversity

Shannon diversity	**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
	130		145		700		120		780		230		710	
β-AOB	t	P	t	P	t	P	t	P	t	P	t	P	t	P
April-June	1.16	0.322	4.12	**0.025**	0.02	0.981	0.83	0.440	1.40	0.232	0.55	0.594	1.97	0.100
April-Sept	0.35	0.761	3.36	**0.037**	0.08	0.932	1.27	0.275	7.44	**0.004**	1.86	0.138	1.51	0.223
June-Sept	0.91	0.433	1.95	0.137	0.09	0.932	3.20	**0.039**	6.01	**0.005**	0.94	0.399	17.56	**0.001**
AOA														
April-June	1.57	0.180	1.13	0.381	1.13	0.304	2.00	0.130	0.05	0.969	0.60	0.565	7.12	**0.004**
April-Sept	1.04	0.367	1.58	0.233	1.81	0.151	2.37	0.087	2.36	0.118	1.46	0.231	2.76	0.052
June-Sept	0.79	0.489	2.61	0.062	9.65	**0.002**	12.50	**0.001**	2.07	0.105	0.76	0.511	23.35	**0.001**
Bacteria	t	P												
April-June	5.89	**0.001**												
April-Sept	7.62	**0.001**												
June-Sept	11.27	**0.001**												
Archaea														
April-June	1.06	0.315												
April-Sept	1.76	0.086												
June-Sept	0.86	0.388												

P-values for β-AOB and AOA obtained from Monte-Carlo test, P (MC) while those for bacteria and archaea obtained from permutation, P (Perm).

Table S8. Pairwise test results from PERMANOVA analysis for spatial differences of microbial OTU richness

OTU richness		**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
		130		145		700		120		780		230		710	
Bacteria		t	P	t	P	t	P	t	P	t	P	t	P	t	P
	130														
	145	1.97	0.121												
April	700	0.02	**0.041**	0.65	0.553										
	120	2.30	0.084	0.14	0.895	0.87	0.440								
	780	11.00	**0.000**	1.86	0.135	1.06	0.343	2.50	0.066						
	230	1.44	0.224	0.24	0.822	0.86	0.442	0.13	0.899	1.98	0.120				
	710	1.73	0.160	2.40	0.072	3.41	**0.025**	2.77	0.053	9.45	**0.000**	1.85	0.137		
	130														
	145	2.52	0.065												
June	700	5.50	**0.005**	5.33	**0.007**										
	120	4.33	**0.012**	0.80	0.469	8.22	**0.001**								
	780	1.89	0.130	3.28	**0.032**	0.20	0.855	4.29	**0.013**						
	230	7.35	**0.001**	1.61	0.180	14.50	**0.000**	0.86	0.436	5.30	**0.006**				
	710	1.71	0.163	0.51	0.634	4.25	**0.013**	1.33	0.251	2.71	0.053	2.13	0.101		
	130														
	145	0.78	0.477												
Sept	700	0.00	1.000	0.76	0.488										
	120	8.50	**0.001**	0.70	0.530	4.71	**0.008**								
	780	0.17	0.870	0.63	0.550	0.15	0.892	2.83	0.051						
	230	2.29	0.080	0.08	0.940	1.89	0.129	1.70	0.162	1.31	0.255				
	710	2.40	0.072	0.46	0.660	2.16	0.095	0.33	0.760	1.71	0.161	0.70	0.536		
AOA															
	130														
	145	1.03	0.358												
April	700	2.33	0.086	0.35	0.761										
	120	5.92	**0.004**	0.10	0.438	0.84	0.435								
	780	5.83	**0.005**	0.85	0.478	0.68	0.530	0.26	0.835						
	230	1.55	0.179	0.37	0.752	0.14	0.890	0.37	0.730	0.27	0.811				
	710	2.06	0.119	0.07	0.950	0.43	0.680	1.51	0.220	1.34	0.240	0.44	0.652		
	130														
	145	0.80	0.483												
June	700	2.68	0.052	0.43	0.679										
	120	21.50	**0.001**	8.51	**0.001**	37.00	**0.001**								
	780	3.88	**0.013**	2.89	0.051	3.14	**0.026**	2.16	0.101						
	230	0.86	0.438	0.57	0.559	0.46	0.684	2.00	0.126	0.91	0.433				
	710	21.50	**0.001**	8.51	**0.003**	37.00	**0.001**	0.45	0.674	2.16	0.096	2.00	0.133		
	130														
	145	1.79	0.134												
Sept	700	2.79	**0.046**	3.62	**0.028**										
	120	0.92	0.453	0.51	0.644	1.52	0.211								
	780	1.06	0.310	0.35	0.721	1.40	0.234	0.12	0.917						
	230	0.60	0.618	0.90	0.423	1.87	0.141	0.30	0.790	0.43	0.701				
	710	2.28	0.087	1.41	0.242	0.67	0.538	1.10	0.351	0.97	0.398	1.45	0.216		
Archaea															
	130														
	145	1.86	0.082												
	700	2.53	**0.031**	2.95	**0.014**										
	120	0.79	0.458	1.98	0.068	0.25	0.792								
	780	0.13	0.885	1.37	0.212	1.30	0.206	0.71	0.479						
	230	0.05	**0.011**	3.25	**0.007**	0.90	0.385	0.61	0.536	1.66	0.102				
	710	1.93	0.079	2.84	**0.025**	0.86	0.417	0.80	0.431	1.58	0.160	0.45	0.666		
β-AOB															
	130														
	145	3.27	**0.017**												
	700	3.35	**0.011**	9.70	**0.001**										
	120	0.51	0.643	0.74	0.524	1.90	0.075								
	780	4.12	**0.002**	1.75	0.099	8.31	**0.001**	1.43	0.183						
	230	1.89	0.081	2.68	**0.027**	0.91	0.369	1.90	0.085	3.34	**0.014**				
	710	5.93	**0.001**	7.84	**0.001**	4.36	**0.004**	4.20	**0.002**	8.64	**0.001**	1.21	0.237		

P-values for bacteria and AOA obtained from Monte-Carlo test, P (MC) while those for archaea and β-AOB obtained from permutation, P (Perm).

Table S9. Pairwise test results from PERMANOVA analysis for spatial differences of microbial Shannon diversity

Shannon diversity		**Muddy stations**						**Fine sandy stations**				**Permeable stations**			
		130		145		700		120		780		230		710	
β-AOB		t	P	t	P	t	P	t	P	t	P	t	P	t	P
	130														
	145	1.05	0.364												
April	700	0.15	0.875	1.68	0.195										
	120	0.70	0.502	0.67	0.566	0.68	0.541								
	780	2.01	0.125	1.53	0.235	3.10	**0.037**	0.95	0.409						
	230	1.89	0.148	1.64	0.194	1.88	0.133	0.65	0.520	2.22	0.087				
	710	1.36	0.237	1.22	0.326	1.34	0.240	0.31	0.790	1.67	0.172	0.38	0.724		
	130														
	145	3.52	**0.021**												
June	700	3.08	**0.047**	13.40	**0.001**										
	120	0.18	0.856	2.45	0.063	1.85	0.132								
	780	2.65	0.058	0.87	0.434	8.66	**0.001**	2.00	0.103						
	230	1.11	0.369	1.30	0.241	0.92	0.400	1.08	0.337	1.28	0.292				
	710	57.40	**0.001**	256. 92	**0.001**	115.70	**0.001**	35.82	**0.001**	103.80	**0.001**	2.00	0.115		
	130														
	145	3.54	**0.028**												
Sept	700	0.54	0.604	4.74	**0.009**										
	120	3.54	**0.026**	1.03	0.404	4.029	**0.019**								
	780	5.05	**0.006**	1.66	0.167	6.83	**0.003**	0.20	0.857						
	230	0.11	0.916	1.64	0.180	0.14	0.892	2.01	0.134	2.10	0.090				
	710	0.46	0.681	1.77	0.153	0.85	0.483	2.20	0.096	2.51	0.073	0.39	0.719		
AOA															
	130														
	145	1.26	0.293												
April	700	1.41	0.231	0.14	0.890										
	120	2.51	0.066	0.53	0.635	0.40	0.693								
	780	2.22	0.099	0.44	0.669	0.31	0.774	0.09	0.934						
	230	1.74	0.171	0.52	0.663	0.47	0.639	0.18	0.856	0.24	0.822				
	710	1.84	0.145	0.45	0.704	0.65	0.524	1.41	0.238	1.22	0.131	1.12	0.339		
	130														
	145	0.25	0.815												
June	700	2.70	**0.049**	1.11	0.356										
	120	189.68	**0.001**	22.23	**0.001**	66.40	**0.001**								
	780	2.08	0.114	2.10	0.125	1.91	0.130	1.85	0.115						
	230	0.90	0.416	0.92	0.397	0.77	0.461	2.00	0.135	0.51	0.611				
	710	189.68	**0.001**	22.23	**0.001**	66.40	**0.001**	2.00	0.137	1.86	0.130	2.00	0.110		
	130														
	145	1.59	0.191												
Sept	700	1.78	0.153	1.69	0.164										
	120	0.93	0.412	1.09	0.331	1.48	0.194								
	780	0.87	0.449	0.83	0.442	1.12	0.345	0.00	0.998						
	230	0.50	0.623	0.92	0.434	1.12	0.331	0.32	0.794	0.30	0.762				
	710	1.75	0.148	0.77	0.497	0.12	0.906	1.36	0.257	1.08	0.353	1.12	0.330		
Bacteria															
	130														
	145	1.25	0.216												
	700	1.07	0.335	1.75	0.108										
	120	3.70	**0.003**	1.10	0.302	3.51	**0.004**								
	780	0.38	0.739	0.70	0.487	1.07	0.280	2.04	0.069						
	230	4.31	**0.003**	0.99	0.339	3.66	**0.003**	0.27	0.775	2.02	0.065				
	710	3.49	**0.011**	1.36	0.206	3.52	**0.006**	0.45	0.694	2.22	0.065	0.72	0.474		
Archaea															
	130														
	145	1.46	0.172												

700	3.68	**0.005**	2.61	**0.023**								
120	0.72	0.490	1.66	0.128	0.70	0.506						
780	0.77	0.439	1.71	0.108	0.84	0.457	0.03	0.973				
230	3.66	**0.006**	2.65	**0.025**	0.18	0.852	0.75	0.494	0.90	0.418		
710	3.04	**0.019**	2.82	**0.016**	0.93	0.350	1.12	0.331	1.27	0.228	0.82	0.451

P-values for β-AOB and AOA obtained from Monte-Carlo test, P (MC) while those for bacteria and archaea obtained from permutation, P (Perm).

Addendum 3. Appendices to Chapter 4

The effect of bio-irrigation by the polychaete *Lanice conchilega* on denitrifiers: distribution, diversity and composition of *nosZ* transcripts

Table S1: Primers for paired-end nosZ sequencing on the Illumina Miseq platform. Adaptor, Multiple Identifier (MID), pad, linker and primer sequences for the forward and reverse data sets are given.

FORWRD PRIMER

5' Illumina Adapter	*Pad*	*Linker*	*Forward Primer (nosZ-2F)*
AATGATACGGCGACCACCGAGATCTACAC	TATGGTAATT	GT	CGCRACGGCAASAAGGTSMSSGT

REVERSE PRIMER

Reverse complement of 3' Illumina Adapter	*MID*	*Pad*	*Linker*	*Reverse primer (nosZ-2R)*
CAAGCAGAAGACGGCATACGAGAT	TACCGCTTCTTC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TGTGCGATAACA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GATTATCGACGA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCCTAGCCCAAT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GATGTATGTGGT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ACTCCTTGTGTT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GTCACGGACATT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCGAGCGAAGTA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ATCTACCGAAGC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ACTTGGTGTAAG	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TCTTGGAGGTCA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TCACCTCCTTGT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCACACCTGATA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCGACAATTACA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TCATGCTCCATT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	AGCTGTCAAGCT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GAGAGCAACAGA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TACTCGGGAACT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	CGTGCTTAGGCT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TACCGAAGGTAT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	CACTCATCATTC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GTATTTCGGACG	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TATCTATCCTGC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TTGCCAAGAGTC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	AGTAGCGGAAGA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCAATTAGGTAC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	CATACCGTGAGT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ATGTGTGTAGAC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	CCTGCGAAGTAT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	TTCTCTCGACAT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCTCTCCGTAGA	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GTTAAGCTGACC	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ATGCCATGCCGT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GACATTGTCACG	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	GCCAACAACCAT	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA
CAAGCAGAAGACGGCATACGAGAT	ATCAGTACTAGG	AGTCAGTCAG	CC	CAKRTGCAKSGCRTGGCAGAA

Table S2: Results from PERMANOVA analysis for differences in O_2 concentrations (depth and time profiles and max OPD) among treatments (high and low *Lanice* densities and control) based on a Euclidean distance similarity matrix (df=2). Where the treatment effect was significant, pairwise tests were performed within treatments. P-values were obtained by permutation and drawn from Monte-Carlo samplings ($p_{(MC)}$) if the number of unique permutations was less than 100 (Anderson *et al*., 2008). Max OPD indicates maximum oxygen penetration depth. Bold values indicate significant differences at $p < 0.05$.

Main test			
	factor	*Pseudo-F*	$P_{(perm)}$
O_2 depth profiles	treatment	5.33	**0.006**
Max OPD	treatment	12.19	**0.000**
O_2 time profiles	treatment	2.11	0.178
Pairwise test			
		t	$P_{(perm)}$
O_2 depth profiles	High – Low density	1.36	0.168
	High density - Control	3.39	**0.002**
	Low density - Control	2.03	**0.039**
Max OPD		*t*	$P_{(MC)}$
	High – Low density	3.11	**0.008**
	High density - Control	4.08	**0.001**
	Low density - Control	1.88	0.079

Table S3: Numbers of reads and total numbers of *nosZ*-UATs per sample as well as the numbers of abundant *nosZ*-UATs (>1% relative abundance) in treatment-depth combinations are provided.

	Number of Reads	Number of total *nosZ*-UATs	Number of abundant (> 1%) *nosZ*-UATs
h1d1	6,945	296	Hd1=3
h2d1	16,296	368	
h3d1	31,954	425	
h1d2	5,639	284	Hd2=10
h2d2	3,007	220	
h3d2	1,322	114	
h1d3	4,741	250	Hd3=10
h2d3	1,455	121	
h3d3	14,570	373	
h1d4	1,242	123	Hd4=12
h2d4	1,022	104	
h3d4	1,956	162	
L1d1	9,051	320	Ld1=5
L2d1	38,734	449	
L3d1	8,485	315	
L1d2	4,003	245	Ld2=5
L2d2	25,073	402	
L3d2	7,652	316	
L1d3	1,143	128	Ld3=8
L2d3	15,863	348	
L3d3	7,194	290	
L1d4	24,498	416	Ld4=3
L2d4	33,194	406	
L3d4	20,627	349	
C1d1	15,082	343	Cd1=5
C2d1	38,098	439	
C3d1	28,538	430	
C1d2	12,906	357	Cd2=5
C2d2	13,119	363	
C3d2	15,272	374	
C1d3	15,166	381	Cd3=3
C2d3	14,870	377	
C3d3	29,275	416	
C1d4	31,399	400	Cd4=3
C2d4	13,554	361	
C3d4	32,797	388	

Table S4: Results from PERMANOVA analysis pairwise tests for differences in composition of *nosZ* transcripts (based on Generalized UniFrac distances; α= 0.5) influenced by the single effects of "treatment" and "depth" on the data sets using all *nosZ*-UATs and only abundant *nosZ*-UATs (with relative abundance >1%.). Treatments: high and low *Lanice* densities and control; Depths: 0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm

All nosZ-UATs							
Treatments		**High / Low**	**High / Control**	**Low / Control**			
	Pseudo-F	2.33	3.23	0.79			
	P value	0.058	**0.036**	0.455			
Depth (cm)		**0-0.5 / 0.5-1**	**0-0.5 / 1-1.5**	**0-0.5 / 2.5-3**	**0.5-1 /1-1.5**	**0.5-1 / 2.5-3**	**1-1.5 / 2.5-3**
	Pseudo-F	7.63	3.45	3.37	0.75	1.64	1.51
	P value	**0.001**	**0.018**	**0.020**	0.476	0.168	0.640
Abundant (> 1%) nosZ-UATs							
Treatments		**High / Low**	**High / Control**	**Low / Control**			
	Pseudo-F	2.64	3.96	0.51			
	P value	0.077	**0.034**	0.608			
Depth (cm)		**0-0.5 / 0.5-1**	**0-0.5 / 1-1.5**	**0-0.5 / 2.5-3**	**0.5-1 /1-1.5**	**0.5-1 / 2.5-3**	**1-1.5 / 2.5-3**
	Pseudo-F	7.46	3.23	3.70	0.66	1.35	0.33
	P value	**0.004**	**0.045**	**0.040**	0.506	0.266	0.712

Table S5: Results from PERMANOVA analysis main tests for differences in diversity indices of *nosZ* transcripts among treatments (high and low *Lanice* densities and control) and depths (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm). Diversity indices (richness, Shannon-Wiener [log e] and inverse Simpson) were calculated from the average values obtained from 1000 sub-samples of the data matrix to the minimum number of reads (1022). Univariate analyses of diversity indices were based on Euclidean distance similarity matrix. P-values obtained by permutation.

	*factor**	df_{term}	*Pseudo-F*	*P*
Richness	treatment x depth	6	5.45	**0.043**
Shannon diversity	-	-	-	No sig. **
Inverse Simpson	-	-		No sig. **

* indicates only significant factors

**p > 0.05

Table S6: Results from PERMANOVA analysis pairwise tests for differences in richness of *nosZ* transcripts influenced by the interaction effect "treatment x depth". Three treatments (High: high *Lanice* treatment, Low: low *Lanice* treatment, Control) and four depths (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm). Richness was calculated from the average values obtained from 1000 sub-samples of the data matrix to the minimum number of reads (1022). Analyses were based on Euclidean distance similarity matrix. P-values obtained by permutation

Treatments		**High / Low**	**High / Control**	**Low / Control**			
0-0.5 *cm*	t	2.57	2.13	0.55			
	$P_{(MC)}$	0.624	0.105	0.608			
0.5-1 *cm*	t	0.92	3.49	1.65			
	$P_{(MC)}$	0.408	**0.026**	0.182			
1-1.5 *cm*	t	0.19	2.11	2.44			
	$P_{(MC)}$	0.859	0.098	0.072			
2.5-3 *cm*	t	3.99	4.13	0.28			
	$P_{(MC)}$	**0.016**	**0.015**	0.795			
Depth (cm)		**0-0.5 / 0.5-1**	**0-0.5 / 1-1.5**	**0-0.5 / 2.5-3**	**0.5-1 /1-1.5**	**0.5-1 / 2.5-3**	**1-1.5 / 2.5-3**
High	t	0.68	1.39	6.30	0.15	1.06	2.87
	$P_{(MC)}$	0.570	0.294	**0.022**	0.894	0.403	0.104
Low	t	1.74	2.50	0.94	1.60	0.31	0.93
	$P_{(MC)}$	0.220	0.128	0.452	0.244	0.783	0.462
Control	t	2.49	0.42	2.93	0.79	2.29	1.76
	$P_{(MC)}$	0.137	0.729	0.103	0.513	0.124	0.145

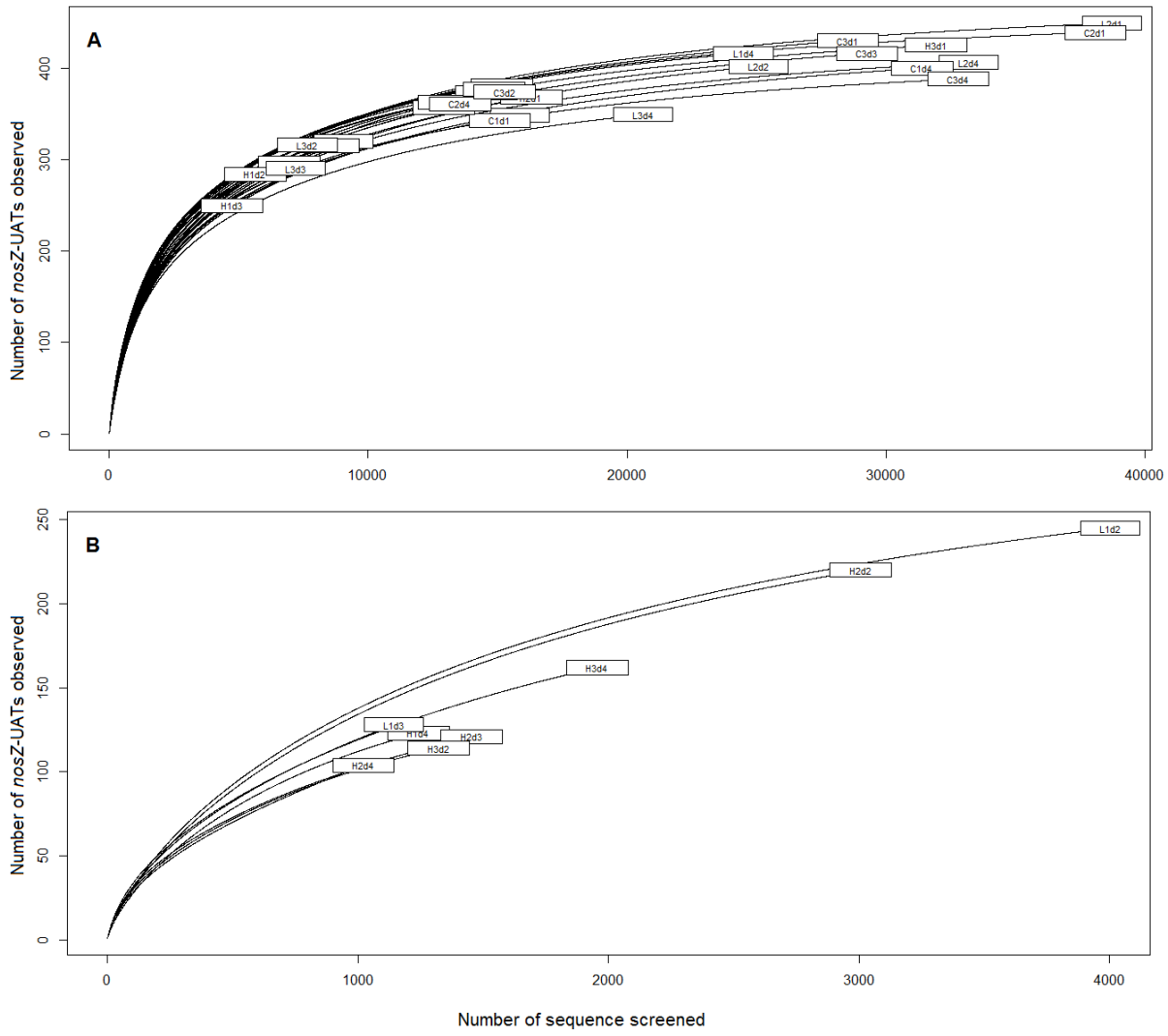


Figure S1: Rarefaction curves of the *nosZ* transcripts plotting the number of unique transcripts (*nosZ*-UAT) observed as function of the number of sequences screened in samples with > 4000 reads (A) and ≤ 4000 reads (B). “H” indicates high *Lanice* treatment. “L”: low *Lanice* treatment, “C”: control treatment, “d1”: 0-0.5 cm depth, “d2”: 0.5-1 cm, “d3”: 1-1.5 cm, “d4”: 2.5-3 cm.


High
Low
Control
Hd1 Hd2 Hd3 Hd4 Ld1 Ld2 Ld3 Ld4 Cd1 Cd2 Cd3 Cd4
nosZ-UAT 29
nosZ-UAT 32
nosZ-UAT 46
nosZ-UAT 37
nosZ-UAT 10
nosZ-UAT 28
nosZ-UAT 26
nosZ-UAT 18
nosZ-UAT 4
nosZ-UAT 38
nosZ-UAT 68
nosZ-UAT 42
nosZ-UAT 53
nosZ-UAT 52
nosZ-UAT 44
nosZ-UAT 1
nosZ-UAT 35
nosZ-UAT 33
nosZ-UAT 30
nosZ-UAT 83
nosZ-UAT 80
nosZ-UAT 25
nosZ-UAT 58
nosZ-UAT 48
nosZ-UAT 2
nosZ-UAT 3
nosZ-UAT 195
nosZ-UAT 12
nosZ-UAT 7
nosZ-UAT 70
nosZ-UAT 20
nosZ-UAT 425
nosZ-UAT 67
nosZ-UAT 64
nosZ-UAT 77
nosZ-UAT 24
nosZ-UAT 16
nosZ-UAT 6
nosZ-UAT 84
nosZ-UAT 98
nosZ-UAT 51
nosZ-UAT 17
nosZ-UAT 11
nosZ-UAT 14
nosZ-UAT 9
nosZ-UAT 102
nosZ-UAT 19
nosZ-UAT 137
nosZ-UAT 8
0.1 subst./site
0
8% 40-70%

Figure S2: Maximum likelihood phylogeny of *nosZ* amino acid sequences retrieved from our samples. 10% of *nosZ*-UATs (50 *nosZ*-UATs out of 502) making up around 84% of the total reads are shown. Abundant nosZ-UAT (>1% relative abundance in at least one treatment-depth cobination) are indicated in red. The heat map (on the right) illustrates the average relative abundance of each *nosZ*-UAT per sample. The dominant *nosZ*-UAT 1 is shown in red. Numbers at nodes are bootstrap values (values < 60% not shown). The scale bar represents 10% sequence divergence (10 mutations per 100 sequence positions).


Gammaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Gammaproteobacteria
Alphaproteobacteria
0.3

Figure S3: Maximum likelihood phylogeny of *nosZ* amino acid sequences retrieved from our samples and reference sequences. To construct a phylogeny with reference sequences, protein BLAST searches of 25 *nosZ*-UAT representatives selected randomly across the phylogenetic tree in Figure S2 were performed against the NCBI non-redundant protein database. Abundant *nosZ*-UAT (>1% relative abundance) are shown in red. Numbers at nodes are bootstrap values (values < 50% not shown). The scale bar represents 30% sequence divergence (30 mutations per 100 sequence positions).

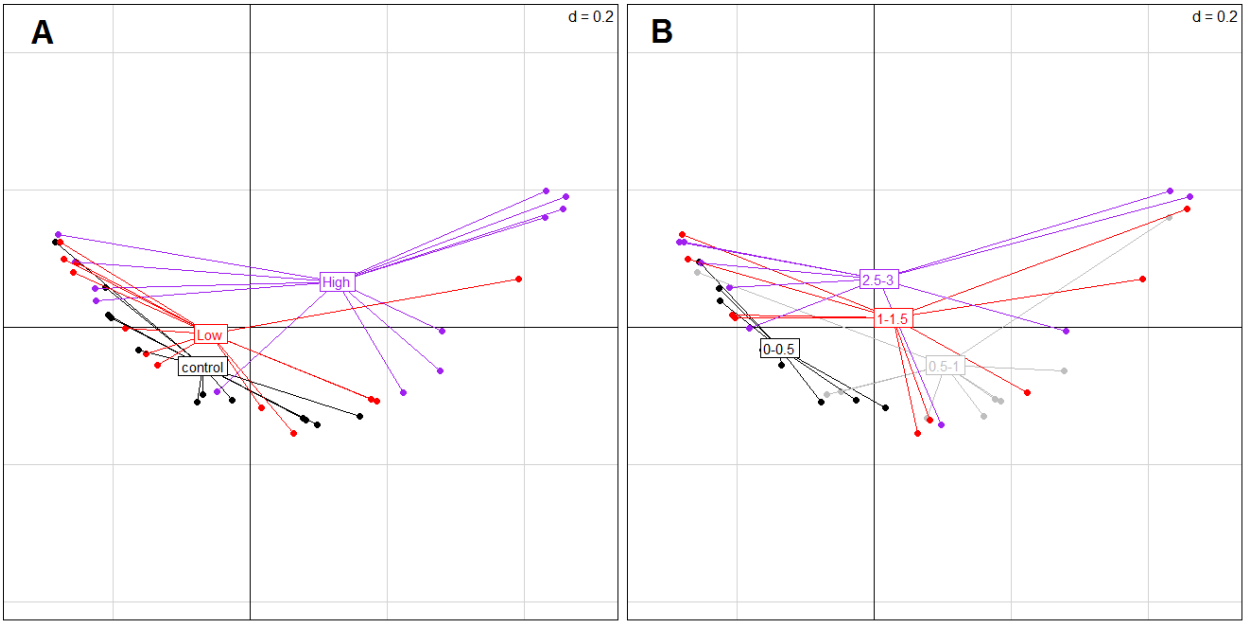


Figure S4: Principal coordinates analysis plot (PCoA) based on generalized UniFrac distances on the normalized data including only abundant *nosZ*-UATs (A: per treatment; B: per depth). Each point represents a sample. Three treatments (High: high *Lanice* treatment, Low: low *Lanice* treatment, Control) and four depth layers (0-0.5, 0.5-1, 1-1.5 and 2.5-3 cm).

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Publication list

A1- peer reviewed articles

Yazdani Foshtomi, M., Braeckman, U., Derycke, S., Sapp, M., Van Gansbeke, D., Sabbe, K., Willems, A., Vincx, M., Vanaverbeke, J. (2015). The link between microbial diversity and nitrogen cycling in marine sediments is modulated by macrofaunal bioturbation. *PLoS One 10(6)*: e0130116. hdl.handle.net/10.1371/journal.pone.0130116

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Article in prepration

Yazdani Foshtomi, M., Leliaert, F., Derycke, S., Willems, A., Vincx, M., Vanaverbeke, J., The effect of bio-irrigation by the tube-building polychaete *Lanice conchilega* on denitrifiers: distribution, diversity and composition of *nosZ* transcripts".

Presentations

Maryam Yazdani Foshtomi, Ulrike Braeckman, Sofie Derycke, Frederik Leliaert, Anne Willems, Magda Vincx, Jan Vanaverbeke. 2016. The interaction between macro and micro-organisms affects biogeochemical cycles in coastal sediments. The event, organised by OD Nature of the Royal Belgian Institute of Natural Sciences and the Belgian Biodiversity Platform, takes place in Ostend, Belgium from 7-10 November 2016. North Sea Open Science Conference

Yazdani Foshtomi, M., Braeckman, U., Derycke, S., Sapp, M., Willems, A., Vincx, M., Vanaverbeke, J. (2015). Effect of natural populations of the ecosystem engineering polychaete, Lanice conchilega, on abundance and diversity of nitrifying and denitrifying organisms, in: Mees, J. et al. (Ed.) Book of abstracts – VLIZ Young Scientists' Day. Brugge, Belgium, 20 February 2015. VLIZ Special Publication, 71: pp. 150

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