Microbial – deposit feeder aquaculture bioremediation systems

A Thesis Submitted By

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Abstract

Land-based intensive aquaculture produces large volumes of particulate organic waste that can be upcycled into high value secondary biomass. In this research, the application of two key principles underpinning low-cost bioremediation technologies, namely the addition of rate limiting (i) electron acceptors (oxygen), and (ii) donors (carbon) is investigated in a sediment-based aquaculture effluent treatment system integrating the sea cucumber, *Holothuria scabra*. Growth trials of *H. scabra,* combined with next generation sequencing (NGS) technologies, were used to examine the response of sea cucumbers and sediment bacterial communities under contrasting redox regimes, describing fully oxic and redox-stratified sediments. The oxic system resulted in high taxonomic and functional diversity of bacteria with a range of dissimilatory metabolisms required for successful bioremediation of aquaculture wastes; however, the final biomass of *H. scabra* was significantly lower than the redox-stratified sediments (449.22 ± 14.24 g m^{-2} versus 626.89 ± 35.44 g m^{-2}). Improving the resource quality of aquaculture waste through carbon supplementation was investigated. Increasing the carbon/nitrogen ratio from 5:1 to 20:1 with soluble starch significantly increased the biomass production of *H. scabra* on redox-stratified sediments compared to controls (1011.46 ± 75.58 g m^{-2} versus 702.12 ± 35.93 g m^{-2}). A benthic flux incubation study, combined with NGS, demonstrated that carbon supplementation did not change the pathway of nitrogen cycling by mediating a shift from net release of ammonium to net assimilation, as hypothesised. A final study elucidated the critical role of the sea cucumber microbiome during aquaculture waste bioremediation, demonstrating that endogenous bacteria are primed, at ecological and genomic levels, to respond to nitrogen - a key nutrient limiting deposit feeder growth. Deposit feeder–microbial aquaculture bioremediation systems have the potential to rectify current inefficiencies of nitrogen use in the aquaculture production chain by offering a more economically and environmentally sustainable alternative to closing the nitrogen cycle loop.

We've got to learn to

Reduce, Reuse, Recycle

- *Jack Johnson, "The 3 R's"*

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Chapter 1. General Introduction

1.1 Aquaculture

1.1.1 Current trends in global aquaculture production

Aquaculture is one of the world's fastest growing food production sectors (Béné *et al.*, 2015). Over the past 50 years, global fish production has increased steadily, outpacing world population growth and recently surpassing the total supply from capture fisheries (FAO, 2016b). Global aquaculture production is projected to increase by a further 60 –100 % over the next 20–30 years (FAO, 2016b), and the continued expansion of the sector will be necessary to help feed a global population of nine billion people by 2050 (Béné *et al.*, 2015; UN, 2015).

A key issue that has not yet been fully acknowledged and adequately addressed is that societal needs for protein is leading to inefficient nitrogen use in the aquaculture production chain. As capture fisheries production has stagnated, the general trend has been to fish down the food web and farm up the food web, by increasing production of higher trophic groups, such as finfish and crustacean (Naylor *et al.*, 2000). Inefficiencies of nitrogen use occur at multiple levels within aquaculture production systems; however, they stem from the use of wild caught fish to produce farmed fish (on average, 1.9 kg of wild fish are used to produce 1 kg of farmed fish (Naylor *et al.*, 2000)). Furthermore, nitrogen assimilation in higher organisms is extremely inefficient; as pathways of energy production in finfish are based on the oxidation and catabolism of proteins, only ~25 % of the nitrogen in feed is retained in the organism (Hepher, 1988; Hargreaves, 1998).

Globally, aquaculture practices are contributing to a net increase in nutrient loading, which equates to approximately 0.9 % of anthropogenic inputs to the global nitrogen cycle (Verdegem, 2013). However, this is due to the proportion of intensive 'fed' versus extensive 'unfed' aquaculture. Species produced commercially by the addition of formulated feeds e.g. finfish and crustaceans contribute to net nitrogen loading, while lower trophic levels such as bivalves and algae are 'extractive' species that contribute to net nitrogen removal (Verdegem, 2013). One solution adopted to re-dress this imbalance is integrating the production of 'fed' species with 'extractive' organisms, in integrated multi-trophic aquaculture (IMTA) systems (Chopin *et al.*, 2001; Troell *et al.*, 2003; Verdegem, 2013). Deposit feeding sea cucumbers are gaining increasing recognition as candidate extractive organisms in IMTA systems in the open ocean (Cubillo *et al.*, 2016; Zamora *et al.*, 2016); however, to date, their integration into

land-based aquaculture has received limited attention (MacDonald *et al.*, 2013; Bossers, 2015).

1.1.2 Marine land-based aquaculture

Marine aquaculture, which includes land-based pump-ashore production facilities, in addition to production operations in the sea and intertidal zones, represents 36 % of the global aquaculture production of 74 million tonnes (FAO, 2016b). Land-based aquaculture production systems include; ponds, tanks and raceways operated as extensive, semi-intensive or intensive systems for the production of algae, molluscs, crustaceans, and finfish. Extensive aquaculture systems are characterised by low stocking densities (0.5 kg m^{-3}) and productivity (20-50 kg ha^{-1} y^{-1}) with a reliance on natural productivity to provide supplemental feed to the primary culture species (Lucas and Southgate, 2012). As aquaculture has intensified, formulated feeds have been introduced that aim to completely satisfy the nutritional requirements of the cultured species and increase stocking densities beyond the carrying capacity of natural food webs. Land-based intensive aquaculture is characterised by the use of high quality, high protein formulated feeds and high stocking densities that result in large volumes of waste as approximately 75 % of the nitrogen and phosphorous in feed remains as waste (Hargreaves, 1998).

Land-based intensive aquaculture production generates high volumes of wastewater containing suspended solids as waste (residual food and faecal matter), dissolved inorganic nutrients (N and P) and residues of chemical therapeutants and hormones (Antony and Philip, 2006; Turcios and Papenbrock, 2014). Traditionally, the maintenance of water quality in pond culture or flow-through systems (FTS) has been dealt with through water exchange; however, in many countries this is no longer viable due to the introduction of stringent discharge limits to minimise environmental impacts (van Rijn, 2013). The discharge of aquaculture effluents leads to a number of negative environmental impacts on the marine benthos associated with nutrient loading and organic enrichment, including; increased sedimentation rates, depletion of dissolved oxygen, and increased levels of ammonium and sulphide (Nixon, 1995; Katz *et al.*, 2002; Lehtoranta *et al.*, 2009). As the quantity of waste generated is directly proportional to the increasing volume of global aquaculture production, it is now recognised that it is necessary to develop efficient and innovative systems for aquaculture bioremediation (Chávez-Crooker and Obreque-Contreras, 2010; Martinez-Porchas *et al.*, 2014; Turcios and Papenbrock, 2014).

1.1.3 Treatment of dissolved inorganic nitrogen

The use of formulated feed generally results in high levels of ammonium-nitrogen (NH_4^+-N) excretion. The accumulation of total ammonia nitrogen (TAN), which includes ammonium (NH_4^+) and ammonia (NH_3), is considered to be the main risk in intensive aquaculture since in its unionised form, ammonia is extremely toxic to marine organisms at concentrations > 1.5 mg L^{-1} (Avnimelech, 1999; Crab *et al.*, 2007). Sources of NH_4^+ include: 1) excretion from fish as the end-product of protein catabolism; and 2) the microbial degradation of particulate organic nitrogen from uneaten feed and faeces (ammonification) (Ebeling *et al.*, 2006). The intensification of land-based aquaculture systems has been accompanied by technological developments to deal with the increasing quantities of NH_4^+. As microorganisms are central to all aquaculture bioremediation technologies, the chronological development of aquaculture bioremediation technologies has been based on a transition through different functional groups of microorganisms and nitrogen transformation pathways (Figure 1.1).

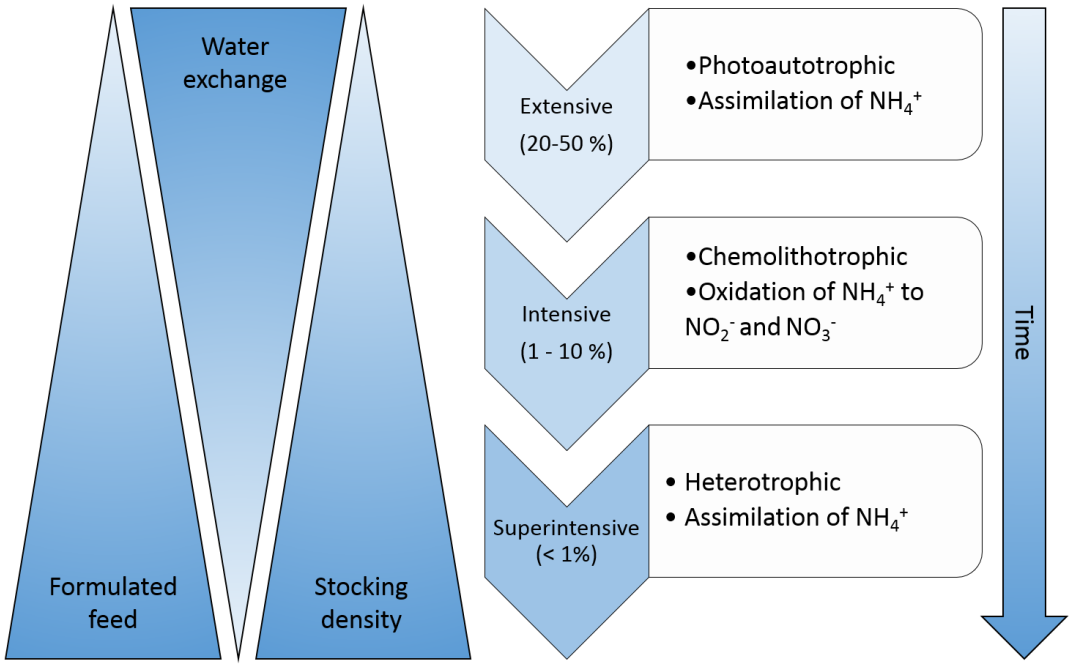

Figure 1.1. Chronological development of aquaculture bioremediation technologies based on the exploitation of different functional groups of microorganisms for the removal of ammonium (NH_4^+) during the progressive intensification of aquaculture.

Traditionally, extensive aquaculture in ponds relied on water exchange and the addition of fertilizers to promote primary production to assimilate dissolved inorganic nitrogen (DIN) through photoautotrophic pathways (Ebeling *et al.*, 2006). As aquaculture has intensified and greater controls over environmental parameters have been required, there has

been a move towards re-circulating aquaculture systems (RAS). Conventional RAS rely on mechanical filters for solids removal and the supply of oxygen to fixed-film biological filtration systems where chemolithotrophic bacteria mediate the stepwise conversion of NH_4^+ to nitrite and nitrate (Ebeling *et al.*, 2006; Badiola *et al.*, 2012). Minimal or zero exchange aquaculture technologies (< 1 % water exchange) have shifted towards exploiting heterotrophic pathways for the *in situ* treatment of inorganic nitrogen. In biofloc technologies, labile organic carbon sources are added directly to the culture tanks to induce microbial protein synthesis via the heterotrophic bacterial conversion of NH_4^+ directly to microbial biomass (Avnimelech, 1999; Avnimelech, 2014).

1.1.4 Treatment of particulate organic wastes

Of the total nitrogen that is lost in intensive aquaculture, approximately 62 % is dissolved inorganic nitrogen and 13 % is particulate organic nitrogen (Folke and Kautsky, 1989; Hargreaves, 1998). Accordingly, while a wide range of bioremediation technologies exist to treat dissolved inorganic nutrients, the treatment of particulate organic wastes has received considerably less attention (Luo *et al.*, 2015). Total suspended solids (TSS) are difficult to treat, particularly in marine systems where their dilute nature is further exacerbated by decreased settling velocity at high salinities (Castine, 2013). Consequently, pre-treatment methods such as the use of mechanical filters that are capable of recovering more than 97 % of TSS loads, are used to trap and concentrate solid wastes (Castine, 2013; Turcios and Papenbrock, 2014).

Conventional RAS rely on the chemolithotrophic conversion of NH_4^+; therefore, system management aims to minimise particulate organic waste accumulation through sludge removal to reduce competition from heterotrophic bacteria in the biofilter (Ebeling *et al.*, 2006; Rurangwa and Verdegem, 2015). The removal of accumulated solids or sludge is also an important management practise in flow-through or zero exchange systems, since the accumulation and decomposition of solid wastes represent a net source of ammonia to the system (Avnimelech, 1999; Ebeling *et al.*, 2006).

The accumulation of particulate organic matter or sludge, together with its removal and disposal, remains a major constraint to the future development of RAS and zero exchange systems. In a land-based RAS, solid wastes (sludge) comprising uneaten feed and faeces, accounts for ~ 30 % of the daily feed input (Cripps and Bergheim, 2000; Brown *et al.*, 2011; van Rijn, 2013). Thus, a 50 tonne fin-fish RAS, would generate 18.75 tonnes of solid waste over a production cycle, based on an average feed conversion ratio of 1.5 kg feed kg^{-1} gain. This represents a significant waste of valuable nutrients, and cost for removal and disposal or

downstream treatment. In freshwater land-based aquaculture, a number of options exist for the downstream treatment of wastes including constructed wetlands, aquaponics, hydroponics, composting and the application as a terrestrial farming input, vermiculture, and reed drying beds (Cripps and Bergheim, 2000; Verdegem, 2013; Turcios and Papenbrock, 2014). However, due to the high salt content, marine aquaculture effluents are unsuitable for agricultural applications, although some potential exists to plant halophyte plants such as *Salicornia spp* (Castine *et al.*, 2013b). Anaerobic digestion is being developed to treat saline sludge from marine RAS (Luo *et al.*, 2015). However, given the limited downstream options for sludge remediation, the majority of sludge recovered from land-based aquaculture is disposed of in landfill, municipal sewers or, in the absence of stringent discharge limits, is discharged to the marine environment (Summerfelt *et al.*, 1999; Cripps and Bergheim, 2000; van Rijn, 2013).

1.1.5 Sustainability issues in land-based intensive aquaculture

In this era of increasing global aquaculture production, that utilises finite resources such as water, land and fishmeal, resource recycling is becoming an increasingly important issue in the aquaculture sector development (Verdegem, 2013). Consequently, future production systems without any nutrient and energy recycling are not considered to be economically and ecologically successful (Troell *et al.*, 2003). The FAO Code of Conduct for Responsible Fisheries recommends that all aquaculture operations take an "Ecosystems Approach to Aquaculture" (EAA; FAO, 2010), which by definition requires a holistic development approach focusing on maintaining ecosystem function and integrity. In line with the EAA and Agenda 2030, modern aquaculture systems should aim to sustainably use marine resources and promote comprehensive nutrient recycling within the culture system, thereby reducing effluent streams and minimising feed inputs including fishmeal (FAO, 2010; UN, 2015).

While a main driver behind aquaculture intensification is to increase production, efficiency and profitability, intensification has been characterised by a move towards minimal or zero exchange aquaculture systems in response to biosecurity concerns. Due to their minimal requirements in terms of space and water, their potential to exert greater control over production parameters and minimise discharges to the environment, RAS are seen as the future for land-based aquaculture production systems (Badiola *et al.*, 2012; Verdegem, 2013). However, life-cycle analysis has revealed that energy and feed requirements and waste generation remain major components that contribute to the ecological impact of RAS (Martins *et al.*, 2010). Previously, RAS were designed to recycle only water; key nutrients such as

nitrogen, comprising some of the most costly inputs, were not recycled but converted into non-toxic forms by different functional groups of microorganisms and exported from the system (Schneider *et al.*, 2005; van Rijn *et al.*, 2006). However, the increasing awareness of the environmental impacts, unsustainable practices, and dependency of the global population on aquaculture, are leading to a new era in the development of sustainable aquaculture technologies (Antony and Philip, 2006; Martins *et al.*, 2010; Martinez-Porchas *et al.*, 2014). The development of innovative technologies that aim to improve the sustainability of RAS is now the focus of considerable research effort (Martins *et al.*, 2010; Badiola *et al.*, 2012). Research is focusing on two key areas: 1) ecologically-driven integrated systems to re-use waste; and, 2) technological developments that integrate new nitrogen transformation pathways to increase water re-use and reduce waste (Martins *et al.*, 2010), with the overall aim of creating "zero discharge" systems (Hamlin *et al.*, 2008).

1.1.6 Ecologically-based systems

Ecologically-based systems are designed to resemble natural aquatic environments that recycle nutrients through the food web. Microbial-based systems and IMTA, are becoming increasingly popular aquaculture bioremediation technologies for the *in situ* removal of particulate suspended and dissolved inorganic nutrients (Chávez-Crooker and Obreque-Contreras, 2010; Martins *et al.*, 2010; Martínez-Córdova *et al.*, 2015). Typically, IMTA systems combine an aquaculture species that requires external feeding (e.g. finfish) with 'extractive' species capable of deriving nutrients from the wastes of the 'fed' species. In IMTA, the emphasis is on the direct assimilation and recycling of wastes by economically valuable extractive species, which can be harvested as secondary organisms, thus resulting in a net export of nutrients from the system. Microbial-based systems, which comprise biofloc technology and periphyton-based aquaculture, are based on promoting the *in situ* proliferation of microorganisms to recycle and transform excess nutrients into biomass, which can be consumed by the culture organism (Avnimelech, 2014; Martínez-Córdova *et al.*, 2015).

1.1.7 Technological developments

In conventional RAS, daily water exchange rates of 1-10 % of the total system volume are necessary to maintain nitrate levels produced by nitrification to < 10 mg L^{-1} (Pillay and Kutty, 2005; Badiola *et al.*, 2012; Verdegem, 2013). 'Next generation' RAS technologies are being developed that aim to reduce water usage from < 1 to <0.01 m^3 water kg^{-1} feed by introducing new nitrogen transformation pathways for nitrate removal (Hamlin *et al.*, 2008; Martins *et al.*, 2010; Verdegem, 2013). These aquaculture technology developments are currently focused on integrating additional filtration systems to house the processes of

denitrification and anaerobic oxidation of ammonia (anammox) that aim to permanently remove nitrogen from the culture system by conversion to dinitrogen gas (N_2) (Tal *et al.*, 2006; van Rijn *et al.*, 2006). In order to reduce solid wastes, anaerobic digestors are being developed to produce biogas from sludge (Mirzoyan *et al.*, 2012).

1.1.8 Particulate organic waste – an increasing problem

The quantity of particulate organic matter waste is increasing in direct proportion to the growth of intensive land-based aquaculture. Furthermore, minimal exchange, next generation RAS, and zero exchange technologies are adding to the volume of particulate waste, since they lead to the production of additional quantities of particulate waste, in the form of heterotrophic bacterial biomass, that requires removal and disposal (Martins *et al.*, 2010; Turcios and Papenbrock, 2014). Due to the high growth efficiencies of heterotrophic bacteria, technologies that employ the continual addition of carbon to promote nitrogen removal – either by denitrification (anaerobic nitrate respiration) or assimilation (NH_4^+) – leads to the production of bacterial solids (Ebeling *et al.*, 2006). Biofloc systems are operated on 'zero-exchange'; however, 0.5-1% of the total water volume has to be replaced on a daily basis to enable solids removal. In biofloc systems, total suspended solids (TSS) are generally maintained at 200 – 500 mg L^{-1} (Hargreaves, 2013). Solids management is an important process in biofloc systems since the accumulation of TSS beyond these concentrations can lead to operational problems, including; 1) increased mixing and aeration costs to maintain the floc in suspension; 2) gill clogging in finfish; and 3) increased consumption of dissolved oxygen and the release of toxic metabolites from anaerobic metabolism such as hydrogen sulphide and ammonia (Hargreaves, 2013). Current techniques for solids removal include: flushing; siphoning; pumping sludge from the centre of ponds; and cyclone filters (swirl separators).

Solids removal remains one of the key constraints in land-based intensive aquaculture (Cripps and Bergheim, 2000); however, land-based systems, where effluent streams can be separated for the point source collection of particulate organic matter, offer the greatest scope for the development of bioremediation technologies. The effective and affordable treatment of waste solids is a key sustainability challenge for the aquaculture industry (Robinson *et al.*, 2015). Minimal or zero exchange systems represent expensive investments in terms of both construction and operation, due to high demands for aeration and/or pumping (Rurangwa and Verdegem, 2015); therefore, future developments that focus on removal and upcycling of particulate wastes should be seen as key to improving their economic viability (Verdegem, 2013). Furthermore, in order to promote nutrient recycling and minimise discharge, it is

recommended that future production systems should take an integrated approach (Verdegem, 2013). Given the limited opportunities that currently exist to valorise these wastes, there is significant scope to develop innovative approaches to aquaculture bioremediation technologies. In this thesis, the overall aim is to develop integrated aquaculture production and bioremediation systems for the sustainable treatment of particulate organic waste from land-based aquaculture.

1.2 Integration of deposit feeding sea cucumbers into land-based aquaculture systems

1.2.1 Use of particulate organic matter for the production of secondary organisms

Particulate organic waste originating from intensive land-based aquaculture, (a mixture of uneaten feed and faecal material) is a potential feed source for secondary culture species (Erler *et al.*, 2004; Brown *et al.*, 2011). Furthermore, there is potential to utilise heterotrophic biomass produced as a by-product in RAS filtration systems to farm secondary organisms. Organisms that could potentially feed on this waste include deposit feeding invertebrates such as polychaete worms (Brown *et al.*, 2011; Bischoff, 2012; Palmer *et al.*, 2014) and sea cucumbers (MacDonald *et al.*, 2013; Bossers, 2015). The integration of detritivores into land-based intensive aquaculture has been identified as an area for further research (Verdegem, 2013). To date, the majority of research on the integration of deposit feeding invertebrates, has focused on polychaetes; however, due to their high market value and demand, sea cucumbers are also excellent candidate extractive organisms for integration into aquaculture bioremediation systems (Cubillo *et al.*, 2016; Zamora *et al.*, 2016).

1.2.2 Deposit feeding sea cucumbers as candidate species for aquaculture bioremediation

Sea cucumbers are a traditional delicacy prized by the Chinese for their medicinal properties, and known locally as *bêche-de-mer* (the dried body wall) (Purcell, 2014). *Bêche-de-mer* fall into the same niche market as other luxury seafood products including shark fin, fish maw and abalone (Robinson, 2013). The expansion of trade and the rise in demand for *bêche-de-mer* in the latter part of the 20th century has led to the serial over-exploitation of global sea cucumber stocks, and aquaculture is now considered as the only means of meeting consumer demand (Anderson *et al.*, 2011; Robinson, 2013). Sea cucumber production is one aquaculture sector that has recently shown a marked increase (FAO, 2012); however, global sea cucumber production is less than one percent of global marine aquaculture production. Global annual sea production was ~209,000 tonnes in 2014, which comprised mostly the production of the temperate species *Apostichopus japonicus* (FAO, 2016a).

1.2.3 **Holothuria scabra** – ***a candidate species for aquaculture bioremediation***

Holothuria scabra, commonly known as sandfish, is the highest value tropical sea cucumber species, with an average market price of US$ 303 kg^{-1} with prices reaching as high as US$1,668 kg^{-1} for premium Grade A product (Purcell, 2014). The strong demand for *bêche-de-mer* led to the overexploitation of stocks in traditional Indo-Pacific fishing grounds adjacent to the main market, Hong Kong SAR and the species was recently placed on the International Union for Conservation of Nature (IUCN) red list as 'endangered' (Purcell *et al.*, 2014). *Holothuria scabra* is the only tropical species for which the aquaculture technology has been fully developed. While commercial production is gaining momentum, most of global aquaculture production of 63 tonnes per annum (FAO, 2016a) is derived from extensive production systems including sea ranching, sea pen farming and pond culture in countries ranging from Madagascar (Figure 1.2) to Fiji (Purcell *et al.*, 2012a; Robinson, 2013).

Figure 1.2. Community-based farming of *Holothuria scabra* in south-west Madagascar : a) delivery of hatchery-reared juveniles; b) release into protected nursery pens; c) cleaning and maintenance of pens; and, d) market-size H. scabra. Photos G. Robinson.

As an epibenthic deposit feeder that ingests sediment to extract organic matter (Yingst, 1976; Moriarty, 1982; Conand, 1990; Baskar, 1994), *H. scabra* is well suited to extensive production as it requires no additional feed, has fast growth rates and reaches market size (350 g) within 8-12 months (Figure 1.2c). Growth rates are linked to food availability and

carrying capacities in the wild are site specific, with yields ranging from 100 g m^{-2} to 700 g m^{-2} (Robinson and Pascal, 2012). The ecological attributes of *H. scabra* make them ideally suited for integration into land-based aquaculture bioremediation systems. In the wild, *H. scabra* inhabits low energy nearshore environments, including seagrass beds, mangrove areas, inner reef flats and estuaries that are subject to high terrigenous inputs and are natural sinks of organic matter (Hamel *et al.*, 2001). Furthermore, laboratory studies have indicated that *H. scabra* has strong chemosensory abilities, is attracted to mud or muddy-sandy habitats, and can rapidly identify areas of sediment that are enriched with organic matter (Baskar, 1994; Mercier *et al.*, 1999). *Holothuria scabra* has one of the highest sediment re-working rates of all tropical and temperate shallow-water deposit feeding sea cucumbers, and one adult can process on average 196.7 g d^{-1} (Yamanouchi, 1939; Roberts *et al.*, 2000). Studies have demonstrated that *H. scabra* can remove ~70 % of the organic matter present in organic rich sediments during one 12 hour feeding period (Mercier *et al.*, 1999).

Deposit feeding sea cucumbers are large-scale bioturbators that deliver critical ecosystem services that link to other trophic levels, through the regeneration of inorganic nutrients to support primary production. Deposit feeders that bioturbate the sediment through their movement, burrowing and feeding activities are prime candidates for inclusion into aquaculture bioremediation systems. However, research to date on the bioremediation potential of temperate sea cucumbers has focused on species with limited bioturbation potential (e.g. *Apostichopus japonicus*, *Australostichopus mollis* and *Holothuria forskali*), due to their rocky reef habitat and lack of a burial cycle (Sewell, 1990; Tuwo and Conand, 1992; Slater and Jeffs, 2010; Yamana *et al.*, 2010). *Holothuria scabra* is one of the few sea cucumber species that displays a diurnal burial cycle in which burying alternates rhythmically with feeding activities. This behaviour increases oxygen penetration to sediments, enhancing microbial activity and increasing the rate of organic matter degradation and nutrient remineralisation (Aller and Aller, 1998; Kristensen, 2000; Mermillod-Blondin and Rosenberg, 2006).

There has been considerable research on the potential to integrate *H. scabra* into land-based prawn aquaculture. Co-culture and IMTA models that integrate sea cucumber production are conceptually attractive, since spatial use is maximised; however, the potential to integrate *H. scabra* into extensive shrimp production systems has met with mixed results (Purcell *et al.*, 2006; Bell *et al.*, 2007; Watanabe *et al.*, 2012a). Initial trials in Vietnam demonstrated that *H. scabra* grew well in prawn ponds at low density, with growth rates of 2.2 – 3.2 g d^{-1} and exceeded growth in the natural environment (Pitt *et al.*, 2004; Agudo, 2006). Additionally, tank-based feeding trials have demonstrated that *H. scabra* can consume

and bioremediate particulate organic waste, including waste feed and faeces, originating from commercially important shrimp species, including *Litopenaeus stylirostris* and *Penaeus monodon* (Purcell *et al.*, 2006; Bell *et al.*, 2007; Watanabe *et al.*, 2012a). Watanabe *et al.* (2012a) found that *H. scabra* contributed to the bioremediation of hydrogen sulfide toxicity by reducing acid volatile sulfur (hydrogen sulfide and iron sulfide) in shrimp tank detritus by 55 %. However, despite promise to develop co-culture with shrimp and finfish, the majority of studies have concluded that co-culture is not viable due to the antagonist effects of the shrimp predating on the sea cucumbers, and that some form of physical or temporal separation is needed (Pitt *et al.*, 2004). Given the cost and practicality of physically protecting *H. scabra* from predation, rotational culture has become popular in countries such as Vietnam (Mills *et al.*, 2012).

To date, there are no published studies demonstrating the production of sea cucumbers, solely on aquaculture effluents, at commercially viable densities. This may be since, to date, the majority of studies examining the bioremediation potential of sea cucumbers have been conducted in bare tanks (

Figure 1.3), thus failing to adequately simulate the natural environmental conditions. Watanabe *et al.* (2012a) observed the fastest growth rates of *H. scabra* in bare tanks when fed shrimp faeces, however overall growth rates were slow and only marginally positive, ranging from 0.018 g d^{-1} to 0.052 g d^{-1} (Watanabe *et al.*, 2012a). The importance of providing sand as a substrate to support positive growth of *H. scabra* reared in nursery and grow-out tanks has been previously demonstrated (Battaglene *et al.*, 1999; Pitt *et al.*, 2001; Pitt *et al.*, 2004; Lavitra *et al.*, 2010; Watanabe *et al.*, 2012a; Robinson *et al.*, 2013).

Figure 1.3. Integrated multi-trophic aquaculture of *Holothuria forskali* with the starry flounder *Platichthys stellatus* in bare tanks in Germany. Photo courtesy of Matthew Slater (Zamora *et al.*, 2016).

Neglecting the inclusion of a sediment component has undoubtedly reduced the overall bioremediation potential and foregone the critical role played by sediment microbial communities in organic matter mineralisation, nutrient cycling, and the productivity gains attributable to *in situ* benthic recycling. Sediments are critically important for: 1) supporting the food sources of sea cucumbers; 2) mediating the biogeochemical processes inherent in mineralising aquaculture wastes; 3) supporting benthic productivity; and, 4) sustaining positive growth rates (Robinson *et al.*, 2013). Until studies are underpinned by strong survival and growth performance, citing palatability of aquaculture waste or decreases in carbon or nitrogen content alone (Slater *et al.*, 2009; MacDonald *et al.*, 2013), will not engender support for the integration of deposit feeding sea cucumbers into land-based aquaculture systems, since the economic viability is directly dependent on maximising outputs (biomass) and minimising the footprint. This thesis takes an ecological, systems-based approach to bioremediation by recognising the ecological attributes of *H. scabra*, its role in biogeochemical cycling, and interaction with its food resources.

1.3 Marine sediments

1.3.1 Biogeochemical cycling of carbon and nitrogen

Marine sediments represent a natural biological filter, housing microbial communities, comprising bacteria, archaea, protozoa, microalgae, eukaryotes, viruses and fungi, that mediate the biogeochemical cycling of key elements (C, N and P). Organic matter is degraded (mineralized) by an array of aerobic and anaerobic microbial processes with a concurrent release of inorganic nutrients (Kristensen, 2000). In naturally stratified sediments, aerobic and anaerobic processes are partitioned into oxic and anoxic layers respectively (Figure 1.4a). In the oxic layer, oxygen is used preferentially as the electron acceptor to support aerobic microbial oxidation - since this is the most thermodynamically efficient mechanism (Kristensen *et al.*, 1995). The availability of oxygen determines the metabolic pathway (aerobic or anaerobic) used by bacteria. In the anoxic layers, bacteria switch to the metabolic pathways of fermentation and anaerobic respiration utilising chemically bound forms of oxygen as terminal electron acceptors (e.g. NO_3^-, SO_4^{2-}). These electron acceptors are used in a sequential manner according to their thermodynamic potential, which dictates the vertical dominance of microbial communities (Middelburg *et al.*, 1993; Kristensen, 2000; Fenchel *et al.*, 2012). As aerobic and anaerobic respiration are reduction reactions, an electron donor is required; however, in fermentation the organic substrate is oxidised and reduced simultaneously; therefore, the redox state of the substrate remains unchanged and no external electron acceptors are involved (Fenchel *et al.*, 2012).

Since carbon and nitrogen comprise the bulk of all living organisms, the nitrogen cycle is closely tied to the carbon cycle. The nitrogen cycle is one of the most important and complex biogeochemical cycles on earth, since nitrogen exists in eight different oxidation states, ranging from -3 in ammonium (NH_4^+) to +5 in nitrate (NO_3^-) (Thamdrup and Dalsgaard, 2008). Nitrogen is a critically important element since it is used in assimilatory (anabolic; biomass-producing) reactions for the biosynthesis of cellular components, such as nucleic acids and proteins (Capone, 2000; Kirchman, 2012); and, as an energy source by microorganisms in dissimilatory (catabolic; energy-producing) reactions (Thamdrup and Dalsgaard, 2008; Fenchel *et al.*, 2012).

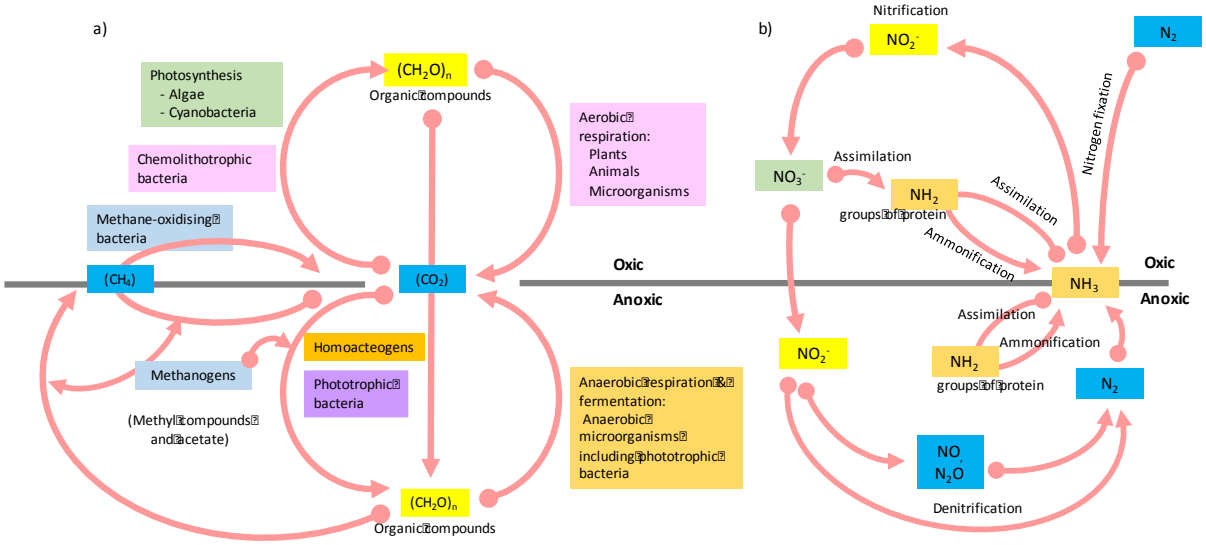

Figure 1.4. Reduction-oxidation (redox) cycles for a) carbon and b) nitrogen. Adapted from Madigan *et al.* (2003).

Biogeochemical cycling of nitrogenous wastes, originating from the decomposition of organic nitrogen (ammonification), are redox reactions involving electron acceptors and electron donors. As in the carbon cycle, these redox reactions are similarly partitioned into the oxic and anoxic layers of marine sediments (Figure 1.4b). In the aerobic sediment surface layer, ammonium, a primary decomposition product from particulate organic matter, is oxidised to nitrite (NO_2^-) and then nitrate (NO_3^-) in a step-wise reaction of nitrification, mediated by chemoautotrophic ammonia-oxidising bacteria and nitrite-oxidising bacteria respectively (Blackburn and Blackburn, 1992).

Under anoxic conditions, NO_3^- and NO_2^- can be further transformed to nitrogen gas (N_2) by denitrification; thus, in marine sediments, coupled nitrification-denitrification occurs naturally across the oxic-anoxic interface. Anaerobic oxidation of ammonia (anammox) is a more recently discovered microbially-mediated anaerobic process, which like denitrification

leads to the permanent removal of nitrogen by conversion to N_2. Dissimilatory reduction of nitrate to ammonia (DNRA) is an alternative form of anaerobic nitrate respiration that transitions nitrogen from the +5 to the -3 oxidation state (Thamdrup, 2012; Song *et al.*, 2014). Nitrogen fixation, is the only reaction that adds new 'unfixed' nitrogen to the cycle, carried out by only a few microbial groups due to the high energy required to break the triple bond in dinitrogen gas (Capone, 2000).

1.3.2 Deposit feeding sea cucumbers as components of the benthic recycling system

Deposit feeding sea cucumbers are an important component of this benthic recycling system in marine sediments, that degrades organic matter and releases inorganic nutrients for primary production (Uthicke, 2001; Fenchel, 2008). In the natural environment, sea cucumbers are major components for sustaining coastal ecosystems in tropical areas, and act as ecosystem "engineers" that increase the structural complexity of the habitat (Coleman and Williams, 2002; Robinson and Pascal, 2009). The feeding, locomotory, burial and respiratory activities of deposit feeders, collectively referred to as 'bioturbation', result in physico-chemical habitat modifications of marine sedimentary environments including increasing sediment permeability, particle transport, and pore-water exchange. Bioturbation helps to maintain an oxidised layer in the sediment and increases oxygen penetration to reduced sediments, enhancing microbial activity and increasing mineralisation rates (Aller and Aller, 1998; Kristensen, 2000; Mermillod-Blondin and Rosenberg, 2006). In shallow water ecosystems, these interactions at the sediment-water interface, play a critical role in influencing the benthic-pelagic exchange of oxygen and nutrients (Rice and Rhoads, 1989; Aller, 1994; Burford and Longmore, 2001). The net effect of particle re-working and increased water and solute exchange is an enhancement of microbial metabolism and growth rates, and a concomitant increase in the rate of organic matter degradation and nutrient remineralisation (Kristensen, 2000; Mactavish *et al.*, 2012). The potential to harness this important ecosystem function of 'biostimulation' by exploiting the link between the organism and sediment microbial communities that mediate nutrient cycling is the fundamental basis underpinning microbial—deposit feeder aquaculture bioremediation systems.

Studies conducted in the natural habitat that have taken the sediment into account have indicated that sea cucumbers are beneficial to sediment health (Uthicke, 1999; Wolkenhauer *et al.*, 2010). *Holothuria scabra* is able to biomitigate sediment eutrophication and improve sediment quality by reducing dissolved oxygen consumption, increasing oxygen penetration, increasing the depth of the oxic-anoxic interface, and increasing the sediment reduction-oxidation potential (Wolkenhauer *et al.*, 2010). Deposit feeders stimulate the rates of carbon

mineralisation, evidenced by decreased organic carbon contents, increased oxygen consumption, and increased effluxes of dissolved inorganic carbon (Kristensen, 2001; Papaspyrou *et al.*, 2007; Mactavish *et al.*, 2012). In addition, due to the tight coupling between carbon and nitrogen cycles, bioturbation also has a significant impact on marine nitrogen cycling (Laverock *et al.*, 2011). Deposit feeding invertebrates have been shown to enhance the efflux of ammonium in marine sediments, and increase rates of nitrification by increasing the depth of the oxic-anoxic interface (Blackburn and Henriksen, 1983). Importantly, the influence of sea cucumbers on benthic-pelagic exchange is significantly enhanced in diffusion-dominated systems such as marine sediments subject to high organic loading (Mermillod-Blondin and Rosenberg, 2006).

1.3.3 Food resources and limitation of deposit feeder growth

Sea cucumber growth is limited by the availability of food resources (Battaglene *et al.*, 1999; Robinson and Pascal, 2012). In the wild, sediments ingested by holothurians comprise a diverse mix of organic and inorganic compounds, comprising living and non-living components (Lopez and Levinton, 1987). As deposit feeding animals meet their nutritional requirements from the organic fraction of the sediment, the inorganic fraction passes relatively unmodified through the gut with minimal impact on either particle size or dissolution (Lopez and Levinton, 1987). Food resources that potentially limit deposit feeder growth, namely sediment particles and microbes (bacteria, diatoms and other microorganisms) can be considered as renewable resources (Levinton and Lopez, 1977). To better understand factors that limit deposit feeder growth, the exploitation of the resource can be considered as a balance of exploitation and renewal; thus, the greater the rate of renewal, the greater the population that can be supported (Levinton and Lopez, 1977).

In shallow marine sediments, deposit feeders in concert with microbes play a major role in enhancing the rates of nutrient cycling and support the renewal of their food resources (Uthicke, 2001). Sea cucumbers therefore have the advantage that they can enhance the productivity of a renewable resource, and hence the rate of renewal (Levinton and Lopez, 1977). By considering *H. scabra's* food resources (sediment particles, bacteria and benthic microalgae) as renewable resources, the only missing element is the supply of particulate organic matter to drive the sediment biology (Fenchel *et al.*, 2012). The potential to overcome food limitation in sea cucumber aquaculture utilising particulate organic matter from intensive aquaculture is immense when you consider that, 1) the effect of food quantity on sea cucumber growth is a supply phenomenon, and 2) food resources are renewable resources (Jangoux and Lawerence, 1982).

1.3.4 Trophic transfer efficiencies in benthic food webs

Sediment-based systems that integrate deposit feeding sea cucumbers with a high bioturbation potential offer a natural basis for the development of aquaculture bioremediation technologies. The exploitation of microbial assemblages as an essential link in the benthic food web represents a highly efficient mechanism for transferring energy and nutrients (Schroeder, 1987). In natural aquatic environments, nutrients are recycling via the traditional food web, based upon primary production, accompanied in parallel by a microbial loop (Azam *et al.*, 1983; Avnimelech, 2014). In shallow marine sediments, deposit feeding sea cucumbers are an important component of a benthic recycling system, similar to the microbial loop, that degrades organic matter and releases inorganic nutrients for primary production (Uthicke, 2001). Feed efficiencies in this deposit feeder-microbial loop in marine sediments are much higher than in traditional food webs, since on average, bacterial growth utilises 40 - 60 % of substrates (organic carbon sources) assimilated for growth, while higher organisms utilise only about 10 % (Pauly and Christensen, 1995; Avnimelech, 2014). Given the high trophic transfer efficiencies, deposit feeder-microbial food chains offer great potential for manipulation (Moriarty, 1987). Furthermore, as microbial assemblages are the basis of the heterotrophic food web and the link with higher trophic levels (Schroeder, 1987), it is of great interest to exploit bacterial biomass as a direct food source for culture species, and in this way, increase the overall energy transfer efficiency.

Integration of deposit feeding sea cucumbers into sediment-based aquaculture bioremediation systems should increase the assimilative capacity of the system, directly via the consumption of particulate organic matter, and indirectly through the stimulation of benthic metabolism. Sea cucumbers have the capacity to maintain high sediment permeability through the selective ingestion of fine particulate organic matter. In addition, bioturbation will increase mineralisation rates by increasing the availability of energetically favourable electron acceptors such as oxygen. Furthermore, the indirect effects of bioturbation stimulating sediment microbial community metabolism are positively correlated with sea cucumber density (Yuan *et al.*, 2016), indicating the potential to maximise organic loading during high density sea cucumber production. While the addition of deposit feeding sea cucumbers has the potential to balance the input of organic matter, it may not be sufficient to cope with high organic loading rates experienced under *in situ* conditions in integrated land-based aquaculture bioremediation systems.

1.3.5 Impact of organic loading on marine sediments

Marine sediments are primarily heterotrophic systems (Fenchel *et al.*, 2012; Kirchman, 2012); consequently, the quantity of particulate organic matter input to the

sediment is the most important factor controlling microbial processes. The rate of organic enrichment in the sediment is an important variable in controlling the dynamics and metabolic activity of microbial communities (Alongi and Hanson, 1985). The rate of carbon loading is used to define the trophic status of coastal marine ecosystems since carbon is the principal element in organic matter (Nixon, 1995). In marine sediments, the input of organic matter is directly related to the position of the oxic-anoxic interface, hence increasing rates of organic loading increases the sediment oxygen demand, decreases oxygen concentrations and reduces the depth of the oxic-anoxic interface (Brune *et al.*, 2000; Kristensen, 2000). In oligotophic environments (<100 g C m^{-2} y^{-1}), oxygen penetration can range from a few centimetres to a few metres, while in mesotrophic or eutrophic environments (100-500 g C m^{-2} y^{-1}) with high primary productivity, oxygen rarely penetrates more than a few millimetres (Brune *et al.*, 2000; Pinckney *et al.*, 2001).

The upper tolerance for organic loading for benthic macrofauna ranges from 100-400 mmol C m^{-2} day^{-1} (1 200 – 4 800 C m^{-2} day^{-1}), which is defined as 'eutrophic' (Lehtoranta *et al.*, 2009). Particulate organic matter originating from land-based intensive aquaculture is labile and easily degraded. Under rates of high organic loading, as experienced in aquaculture operations, the high chemical and biological oxygen demand causes a progressive movement of anoxic conditions towards the sediment surface. If combined with stagnant conditions in the overlying water, excessive rates of organic loading can induce large-scale bottom water hypoxia (<2 mg O_2 L^{-1}) and anoxia (no detectable dissolved O_2). The critical mean sub-lethal concentration for 50 % of benthic species is considered to be 2.61 mg O_2 L^{-1} (Vaquer-Sunyer and Duarte, 2008). For sea cucumbers that inhabit the sediment-water interface, the risk of crop losses is particularly elevated due to the deterioration of sediment quality, leading to induced water column hypoxia, evisceration and mortality (Zamora and Jeffs, 2011). Furthermore, as poor water and sediment quality are reputed to be one of the key drivers of disease in land-based intensive aquaculture, it is advisable to safeguard and mitigate against this occurring.

Maximising the input of organic matter to the sediment is the key to overcoming density limitation of deposit feeder growth. Within limits, increasing the quantity and frequency of the addition of particulate organic matter will increase the biomass of microbial communities and the density of benthic macrofauna, since the flux of particulate organic matter is a key determinant of the carrying capacity of deposit feeder populations (Olafsson, 1986; Church, 2008; Fenchel *et al.*, 2012). However, the degradative activity of microbial communities is a self-regulatory system, and in intensive aquaculture systems, high rates of organic loading and/or the accumulation of solid wastes, frequently exceed microbial

mineralization capacities in sediments (Hargreaves, 1998; Antony and Philip, 2006). For exhaustive degradation of organic matter, there is a need to increase the assimilative capacity of a sediment-based system by stimulating the natural capacity of microbial communities for organic matter mineralization.

1.4 Application of bioremediation technologies

1.4.1 Factors controlling microbial growth

Stimulation of indigenous microbial communities or microbial processes to degrade environmental contaminants or wastes is the fundamental principal underpinning bioremediation (Baker and Herson, 1994). Accordingly, bioremediation technologies are centred on the manipulation of factors that control the kinetics of substrate uptake by microorganisms and their growth efficiencies in terms of how the substrate utilisation is coupled to growth (Colleran, 1997; Chávez-Crooker and Obreque-Contreras, 2010). The factors regulating decomposition and links between the mineralisation of particulate organic matter and coupling with the remineralisation of inorganic nutrients have been recognised as key to manipulating the transfer to carbon and energy in microbial—deposit feeder systems (Anderson, 1987). Therefore, an understanding of the factors that control and limit microbial growth is critical to the development of microbial-based aquaculture bioremediation systems. A range of factors play a role in regulating microbial communities, including; abiotic factors such as temperature, nutrient availability, pH, light; and biotic factors including predation, disease and competition (Church, 2008). In natural ecosystems, the maximum microbial population size is dependent on the availability of resources, while the actual size is controlled by biotic factors (Church, 2008). In this research, a bottom-up approach to understanding resource control of microbial growth by energy or nutrients is adopted.

Microbial growth depends on the availability of organic substrates in terms of quantity and quality. Organic substrate concentration is the most important factor limiting bacterial growth since these are required for energy and the synthesis of new cellular material. Resource availability can limit growth rates or the standing biomass of microbial populations (Church, 2008). Resource control of microbial growth rates and yields are characterised by a Monod growth model, where the growth rate is a function of the extracellular concentration of the limiting nutrient or energy source (Church, 2008). Bacterial growth efficiency (BGE) is another important concept in understanding how substrate utilisation is coupled to growth. This is defined as the quantity of bacterial biomass synthesised per unit of substrate assimilated (Church, 2008; Fenchel *et al.*, 2012; Kirchman, 2012). It thus describes the relative proportion of carbon that is used for energy production (respired) versus the part that

is used for anabolic reactions leading to the biosynthesis of new cells. While this concept is generally used to described the use of organic substrates, by heterotrophic bacteria (Church, 2008; Fenchel *et al.*, 2012), it can equally be applied to autotrophic processes by considering the fraction of the electron donor used for energy (dissimilatory) or biosynthesis (assimilatory; Ebeling, *et al.*, 2006).

This concept is useful to compare biomass production between autotrophs and heterotrophs and is fundamental to understanding how substrate utilisation is coupled to growth. Compared to heterotrophs, the biomass yield of autotrophic bacteria is extremely low. For example, in nitrification, over 90 % of the electron donor is used for energy production, with only 6.2 % used for biosynthesis, resulting in very slow growth rates and biomass yields (Ebeling *et al.*, 2006). For heterotrophic bacteria, the BGE is affected by a number of factors including: 1) the reduction-oxidation potential (i.e. the availability of electron acceptors); and, 2) the 'quality' of the organic substrate that serves as an electron donor. Thus, under aerobic conditions with a labile carbon source, heterotrophic bacteria use approximately 70 % of the substrate for biosynthesis, which is more efficient than chemolithotrophic production, leading to rapid growth rates and biomass production (Ebeling *et al.*, 2006).

Substrate availability is therefore the main factor controlling the biomass of both microbial and deposit feeder populations (Olafsson, 1986; Church, 2008); however, this factor can be easily controlled in land-based aquaculture bioremediation systems by controlling the volume and frequency of waste additions, to overcome the biomass density limitation of growth. Another important parameter regulating microbial and deposit feeder growth is resource quality. There are two factors that require consideration when defining the 'quality' of the organic substrates: 1) the biochemical composition of the substrate; and, 2) the elemental ratio of C:N:P. Elemental stoichiometry is the domain of science that uses the ratio of carbon to macronutrients such as nitrogen and phosphorous to describe food quality for heterotrophs (Bukovinszky *et al.*, 2012). In the bottom-up approach to understanding resource limitation control of microbial populations, elemental stoichiometry provides a useful framework to determine whether bacteria are limited by a particular substrate (Church, 2008). Bacteria require carbon, nitrogen and phosphorus in a stoichiometric balance set by the physiological state of the cell population (Herbert, 1967). The relative abundance of key elements (C, N and P) is determined by the biochemical composition of the substrate (Kirchman, 2012); however, the biochemical structure determines the lability of the substrate. Carbon compounds can have similar bulk elemental stoichiometry, but differ considerably at the biochemical level, affecting their utilisation as substrates (Schroeder, 1987; Fenchel *et al.*, 2012). Additionally, since the rates of remineralisation of nitrogen and phosphorous are

stoichiometrically related to the organic matter degradation, the rate of inorganic nitrogen regeneration is influenced by the elemental stoichiometry of organic matter inputs (Tezuka, 1990; Aller and Aller, 1998). The amount of substrate assimilated and the amount of nitrogen regenerated are therefore dependent on a combination of redox potential, the element ratio and the biochemical composition of the substrate since these factors directly impact BGE (Goldman *et al.*, 1987; Tezuka, 1990).

1.4.2 Biostimulation

Biostimulation is a technique commonly employed in *in situ* or *ex situ* bioremediation techniques that involves the modification of the existing environment to stimulate microbial metabolism (Baker and Herson, 1994; Colleran, 1997). Since BGE is dependent on the reduction-oxidation potential and microbially mediated nutrient cycles are principally reduction-oxidation reactions, biostimulation typically involves the addition of rate-limiting electron acceptors and electron donors. Substrate concentration and the availability of electron acceptors are two key factors that affect the energy yield of metabolic processes (Fenchel *et al.*, 2012). In land-based aquaculture, the potential for point source collection of waste and transfer to different system components, increases the scope for the development of *ex situ* bioremediation technologies, where factors can be controlled and system performances optimised. Since resource quantity can be controlled, the focus in this research is on the manipulation of microbial communities by increasing the availability of electron donors and electron acceptors. The supply of external electron acceptors, such as oxygen, is one method used by *in situ* bioremediation technologies to alleviate the constraints of the naturally slow process of mineralisation (Alexander, 1994; Colleran, 1997). Conversely, biostimulation technologies may also employ the supply of rate-limiting electron donors. This technique is particularly suited to anaerobic environments, where reduction reactions may be limited by the availability of organic carbon substrates to act as electron donors (Colleran, 1997). Given that microbial remediation of aquaculture wastes is limited by the availability of organic carbon (Schneider *et al.*, 2007b), this technique may be broadly applicable to the manipulation of sediment-based aquaculture bioremediation systems.

1.4.3 Combining integrated multi-trophic aquaculture, microbial-based systems and bioremediation technologies

Recent reviews have advocated microbial-based systems and IMTA — newly emerging technologies based on nutrient recycling within the culture system — as the most promising options for sustainable aquaculture (Chávez-Crooker and Obreque-Contreras, 2010; Martinez-Porchas *et al.*, 2014). However, the potential to combine IMTA and

microbial-based approaches and couple them with existing bioremediation technologies remains a largely unexplored and novel concept. The overall goal of this thesis is to develop innovative approaches to link deposit feeders into bioremediation technologies for the sustainable treatment of particulate organic waste from land-based intensive marine aquaculture. In this thesis, the application of two key principles underpinning low-cost bioremediation technologies, namely the addition of rate limiting (i) electron acceptors (oxygen), and (ii) donors (carbon), to sediment-based aquaculture effluent treatment systems integrating *H. scabra,* is investigated. Understanding the functioning of sediment microbial dynamics and their concomitant effects on the delivery of key ecosystem services, in terms of biogeochemical cycling and secondary (heterotrophic) production is a pre-requisite to their manipulation. Consequently, a coupled approach of combining long-term growth trials with next generation sequencing (NGS) is used to investigate the effects of manipulated sediment-based systems on deposit feeder growth and microbial community structure and function.

1.5 Outline of thesis

Chapter 2 aims to determine the optimum physico-chemical parameters for the design of sediment-based bioremediation systems integrating deposit feeding sea cucumbers in land-based recirculating aquaculture systems (RAS). The effects of sediment redox regime, depth and particle size on the growth, biochemical composition and behaviour of juvenile *H. scabra* are investigated. It is hypothesised that increasing oxidant supply will increase the assimilative capacity of the sediment to process organic matter and prevent the formation of hypoxic conditions at the sediment-water interface. The effect of contrasting redox regimes, describing fully oxic and redox-stratified sediments, on organic matter mineralisation and nutrient cycling are discussed in relation to the quantity and quality of food resources available to sea cucumbers.

Chapter 3 advances the observations detailed in Chapter 2 by characterising the diversity, structure and predicted function of the microbial communities present in the sediment of *H. scabra* culture tanks subjected to contrasting redox regimes (oxic and oxic-anoxic) using high-throughput 454-pyrosequencing of barcoded 16S ribosomal ribonucleic acid (rRNA) genes. It is hypothesised that increasing the availability of oxygen, via the percolation of oxygenated water, will increase the diversity and functional potential of microbial communities for the bioremediation of aquaculture wastes.

Chapter 4 investigates the effect of resource quality on growth of *H. scabra* reared on particulate organic waste from an intensive abalone RAS. The experiment aims to test the application of carbon to nitrogen ratio (C:N) manipulation to sediment-based systems. The

effect of increasing the C:N of aquaculture waste by carbon supplementation is investigated and a range of carbon sources of differing biochemical composition and degradation rates are tested. It is hypothesised that increasing the C:N of aquaculture waste from 5:1 to 20:1 will increase the growth rate and biomass density of *H. scabra* reared on redox-stratified sediments.

Chapter 5 advances the findings of the previous chapter by investigating the effect of C:N manipulation on pathways of benthic nitrogen cycling in manipulated sediment mescocosms. A coupled biogeochemical-molecular approach is used: incubation experiments are conducted to quantify benthic fluxes of gases and nutrients, while sediment microbial communities are examined using 16S rRNA gene sequencing. The study aimed to test the hypothesis that increasing the C:N of particulate aquaculture waste from 5:1 to 20:1 by carbon supplementation would promote the assimilation of NH_4^+ by heterotrophic bacteria.

In Chapter 6 the role of endogenous bacterial communities in the sea cucumber microbiome during the remediation of aquaculture wastes is examined. The experiment is designed to investigate effects on the gut microbiome and long-term growth performance of *H. scabra* reared on two waste streams originating from land-based intensive aquaculture (shrimp pond sediments and particulate organic waste from a RAS). It was hypothesized that microbial communities would exhibit high turnover along the gut due to digestion in the midgut, but that communities present in the gut microbiome would play a role in the provisioning of key nutrients, such as amino acids, to *H. scabra*.

Finally, Chapter 7 provides a synthesis of the key findings of the research conducted in this thesis. The implications of the research are discussed within the wider context of global nitrogen cycles and the sustainable development of the aquaculture industry. An outline of the proposed technology is given and the potential for technology transfer to other species and aquaculture production systems is highlighted. Finally, key priorities and recommendations for future research are outlined.

Chapter 2. Determination of key physico-chemical parameters in the design of sediment-based aquaculture bioremediation systems

2.1 Introduction

The ecological attributes of *Holothuria scabra* are ideally suited for integration into bioremediation systems to treat particulate organic waste from land-based intensive aquaculture. The species displays a strong affinity for organically rich sediment, an ability to rapidly deplete organic matter and a diurnal burial cycle that enhances their role as bioturbators (Mercier *et al.*, 1999; Hamel *et al.*, 2001; Purcell, 2010). Studies have demonstrated that *H. scabra* can be reared successfully using particulate aquaculture wastes (uneaten feed and faeces) as a sole feed source (Watanabe *et al.*, 2012a), and can bioremediate pond sediments when farmed in rotation with shrimp (Purcell *et al.*, 2006; Bell *et al.*, 2007). Harnessing these attributes will likely increase the assimilative capacity of sediment-based aquaculture bioremediation systems, both directly, through the consumption of particulate organic matter, and indirectly, through the overall stimulation of benthic metabolism.

Most studies focusing on the bioremediation potential of sea cucumbers have been conducted in bare tanks and have neglected to recognise the critical role played by the sediment (Yuan *et al.*, 2006; Slater *et al.*, 2009; MacDonald *et al.*, 2013; Bossers, 2015). Previous studies have confirmed the importance of providing sand as a substrate to induce viable growth in nursery and grow-out tanks (Battaglene *et al.*, 1999; Pitt *et al.*, 2001; Lavitra *et al.*, 2010; Watanabe *et al.*, 2012a; Robinson *et al.*, 2013). The origin, type and source of the substrate are important considerations: the source should be sustainable; not cause adverse impacts on the marine environment; and, reflect the natural habitat of the species. Excavation of beach sand (Battaglene *et al.*, 1999; Pitt *et al.*, 2001; Watanabe *et al.*, 2014) or dredging of marine sediments for use as a substrate (Lavitra *et al.*, 2010) or feed ingredient (Liu *et al.*, 2009; Xie *et al.*, 2016) are considered unsustainable. In the wild, *H. scabra* inhabits nearshore seagrass and mangrove ecosystems where the sediment is predominately calcium carbonate, originating from the erosion of coral reef ecosystems (Hamel *et al.*, 2001; Plotieau *et al.*, 2013a). Previous research demonstrates that commercially available calcium carbonate 'bedding' or 'builders' sand, mined from land-based coastal sedimentary deposits, can provide a suitable substrate for land-based culture of deposit feeders such as polychaete worms and sea cucumbers (Palmer, 2010; Robinson *et al.*, 2013).

Determining the optimum physico-chemical sediment parameters is a prerequisite for the successful design of land-based bioremediation systems. From a biogeochemical perspective, a number of factors affect the rate of organic matter mineralisation in sediments. These include; organic matter characteristics, temperature, pH, reduction-oxidation (redox) potential, bioturbation, sediment depth, and grain size (Stahlberg *et al.*, 2006). A number of studies have examined the effects of sediment particle size and depth on *H. scabra* growth and survival (Battaglene *et al.*, 1999; Mercier *et al.*, 1999; Pitt *et al.*, 2001); however, the potential to manipulate the sediment redox regime has not been investigated.

The supply of external electron acceptors, such as oxygen, is one method used by *in situ* bioremediation technologies to alleviate the constraints of the naturally slow process of mineralisation. In sediment-based systems, high rates of organic loading can result in the release of toxic metabolites (e.g. ammonia, nitrite, and hydrogen sulphide) and induce water column hypoxia. For deposit feeding sea cucumbers that live at the sediment-water interface, the maintenance of an oxidised sediment layer is critical as a deterioration in sediment quality may negatively impact *H. scabra* growth and health (Avnimelech, 2003). In sediment-based bioremediation systems, increased oxidant supply through the percolation of oxygen saturated water, is commonly used to stimulate the *in situ* aerobic degradation of organic matter (Colleran, 1997). Increasing oxidant supply could not only further increase the assimilative capacity of the effluent treatment system, but could potentially provide an important safe-guard against mortalities and health issues through the maintenance of an oxidized sediment layer.

Robinson *et al.* (2013) concluded that future research into intensive technologies for *H. scabra* culture should aim to identify the optimum substrate characteristics and develop tank holding systems capable of maintaining favourable substrate conditions. In this chapter, the effects of sediment redox regime, depth and particle size on the growth, biochemical composition and behaviour of juvenile *H. scabra* were investigated. It was hypothesised that increasing oxidant supply would increase the assimilative capacity of the sediment for organic matter and prevent the formation of hypoxic conditions at the sediment-water interface.

2.2 Materials and methods

2.2.1 Study site

The study was conducted at HIK Abalone Farm (Pty) Ltd in Hermanus, on the southwest coast of South Africa (34°26’04.35”S; 19°13’12.51”E) between 20th March and 9th June 2012.

2.2.2 Experimental animals

Two thousand hatchery-reared juvenile *H. scabra* weighing two grams each were imported from a commercial hatchery (Madagascar Holothurie S.A., Madagascar) on November 3rd 2011 and quarantined in a biosecure facility for six weeks in accordance with South African importation and scientific investigations licences. Following the quarantine period and prior to experimentation, the animals were held in a recirculating aquaculture system in tanks filled with 4.0 cm of calcium carbonate sand and fed a 34 % protein commercial abalone weaning diet (Abfeed®-S34, 1.0 mm sugar grain pellet; Marifeed Pty Ltd, South Africa).

2.2.3 Experimental design

A 2 x 2 x 2 factorial design (Table 2.1) was used to investigate the response of *H. scabra* to:

(1) the sediment redox regime (oxic versus oxic-anoxic);

(2) sediment depth (two and four centimetres); and

(3) sediment particle size (fine: 125 – 250 μm; medium: 250 – 500 μm).

Table 2.1. Description of the eight treatments in the multifactorial experimental design.

Treatment no.	Tank structure	Aeration	Factor 1: Redox regime	Factor 2: Sediment depth	Factor 3: Particle size
1	Plenum	Airlift pump	Oxic	2 cm	Fine
2	Plenum	Airlift pump	Oxic	2 cm	Medium
3	Plenum	Airlift pump	Oxic	4 cm	Fine
4	Plenum	Airlift pump	Oxic	4 cm	Medium
5	No plenum	Airstone	Oxic-anoxic	2 cm	Fine
6	No plenum	Airstone	Oxic-anoxic	2 cm	Medium
7	No plenum	Airstone	Oxic-anoxic	4 cm	Fine
8	No plenum	Airstone	Oxic-anoxic	4 cm	Medium

2.2.4 Experimental system and rearing conditions

Eight experimental treatments were allocated to 32 polyethylene tanks (455 x 328 x 175 mm; Figure 2.1d) using a randomised block design of eight tanks distributed into four blocks with one replicate per block (Table 2.1). Tanks were supplied with heated coastal seawater (25.71 ± 0.05 °C) at a flow rate of 0.75 L min^{-1} $tank^{-1}$ filtered through a recirculating system comprising a composite sand filter (Figure 2.1b), protein skimmer and fixed film biological filter (Figure 2.1c). Two three-kilowatt titanium Hotrod heating elements were used to heat the incoming ambient seawater (Hi-Tech Elements Pty Ltd, South Africa).

Figure 2.1. The experimental recirculating aquaculture system comprising the: a) sump containing two Hotrod heating elements to heat the incoming coastal seawater; b) sand filter; c) biological filter; and, d) experimental tanks fitted with an internal shade cloth liner to contain the sand substrate. NB: photos c and d were taken prior to re-roofing the experimental system with clear polycarbonate roof panels to allow natural light to penetrate.

Aeration was supplied continuously, except during feeding when the air and water supplies were interrupted for fifteen minutes to allow the feed to settle. Feeding was standardised across experimental treatments. Sea cucumbers were fed a 34 % protein commercial abalone weaning diet (Abfeed®-S34, 1.0 mm sugar grain pellet; Marifeed Pty Ltd, South Africa) once per day at 16:00 hours. This commercially available formulated feed was selected as a reference diet to provide a baseline for comparison in future feeding trials using particulate organic aquaculture waste. Daily feed rations were calculated at one percent of the total tank biomass and adjusted every two weeks based on predicted biomass gains over the two week period (Battaglene *et al.*, 1999). Decaying uneaten food and any arising white bacterial patches were removed by siphoning every 48 h (present in oxic-anoxic treatments only). All tanks were cleaned once per month, tank walls were manually scrubbed to remove biofilms and any epiphytic algae or cyanobacteria. Experimental tanks were subject to a natural photoperiod which decreased from 12.07:11.53 L:D (0646 to 1854 hours, sunrise to sunset) to 09.53:14.07 L:D (0745 to 1739 hours, sunrise to sunset) as day length decreased with the onset of winter.

The sediment comprised calcium carbonate 'builders sand' sourced from a commercial dune quarry (SSB Mining, Macassar, South Africa). The sediment was sieved to achieve the requisite particle sizes using a series of decreasing nylon mesh sizes (500, 250 and 125 µm). An internal tank liner made from 95 % shade cloth was used to contain the sediment in all the tanks (Figure 2.1d). A manipulated sediment system with a fully oxic redox regime was created by the addition of a plenum and airlift pump to actively circulate oxygen saturated water through the sand sediment (Figure 2.2).

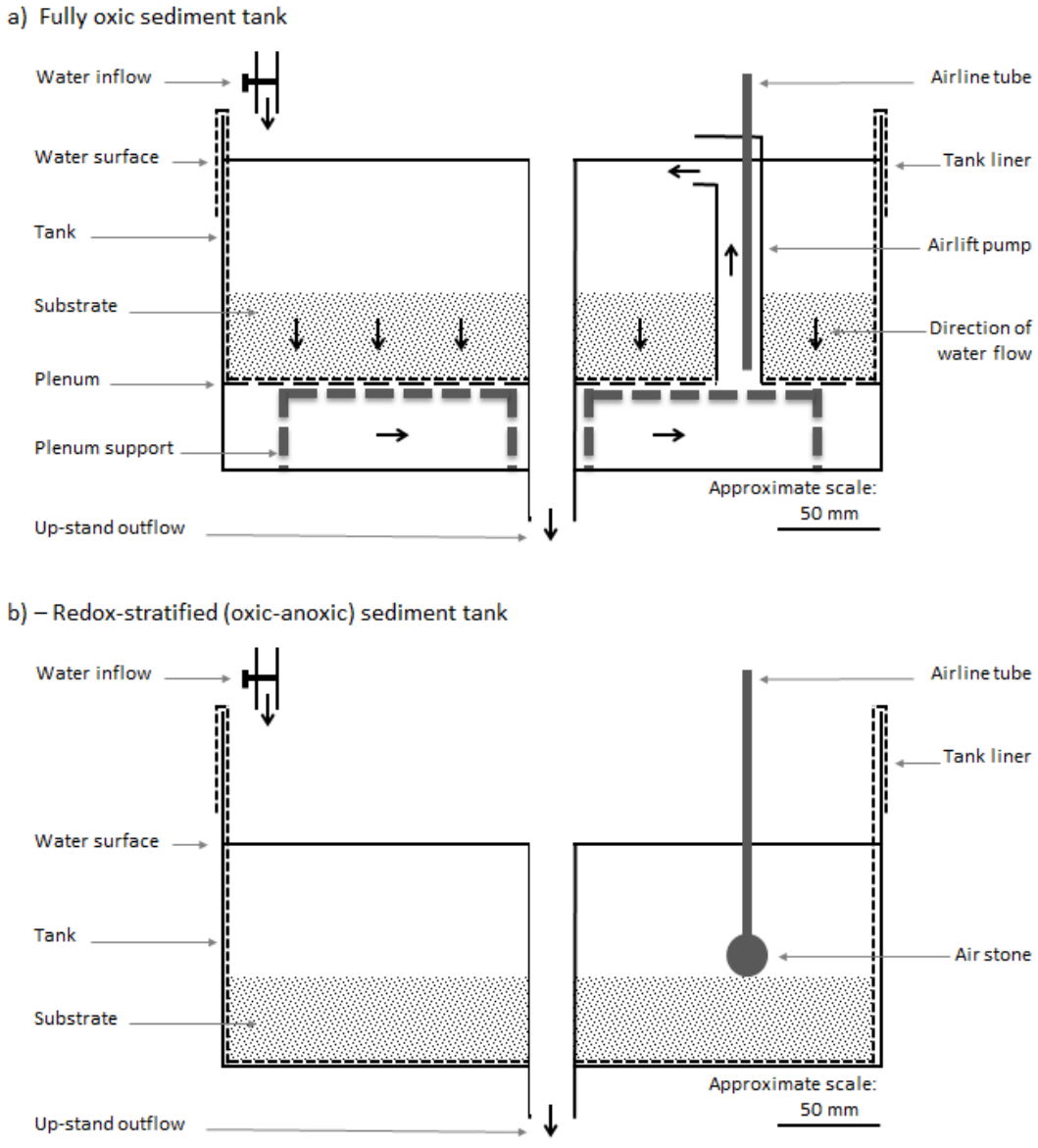

Figure 2.2. Schematic diagram of the tank design used to create the two different sediment redox regimes. a) Fully oxic treatment tanks were fitted with a plenum and an airlift pump was used to circulate oxygenated water downwards through the sediment. b) Redox-stratified oxic-anoxic treatment tanks had no plenum and no water movement through the sediment, so a naturally stratified oxic-anoxic sediment developed; dissolved oxygen levels were maintained with an air stone (Robinson *et al.*, 2015).

Oxic treatment tanks (n = 16) were fitted with a plastic grid partition with perforations of 2.0 cm^2 supported 4.5 cm above the tank base to create a sediment-free plenum (false bottom; Jaubert, 2008) directly under the liner (Figure 2.2a). An airlift pump was used to circulate oxygenated water downwards through the sediment, maintain the sediment under a fully oxic regime and maintain dissolved oxygen levels within the water column. Redox-stratified (oxic-anoxic) sediments were intended to mimic the redox state of sediments in the natural habitat of *H. scabra* which exhibit a shallow oxic-anoxic interface below which the sediment remains anoxic (Wolkenhauer *et al.*, 2010). In the stratified oxic-anoxic treatment tanks, the sediment was directly exposed to the base of the tanks (i.e. no plenum and no water movement through the sediment) so that a redox-stratified oxic-anoxic sediment developed. Aeration was provided to the oxic-anoxic tanks using airstones to maintain dissolved oxygen levels within the water column (Table 2.1 and Figure 2.2b).

2.2.5 Photo-identification and growth of Holothuria scabra

Prior to stocking into experimental tanks, the juvenile sea cucumbers (n = 128) were suspended in mesh bags for 24 h to ensure gut contents were evacuated prior to weighing. They were then drained on a damp cloth for one minute, weighed to the nearest 0.01 gram (g) and photographed for individual photo-identification to permit monitoring of individual growth rates (Raj, 1998; Figure 2.3).

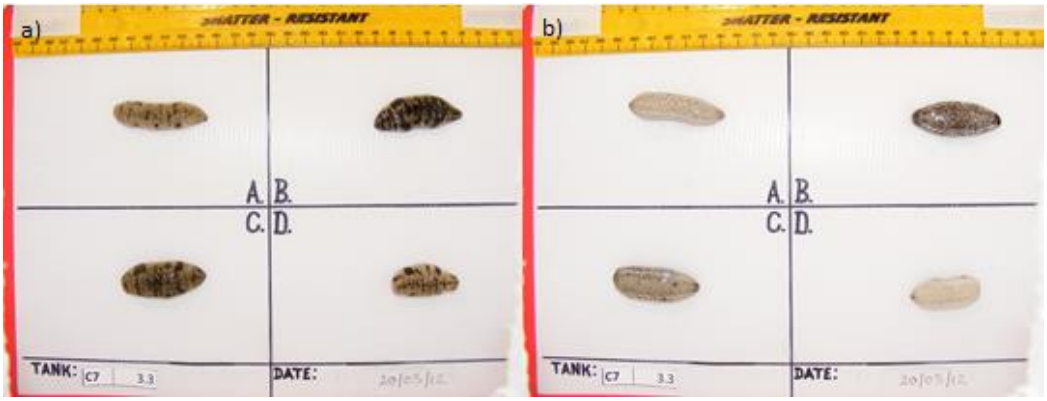

Figure 2.3. Photo-identification of juvenile *Holothuria scabra* including: a) dorsal and b) ventral views to permit monitoring of individual growth rates, according to Raj (1998).

Juvenile sea cucumbers with a mean weight of 7.3 ± 0.07 g (mean ± SE) were allocated randomly to 32 groups of four individuals per group. Each individual was gut-evacuated for 24 h and reweighed every 27 d over the 81 d experimental period. Wet weight (g) data were used to calculated specific growth rate (SGR; Equation 1), growth rate (g day^{-1}; Equation 2) and the co-efficient of variation (CV; Equation 3).

Equation 1 $SGR\ (\%\ d^{-1}) = 100\ (\ln W_2 - \ln W_1) / T$

Equation 2 $\text{Growth rate}\ (g\ d^{-1}) = (W_2 - W_1)/T$

Equation 3 $CV\ (\%) = 100 \times (SD/\bar{x})$

where W_1 and W_2 are initial and final wet body weight of sea cucumbers in each experimental tank (g); T is the duration of the experiment (days); SD is the standard deviation in body weight and $\bar{x}$ is the mean wet weight (g) of sea cucumbers in each experimental tank for a particular sampling period.

2.2.6 Water quality

Water quality parameters were recorded weekly from water sampled adjacent to the outflow of each tank during mid-morning at 10:00. Temperature (0.01 º C) and pH (0.01 pH units were measured using a pH meter (YSI Inc. Model # 60/10 FT; Yellow Springs, Ohio, U.S.A.). Dissolved oxygen concentration (0.01 mg L^{-1}) was measured using an oxygen meter (YSI Inc. Model # 55D; Yellow Springs, Ohio, U.S.A.). Total ammonia nitrogen (0.01 µg L^{-1}; NH_4-N; TAN) was determined using the method of Solorzano (1969). Nitrite (0.01 µg L^{-1}; NO_3^-) concentration was measured using a commercially available test kit (Merck Nitrite Test Kit, Cat. no. 1.14776.0001, Merck, South Africa) with colour absorbance read by a spectrophotometer (Prim Light, Secomam, 30319 Ales, France). Absorbance was converted into the concentration of total ammonia nitrogen or nitrite using the coefficients derived from least-square linear regression standard curves.

2.2.7 Sediment characteristics

At the end of the trial on day 81, the sediment reduction-oxidation (redox) potential was measured in millivolts (0.01 mV) by inserting a redox probe (Eutech Instruments pH 6+ portable meter, USA) to the base of the sand sediment. Readings were taken following stabilisation (after approximately five minutes). In addition, four replicate cores were taken from different positions within each tank using a 10.0 x 1.0 cm (length x diameter) perspex coring device. The depth (cm) from the sediment surface to the oxic-anoxic interface was recorded and mean depth of anoxic sediment in each tank was converted to the percentage of anoxic sediment (0.01 %) in the core to allow direct comparisons between treatments.

Sediment samples were collected from the upper three millimetres of three replicate tanks and dried to a constant weight at 50 °C for 48 h. Samples were analysed for organic carbon (0.01 %) and total nitrogen (0.01 %) content using a Leco TruSpec Micro Elemental Analyzer (Leco, St Joseph, USA) prior to, and after carbonate removal. Carbonates were removed by fuming with 2 M HCl for 48 hours after which the samples were rinsed three

times with distilled water, dried to constant weight and re-analysed for total organic carbon. Carbon to nitrogen mass ratios were then calculated for each replicate sample.

The total chlorophyll concentration (0.01 µg g^{-1}) was determined by the trichromatic equation described by Jeffrey and Welshmeyer (1997) using a variation of the spectrophotometric method initially described by Lorenzen (1967). The extraction step was carried out overnight at four degrees centigrade with 100 % acetone, with the addition of distilled water to return the fluid concentration to 90 % acetone before the first spectrophotometric step. Absorbance was read at wavelengths 630, 647, 664 and 750 nm and substituted into Equations 4-6 respectively;

Equation 4 [chl. *a*]extract = 11.85A664/l - 1.54A647/l - 0.08A630/l

Equation 5 [chl. *b*]extract = 21.03A647/l - 5.43A664/l - 2.66A630/l

Equation 6 [chl. *c*]extract = 24.52A630/l - 1.67A664/l - 7.60A647/l

where A = corrected absorbance, l = cuvette path length (cm). The concentration of each chlorophyll pigment (µg g^{-1}) in the sample was obtained by Equation 7;

Equation 7 [chl.x]sample = [chl.x]extract * (v/V)

where v = volume of acetone (mL) and V = weight of dry sediment (g).

The total concentration of chlorophyll (µg g^{-1}) in the sample was obtained using the trichromatic equation of Jeffrey and Welshmeyer (1997; Equation 8).

Equation 8 [chl.]total = [chl.*a*]sample + [chl.*b*]sample + [chl.*c*]sample

2.2.8 Behavioural observations

Behavioural observations were carried out over three consecutive 24 h periods in the penultimate week of the study. Observations were made at four hour intervals, commencing at noon. Red light was used to facilitate night-time observations. During each observation period, each tank was observed for two minutes and the number of animals in each burial state and their levels of activity were recorded (Table 2.2).

Table 2.2. Definition of sea cucumber behaviours categorised into burial state and activity according to Wolkenhauer (2008).

Behaviour		Definition
Burial state	Buried	Entire body under the sediment surface
	Semi buried	>50 % of the body under sediment surface
	On the surface	Entire body on sediment's surface
Activity	Resting	Animal is inactive with no movement observed
	Feeding	Animal is actively feeding on sediment or walls; tentacles are exposed, and head performs sweeping movements

2.2.9 Proximate composition analysis

At the end of the trial all animals from three of the four replicate tanks for each treatment were pooled and homogenised, thereby creating one composite sample per tank, which was frozen at -20 °C. The composite samples were then lyophilised at -80 °C, ground to a fine powder (~50 µm) with a pestle and mortar and their proximate composition analysed according to the Association of Official Analytical Chemists (AOAC) official methods (AOAC, 2010). Moisture (0.01 %) was determined by weight loss after drying at 95 °C for 72 h (AOAC method 934.01), while ash (0.01 %) was determined by weight loss on combustion after ashing in a furnace for four hours at 550 °C (AOAC method 942.05). Crude protein (0.01 %) was analysed using the Dumas Combustion method (LECO Truspec Nitrogen Analyser, AOAC method 990.03). Crude fibre (0.01 %) was analysed using a Dosi-Fibre machine (AOAC method 978.10). Gross energy (0.01 %) was determined using an automatic bomb calorimeter (LECO AC500, LECO Corporation, USA). Carbohydrate (0.01 %) was calculated indirectly by adding the percentage values determined for crude protein, lipid, crude fibre and ash, and subtracting the total from 100.

2.2.10 Statistical analyses

Mean biomass of individual *H. scabra* (per replicate tank) and mean (per replicate tank) water and sediment characteristics were tested for normality using Shapiro-Wilk's test and homogeneity of variance using Levene's test. Data that met the test assumptions were compared across the eight experimental treatments using multifactor analysis of variance (ANOVA) and Duncan's multiple range tests were used to compare differences among means of dependent variables (Quinn and Keough, 2002). Data that did not meet the test assumptions were log transformed before analysis. Where log transformed data also did not meet the assumption of homogeneity of variance, a Kruskal-Wallis one-way ANOVA was used to test for significant differences in the medians between treatments. Differences were considered significant at $p < 0.05$.

The numbers of animals engaging in each specific behaviour (Table 2.2) were averaged to give the mean number of animals per replicate tank in each burial state or activity at six different time intervals and analysed using repeated measures ANOVA. A multivariate approach was used to establish redox regime, sediment depth and particle size as categorical predictors, and the mean number of animals engaged in a specific behaviour at each time period provided the dependent variable (within effects). Although the assumptions for normality and homogeneity of variance were not met using the Shapiro-Wilk's and Levene's tests, even with transformed data, repeated measures ANOVA was still deemed sufficiently robust to compare treatment means over time (Moser and Stevens, 1992). A Mauchly's test examined sphericity of the variance–covariance matrix. As sphericity was violated in the majority of cases, a Greenhouse–Geisser epsilon correction was used to adjust F statistics conservatively. Significant differences among treatment means were identified using a Tukey's honest significant difference (HSD) post-hoc test. Results are presented as means ± standard errors. All statistical analyses were performed using Statistica version 12.

2.3 Results

2.3.1 Water quality

The mean water temperature was 25.71 ± 0.05 °C and varied between 25.18 and 26.40 °C over the experimental period. The pH ranged from 8.01 to 8.43 and salinity was constant at 35 g L^{-1}. Dissolved oxygen concentration varied between 5.77 and 6.97 mg L^{-1} (6.6 ± 0.04 mg L^{-1}). Total ammonia concentrations varied between 17.06 and 64.21 µg L^{-1} (29.00 ± 1.74 µg L^{-1}) and nitrite concentrations ranged from 17.68 to 37.00 µg L^{-1} (26.01 ± 0.78 µg L^{-1}). There were no significant differences in water quality parameters between treatments (multifactor ANOVA, $p > 0.05$) with the exception of Treatment 4 that exhibited a significantly lower nitrite concentration over the experimental period (multifactor ANOVA, $F_{(1, 24)} = 6.32$, $p = 0.019$), which was explained by the interaction between sediment depth and the manipulated redox regime; sediment particle size made no significant contribution.

2.3.2 Sediment characteristics

The vertical core profiles of sediment confirmed that the active circulation of water using the airlift pump maintained the sediment under fully oxic conditions, indicated by a light brown colour throughout the full vertical core profile (Treatments 1-4); hereafter referred to as oxic. In contrast, in tanks without a plenum, 83.01 ± 3.80% of the sediment profile was anoxic (Treatments 5-8); hereafter referred to as oxic-anoxic (multifactor ANOVA, $F_{(1, 24)} = 465.53$, $p < 0.001$). These redox-stratified sediments, closely resembled the

sediments present in the natural habitat of *H. scabra*, with a shallow oxic-anoxic interface below which the sediment was anoxic, indicated by a grey colour (Michio *et al.*, 2003). The redox potential of the oxic treatments was significantly higher (147.98 ± 21.00 mV) compared with the oxic-anoxic treatments (67.27 ± 21.18 mV; multifactor ANOVA, $F_{(1, 24)} = 5.81$, $p = 0.028$; Table 2.3).

The sediment redox regime significantly affected the total chlorophyll concentration on the sediment surface (multifactor ANOVA; $F_{(1, 21)} = 13.42$, $p = 0.001$; Table 2.3) with higher concentrations in the oxic treatments (10.03 ± 1.51 μg g^{-1}) compared with oxic-anoxic treatments (4.29 ± 0.74 μg g^{-1}) indicating greater microphytobenthic production. Observations of the sediment surface between the contrasting redox regimes also supported this quantitative data since the oxic treatments were characterised by a markedly thicker growth of benthic diatoms (*Nitzschia* and *Navicula* species) and cyanobacteria *Oscillatoria sp.*

Levels of organic carbon in the sediment were generally low, ranging from 0.26 ± 0.01 % to 0.69 ± 0.06 %, although the percentage of organic carbon was significantly higher in sediments with medium particle sizes (0.63 ± 0.03 %) than fine particles (0.42 ± 0.04 %; multifactor ANOVA; ($F_{(1, 16)} = 42.15$, $p < 0.001$; Table 2.3). Sediment redox regime, depth and particle size interacted to affect total nitrogen content (multifactor ANOVA; $F_{(1, 16)} = 8.33$, $p = 0.011$) and the carbon to nitrogen ratio (multifactor ANOVA; $F_{(1, 16)} = 7.85$, $p = 0.013$; Table 2.3). The redox regime explained the oxic-anoxic treatments' higher organic carbon content (0.58 ± 0.04 % versus 0.47 ± 0.05 %; multifactor ANOVA; $F_{(1, 16)} = 10.72$, $p = 0.005$) while oxic treatments had a higher total nitrogen content (0.033 ± 0.001 % versus 0.026 ± 0.001 %; multifactor ANOVA; $F_{(1, 16)} = 27.00$, $p < 0.001$) and resulted in higher carbon to nitrogen ratios in the oxic-anoxic treatments (22.84 ± 1.40 % versus 14.76 ± 1.77 %; multifactor ANOVA; $F_{(1, 16)} = 71.29$, $p < 0.001$).

2.3.3 Growth and survival

There were no significant differences in mean sea cucumber biomass (29.20 ± 2.29 g) between treatments at the start of the experiment (Kruskal-Wallis, $H_{(7, 32)} = 11.17$, $p = 0.13$). Survival was 100 % in all treatments (Table 2.4). The final mean growth rate of animals reared in the oxic-anoxic treatments was significantly higher at 0.20 ± 0.02 g d^{-1} compared with 0.12 ± 0.01 g d^{-1} for animals in oxic treatments (multifactor ANOVA, $F_{(1, 24)} = 19.59$, $p < 0.001$). The growth rate of juveniles in oxic treatments decreased over time from 0.23 ± 0.02 g d^{-1} between days 0 – 27 to a negative growth rate of -0.01 ± 0.02 g d^{-1} between days 54 – 81 (Figure 2.4). Conversely, animals reared in oxic-anoxic treatments maintained

an initial growth rate above 0.2 g d^{-1} with a slight reduction to 0.14 ± 0.04 g d^{-1} between days 54 and 81 (Figure 2.5).

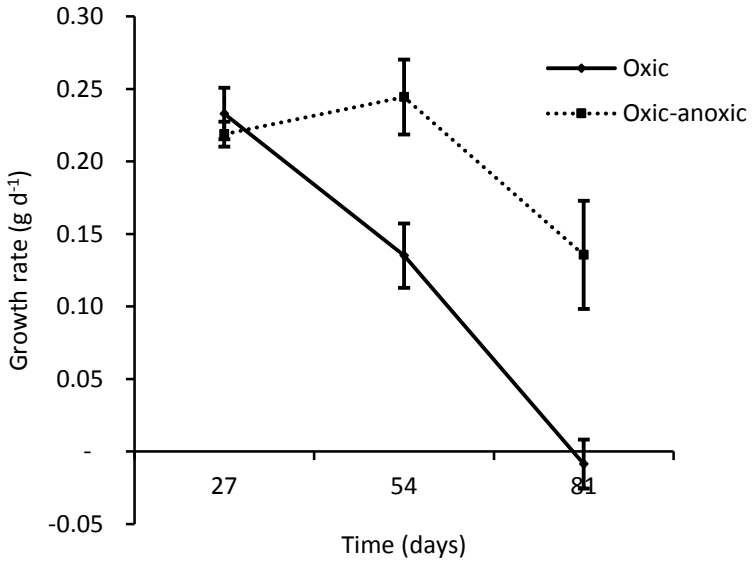

Figure 2.4. The mean (± standard error) growth rate per sampling period of *Holothuria scabra* (n=4) reared under two contrasting sediment redox regimes for eighty-one days; fully oxic and stratified oxic-anoxic.

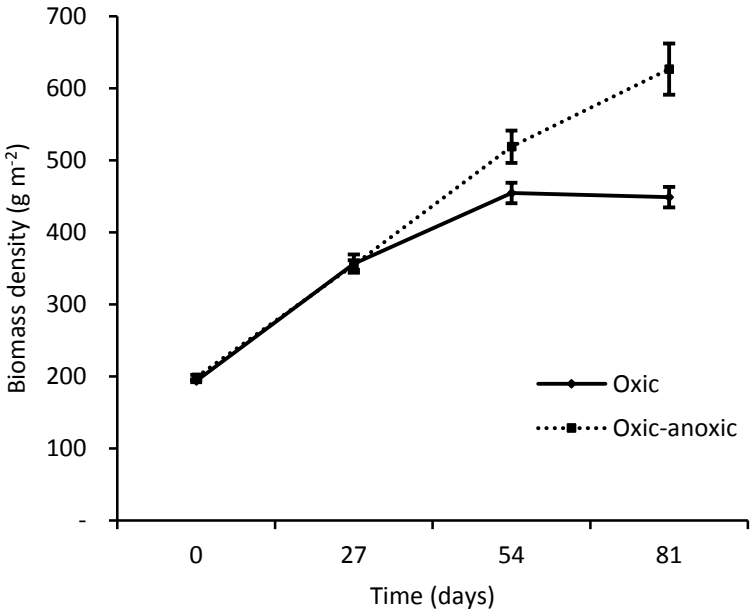

Figure 2.5. The mean (± standard error) cumulative biomass density per sampling period of *Holothuria scabra* (n=4) reared under two contrasting sediment redox regimes for eighty-one days; fully oxic and stratified oxic-anoxic.

Sea cucumber biomass from oxic sediments reached a maximum mean density of 454.84 ± 14.30 g m^{-2} on day 54, after which no further increase in biomass was observed

(Figure 2.5). The final biomass density of animals in the oxic-anoxic treatments was significantly higher at 626.89 ± 35.44 g m^{-2} at the end of the trial compared with 449.22 ± 14.24 g m^{-2} for oxic treatments (multifactor ANOVA, $F_{(1, 24)} = 21.05$, $p < 0.001$; Figure 2.5). Significant differences in growth rates were principally related to the depth of the oxic-anoxic interface (multiple regression, $r^2 = 0.40$; $\beta = 0.63$; $p < 0.001$) and the sediment redox potential (multiple regression, $r^2 = 0.26$; $\beta = -0.51$; $p < 0.001$).

2.3.4 Behaviour

Time and the interaction between sediment redox regime and time, significantly affected all categories of observed burial behaviour and activity level defined in Table 2.2 ($p < 0.05$). Animals reared in oxic-anoxic treatments displayed a shorter burial cycle, remaining on the surface (exposed) for longer in the early morning before burying (repeated measures ANOVA; $F_{(3.43, 82.40)} = 7.27$, $p < 0.001$) and spent significantly more time feeding (repeated measures ANOVA; $F_{(2.59, 62.21)} = 4.85$, $p = 0.006$; Figure 2.6). The most notable differences in burial cycle and feeding activity occurred in the early morning between midnight and 8 am, when animals in oxic treatments ceased feeding and began to bury earlier compared with those animals in oxic-anoxic treatments; for example, at 04:00 hours 2.66 ± 0.27 animals were still feeding on the oxic-anoxic sediment compared with 1.34 ± 0.27 animals feeding on the oxic sediment (Figure 2.6).

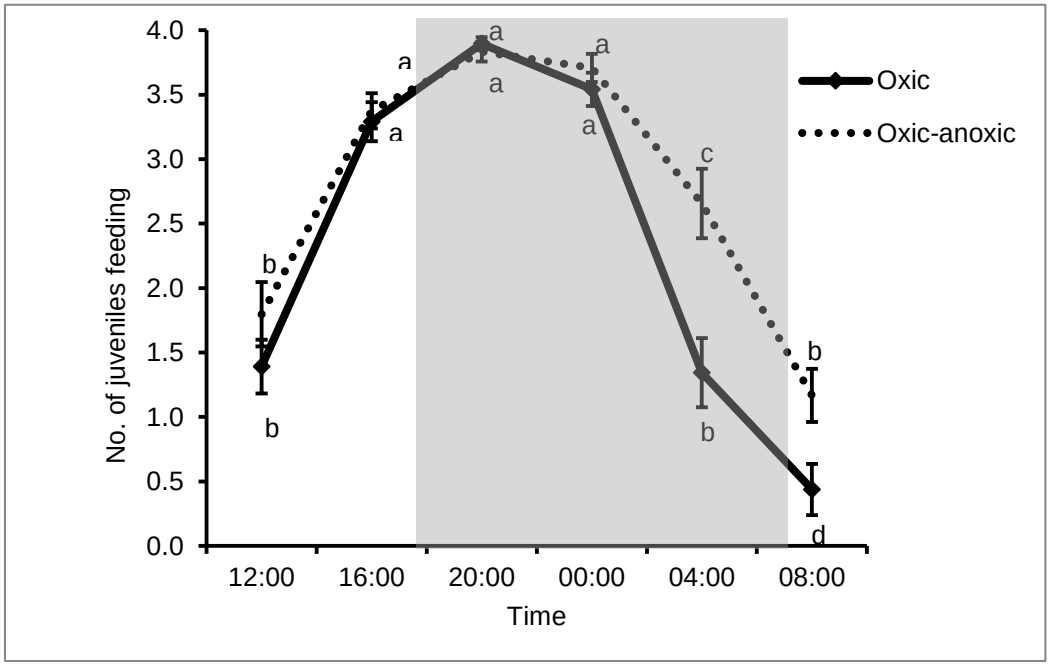

Figure 2.6. The mean (± standard error) number of juvenile *Holothuria scabra* (n=4) observed to be feeding on the sediment surface and the tank walls when reared under two contrasting sediment redox regimes; fully oxic and stratified oxic-anoxic. Different lower case letters indicate significant differences between the numbers of juveniles feeding over time for each redox regime. The shaded area represents dark hours (17:40 – 07:40).

2.3.5 Proximate composition

The sediment redox regime significantly affected the sea cucumber biochemical composition (Table 2.5), with a significantly higher crude lipid and carbohydrate content in animals reared in the oxic treatments (multifactor ANOVA $F_{(1, 16)} = 7.72$, $p = 0.013$ and $F_{(1, 16)} = 8.16$, $p = 0.011$ for lipid and carbohydrate respectively). In contrast, ash content was lower in animals reared in the oxic treatments; 62.82 ± 1.59 % compared with 67.03 ± 1.10 % in oxic-anoxic treatments (multifactor ANOVA $F_{(1, 16)} = 5.01$, $p = 0.04$). Redox regime and sediment depth interacted to account for a significant difference in crude fibre (multifactor ANOVA, $F_{(1, 16)} = 7.34$, $p = 0.015$; Table 2.5); however, there were no significant differences in protein, moisture, or gross energy content (multifactor ANOVA, $p > 0.05$).

Table 2.3. Mean (± standard error) sediment parameters subjected to two contrasting redox regimes, each with different depths and particle sizes (fine particles: 125-250 μm; medium particles: 250-500 μm). Different superscripts indicate significant differences. [1]C:N = Carbon to nitrogen mass ratio

Redox regime	Depth (cm)	Particle size	Total chlorophyll (μg g^{-1})	Redox potential (mV)	Anoxic sediment (%)	Organic carbon (%)	Total nitrogen (%)	[1]C:N
Oxic	2	Fine	15.58 ± 5.05	169.33 ± 40.92	-[a]	0.26 ± 0.01[c]	0.040 ± 0.000[c]	6.49 ± 0.25[c]
Oxic	2	Medium	7.44 ± 0.97	158.08 ± 58.18	-[a]	0.69 ± 0.06[a]	0.030 ± 0.003[a]	20.99 ± 2.54[ab]
Oxic	4	Fine	9.83 ± 3.62	137.44 ± 41.80	-[a]	0.39 ± 0.01[cd]	0.030 ± 0.000[a]	13.03 ± 0.49[d]
Oxic	4	Medium	8.67 ± 1.65	121.88 ± 41.13	-[a]	0.56 ± 0.00[ab]	0.033 ± 0.000[a]	18.52 ± 0.16[a]
Oxic-anoxic	2	Fine	5.72 ± 0.15	95.83 ± 13.60	81.97 ± 8.02[b]	0.45 ± 0.03[bd]	0.027 ± 0.003[a]	17.39 ± 1.78[a]
Oxic-anoxic	2	Medium	6.23 ± 3.06	101.25 ± 57.81	76.98 ± 9.26[b]	0.69 ± 0.05[a]	0.030 ± 0.000[a]	23.09 ± 1.80[b]
Oxic-anoxic	4	Fine	2.24 ± 0.22	41.81 ± 42.40	93.34 ± 1.00[b]	0.59 ± 0.09[ab]	0.027 ± 0.003[a]	21.74 ± 0.83[ab]
Oxic-anoxic	4	Medium	2.93 ± 0.51	24.38 ± 46.38	79.74 ± 9.26[b]	0.58 ± 0.02[ab]	0.020 ± 0.000[b]	29.15 ± 0.91[e]

Table 2.4. Mean (± standard error) growth performance of sea cucumbers subjected to two sediments with contrasting redox regimes, each with different depths and particle sizes (fine particles: 125-250 μm; medium particles: 250-500 μm). Different superscripts indicate significant differences. [2]SGR = Specific growth rate; [3]CV = Co-efficient of variation (wet weight).

Redox regime	Depth (cm)	Particle size	Survival (%)	Initial weight (g)	Final weight (g)	Growth rate (g d^{-1})	[2]SGR (% d^{-1})	Biomass density (g m^{-2})	[3]CV (%)
Oxic	2	Fine	100	7.16 ± 0.22	17.20 ± 0.67^{a}	0.12 ± 0.01^{ab}	1.03 ± 0.06^{a}	461.68 ± 18.01^{a}	23.11 ± 4.34^{ab}
Oxic	2	Medium	100	7.38 ± 0.10	16.09 ± 1.04^{a}	0.11 ± 0.01^{a}	0.95 ± 0.08^{a}	432.00 ± 27.85^{a}	11.58 ± 1.90^{a}
Oxic	4	Fine	100	7.05 ± 0.17	16.90 ± 1.35^{a}	0.12 ± 0.01^{a}	1.00 ± 0.05^{a}	453.67 ± 36.37^{a}	21.28 ± 7.61^{ab}
Oxic	4	Medium	100	7.18 ± 0.10	17.97 ± 1.36^{ab}	0.13 ± 0.02^{ab}	1.10 ± 0.09^{ab}	449.53 ± 38.38^{a}	19.03 ± 4.72^{ab}
Oxic-anoxic	2	Fine	100	7.60 ± 0.06	23.97 ± 2.72^{bc}	0.20 ± 0.03^{ab}	1.36 ± 0.13^{ab}	643.56 ± 72.99^{b}	27.90 ± 7.58^{ab}
Oxic-anoxic	2	Medium	100	7.09 ± 0.09	22.35 ± 2.07^{abc}	0.20 ± 0.03^{bc}	1.30 ± 0.15^{ab}	600.03 ± 55.60^{ab}	51.82 ± 7.34^{c}
Oxic-anoxic	4	Fine	100	7.16 ± 0.33	26.66 ± 2.83^{c}	0.24 ± 0.04^{c}	1.54 ± 0.23^{b}	715.76 ± 75.97^{b}	34.00 ± 5.64^{b}
Oxic-anoxic	4	Medium	100	7.80 ± 0.25	20.42 ± 2.86^{abc}	0.16 ± 0.04^{bc}	1.10 ± 0.20^{ab}	548.20 ± 76.84^{ab}	23.36 ± 6.73^{ab}

Table 2.5. Mean (± standard error) proximate composition of sea cucumbers reared on sediments with two contrasting redox regimes, each with different depths and particle sizes (fine particles: 125-250 μm; medium particles: 250-500 μm). Different superscripts indicate significant differences.

Redox regime	Depth (cm)	Particle size	Crude protein (%)	Crude lipid (%)	Crude fibre (%)	Carbohydrate (%)	Ash (%)	Moisture (%)	Gross energy (kJ g^{-1})
Oxic	2	Fine	21.40 ± 3.21	0.89 ± 0.15^{a}	0.40 ± 0.08^{ab}	13.91 ± 1.41^{ab}	63.39 ± 3.89^{ab}	87.83 ± 1.66	4.89 ± 0.88
Oxic	2	Medium	23.83 ± 0.11	0.84 ± 0.04^{a}	0.50 ± 0.01^{b}	16.39 ± 1.36^{b}	58.45 ± 1.43^{b}	88.82 ± 0.58	5.76 ± 0.50
Oxic	4	Fine	21.91 ± 1.92	0.87 ± 0.15^{a}	0.45 ± 0.17^{ab}	13.82 ± 0.41^{ab}	62.95 ± 1.72^{ab}	88.64 ± 0.53	5.09 ± 0.23
Oxic	4	Medium	18.73 ± 2.61	0.74 ± 0.04^{ab}	0.23 ± 0.04^{a}	13.78 ± 2.10^{ab}	66.51 ± 4.34^{ab}	87.07 ± 1.69	4.07 ± 0.65
Oxic-anoxic	2	Fine	18.35 ± 2.11	0.65 ± 0.08^{ab}	0.22 ± 0.05^{a}	11.47 ± 0.70^{a}	69.32 ± 2.84^{a}	87.61 ± 0.74	4.14 ± 0.59
Oxic-anoxic	2	Medium	21.26 ± 1.61	0.77 ± 0.01^{ab}	0.34 ± 0.03^{ab}	13.26 ± 1.30^{ab}	64.37 ± 1.81^{ab}	90.13 ± 0.17	4.85 ± 0.34
Oxic-anoxic	4	Fine	18.69 ± 1.70	0.51 ± 0.03^{b}	0.39 ± 0.05^{ab}	11.54 ± 0.46^{a}	68.87 ± 1.82^{a}	88.51 ± 0.65	4.00 ± 0.44
Oxic-anoxic	4	Medium	20.96 ± 2.00	0.72 ± 0.07^{ab}	0.54 ± 0.06^{b}	12.24 ± 0.32^{a}	65.55 ± 1.67^{ab}	88.92 ± 0.60	4.89 ± 0.56

2.4 Discussion

This study investigated growth of juvenile *Holothuria scabra* in response to manipulated sediment physico-chemical characteristics, namely sediment depth, particle size, and redox regime. The redox regime was the principal factor affecting sediment characteristics (C:N, total chlorophyll concentration), as well as the growth rate, biomass density, biochemical composition and behaviour of *H. scabra*. The redox regime not only significantly affected the growth rate with overall faster growth on redox stratified oxic-anoxic sediments (0.20 ± 0.02 g d^{-1}) compared to fully oxic sediments (0.12 ± 0.01 g d^{-1}), but also, had a significant effect on the carrying capacity of the sediment in terms of the maximum biomass that could be supported. Maximum production of *H. scabra* in oxic treatments was reached at day 54 at a mean density of 454.84 ± 14.29 g m^{-2} after which no further positive growth was recorded. In contrast, there was no apparent growth limitation on redox stratified oxic-anoxic sediments: density continued to increase until the end of the trial achieving a mean density of 626.89 ± 35.44 g m^{-2} and growth rates remained positive. Deposit feeders are commonly limited by food availability (Lopez and Levinton, 1987; Battaglene *et al.*, 1999; Robinson and Pascal, 2012); however, since rations of the formulated feed were standardised across all treatments and adjusted on a two-weekly basis, the growth limitation in the oxic treatments cannot be explained by differences in the feeding regime. It is hypothesised that the observed growth limitation was driven by the different conditions of organic matter mineralisation and cycling of inorganic nutrients in the manipulated sediment system under fully oxic conditions.

All stages in organic matter degradation involve the interaction of organisms, resource quality, physical, and chemical environmental conditions (Anderson, 1987) with the existence of strong feedback loops between deposit feeders, their food and their chemical environment (Herman *et al.*, 1999). Consequently, deposit feeder nutrition is fundamentally related to particulate organic matter diagenesis (Rice and Rhoads, 1989). The supply of oxygen is the most important chemical influence on the biology of sandy marine sediments since it determines the pathways and rates of organic matter degradation (Reimers *et al.*, 2013; Huettel *et al.*, 2014). When present, oxygen is used preferentially as the electron acceptor in aerobic carbon oxidation since this is the most thermodynamically efficient mechanism for dissimilatory metabolism. Aerobic degradation of organic matter is rapid since almost all heterotrophic organisms with aerobic metabolism are able to completely oxidise complex organic substrates via the tricarboxylic acid cycle (Kristensen, 2001; Fenchel *et al.*, 2012). Thus, under aerobic conditions in the fully oxic treatment, all sources of autochonthous and

allochothnous organic matter may have been rapidly mineralised to CO_2. Where oxic conditions exist, organic matter is largely destroyed during diagenesis, even when organic productivity is high (Schroeder, 1987); thus, the increased mineralisation capacity may have contributed to an overall reduction in food availability to the sea cucumbers.

The presence of molecular oxygen has a secondary effect on the rate of organic matter degradation due its key role in the initial enzymatic attack on substrate molecules and the creation of highly reactive oxygen radicals that can assist extracellular enzymes in the de-polymerisation of complex organic substrates (Kristensen *et al.*, 1995; Kristensen, 2000). In sediment-based aquaculture bioremediation systems, the initial hydrolysis of macromolecules is a potentially rate-limiting step, since particulate organic matter is structurally too large to be readily transported inside bacterial cells (Fenchel *et al.*, 2012). Since there is no equivalent of molecular oxygen in the primary hydrolytic step in organic matter breakdown, the absence of oxygen may therefore result in the retardation of processes in marine sediments (Kristensen *et al.*, 1995). In anoxic sediments, organic matter is generally degraded more slowly by a consortium of bacteria with anaerobic respiratory and fermentative metabolisms, since anaerobic heterotrophs, unlike their aerobic counterparts, do not possess the requisite enzymes for the complete mineralisation of organic matter (Middelburg *et al.*, 1993; Kristensen *et al.*, 1995).

The significantly higher C:N in the oxic-anoxic sediment are indicative of a greater pool of more refractory organic matter, in which bacteria have preferentially hydrolysed nitrogen-rich molecules such as nucleic acids and proteins (Fenchel *et al.*, 2012). It is hypothesised that the slower degradation of organic matter under anaerobic conditions, resulted in the steady release of bioavailable food resources, such as energy-rich fermentation products and dissolved organic matter from incomplete anaerobic mineralisation. This would have contributed to the significantly higher biomass of *H. scabra* (Fenchel *et al.*, 2012). Thus, more refractory organic matter may have functioned as a more durable and persistent food source for deposit feeders, contributing to long-term productivity despite the apparent lower nutritive quality (Schroeder, 1987; Karlsen, 2010). Furthermore, since aspidochirotid sea cucumbers are able to absorb dissolved organics across the epithelium (Jangoux and Lawerence, 1982) and across their respiratory trees as part of a bipolar feeding strategy (Jaeckle and Strathmann, 2013), the benefits may not have been limited only to periods of active feeding behaviour.

The physico-chemical conditions of organic matter degradation interact to determine not only the rate and pathways of organic matter decomposition but also the balance of remineralised nutrients such as nitrogen and phosphorous, since they are stoichiometrically

related to the organic matter degradation (Anderson, 1987; Kristensen *et al.*, 1995; Aller and Aller, 1998). The influence of microbial communities on nitrogen cycling in marine sediments is also sensitive to the absence or presence of oxygen. Under fully oxic conditions, ammonium is oxidised in a step-wise reaction to nitrite and subsequently to nitrate. The oxidation of ammonium is the rate-limiting step since the nitrification rate is regulated by the availability of NH_4^+ and oxygen (Fenchel *et al.*, 2012). The nitrification efficiency would therefore have been high in the manipulated sediment system where oxygen penetration into the sediment was maximised and NH_4^+ production was rapid due to enhanced mineralisation rates. The significantly lower nitrite concentration in tanks with 4 cm of fully oxic sediment indicates that nitrite, as the intermediate product in nitrification, may have been oxidised more efficiently to nitrate by nitrite-oxidising bacteria that proliferated in the sediment under increased oxygen concentrations.

The recycling of nutrients from organic matter mineralisation to supply primary producers is an important ecosystem function; however, the effect of oxygen supplementation appears to have affected this function, by altering pathways of nitrogen cycling resulting in a positive feedback effect. The significantly higher microphytobenthic production (indicated by total chlorophyll concentration) in the oxic sediment system is likely to have resulted from the increased supply of nitrate, which is one of the limiting nutrients for microalgal growth. In shallow euphotic systems where primary producers grow on the sediment surface, there is tight coupling between autotrophic and heterotrophic processes and in shallow, well-mixed systems, this coupling can be intense (Herman *et al.*, 1999; Fenchel *et al.*, 2012). Benthic algal mats are very efficient at taking up nutrients from the sediment surface and an increased supply of nutrients may affect the dominance of bottom-up forces and lead to the overgrowth of algal mats (Levinton and Kelaher, 2004). While nitrate, as a key nutrient for plant growth, is likely to have had the most impact on increased microphytobenthic production, increased concentrations of dissolved inorganic carbon resulting from the rapid, aerobic oxidation of organic matter may have also played a role since increased concentrations of dissolved inorganic carbon have been shown to enhance the production of benthic diatoms (Admiraal *et al.*, 1984; Ludden *et al.*, 1985; Gould and Gallagher, 1990).

In the fully oxic sediment system, where microphytobenthic production was significantly higher, a greater proportion of animals spent less time feeding and longer periods buried, compared to the redox-stratified sediments. In a parallel behavioural study, (Yu, 2012) observed that feeding periods of juvenile *H. scabra* reared on oxic sediments were shorter and individuals tended to be less active. In deposit feeder nutrition, rates of ingestion, digestion, absorption, assimilation and resulting growth all depend critically on food quality (Karlsen,

2010). Deposit-feeding holothurians are broadly adapted to process large quantities of sediments of low nutritional quality (Lopez and Levinton, 1987; Roberts *et al.*, 2000); however, they exhibit a general plasticity of behavioural and feeding strategies in response to variations in resource availability (Roberts *et al.*, 2000). Sea cucumbers have a behavioural capacity to alter their ingestion rate in response to food quality: the 'optimal ingestion rate' predicts that the ingestion rate increases with increasing food value (Taghon, 1981; Taghon and Jumars, 1984), while 'compensatory feeding' predicts that the ingestion rate decreases with increasing food value (Calow, 1977; Cammen, 1979). Apparent compensatory slowdowns in ingestion rates in response to rich food sources such as benthic diatoms have been observed for deposit feeders, including sea cucumbers (Cammen, 1979; Lopez and Levinton, 1987; Zamora and Jeffs, 2011). As their net gain of energy and nutrients is determined by foraging and digestion, sea cucumbers in the oxic treatments may have decreased their feeding rate and activity levels due to the limited need to forage in response to the abundant microphytobenthic production. The demonstration by Phillips (1984) that compensatory regulation of energy intake can be consistent with an optimal foraging model may thus explain the behaviour and growth limitation of sea cucumbers in the oxic treatments.

The biochemical composition of sea cucumbers is significantly influenced by the environment, particularly by seasonal changes related to food availability (Tuwo *et al.*, 2012; Poot-Salazar *et al.*, 2014). The significantly higher lipid and carbohydrate contents of animals reared in the oxic sediment system may be a reflection of the higher quality of food resources, such as the benthic diatoms, which have a relatively high intrinsic food value and assimilation efficiencies (Yingst, 1976; Moriarty, 1982; Watanabe *et al.*, 2012a). As lipids and carbohydrates are believed to be the primary nutrient reserves in sea cucumbers (Krishnan, 1968; Feral, 1985), it would appear that animals were allocating the products of digestion to storage as opposed to using them for maintenance and somatic growth. This type of compensatory feeding is typical in some deposit feeders in the face of abundant food reserves and allows them to regulate their intake of energy and store excess nutrients for later use (Calow, 1977).

2.5 Conclusion

The active circulation of oxygenated water successfully maintained the sediment under fully oxic conditions and appeared to increase the rate of organic matter degradation, as indicated by the significantly lower C:N. Sea cucumbers reared on fully oxic sediments experienced stunted growth, while redox stratified oxic-anoxic sediments, which represent the natural habitat of *H. scabra*, supported a significantly higher biomass. It was concluded that

the differing conditions of carbon oxidation and nitrogen cycling under the different redox regimes affected the quality and quantity of food resources available for growth with concomitant impacts on growth rate and biomass carrying capacity. Positive feedback cycles which affected the quality and quantity of food resources are likely to have resulted from a combination of enhanced oxidant supply, increased solute exchange rates of dissolved inorganic nutrients, and altered pathways of nitrogen cycling. Strictly aerobic detrital systems are rare in nature (Plante *et al.*, 1990) and are unlikely to be a suitable medium for deposit feeder growth in the long term. Naturally stratified oxic-anoxic sediments, resembling the natural habitat of *H. scabra,* that support both aerobic and anaerobic mineralisation pathways appear more suitable for high density production, despite their decreased assimilative capacity for organic matter. It is hypothesised that redox-stratified sediments support a predominately anaerobic microbial community, comprising heterotrophic bacteria operating anaerobic respiratory and fermentation metabolisms, providing a steady release of more nutritionally favourable food resources for deposit feeders than aerobic systems (Schroeder, 1987).

Chapter 3. Taxonomic and functional profiling of sediment microbial communities under contrasting redox regimes

3.1 Introduction

Deposit feeding sea cucumbers are receiving increasing attention as bioremediators in aquaculture systems due to their ability to convert feed and faeces into high value secondary biomass (Slater *et al.*, 2009; Mactavish *et al.*, 2012; Watanabe *et al.*, 2012a; Yokoyama, 2015; Yokoyama *et al.*, 2015; Zamora *et al.*, 2016). In intensive aquaculture systems, high rates of organic loading and/or the accumulation of solid wastes frequently exceed the microbial mineralization capacities in sediments. In sediment-based aquaculture bioremediation systems that integrate deposit feeders, oxygen is likely to be the main constraint since the net accumulation of organic matter and benthic macrofauna are positively correlated with the sediment oxygen demand (Pastor *et al.*, 2011). *In situ* bioremediation technologies frequently employ the addition of external electron acceptors, most frequently oxygen, to enhance the aerobic respiratory breakdown of organic matter and alleviate the constraints imposed by the naturally slow mineralization process in sediments (Colleran, 1997). The percolation of oxygenated water is one of the most cost-effective approaches currently used for *in situ* sediment remediation, which may be applicable to the development of aquaculture bioremediation systems that integrate epibenthic deposit feeders.

The approach of combining bioremediation technologies (increasing oxidant supply) and the production of high value secondary products on aquaculture effluents (addition of deposit feeding sea cucumber species) remains a largely unexplored concept. In Chapter 2 and Robinson *et al.* (2015), the effects of manipulated sediment systems, describing fully oxic and redox-stratified sediments, on the growth and biomass carrying capacity of *Holothuria scabra* were investigated. The active circulation of oxygenated water successfully maintained the sediment under fully oxic conditions and appeared to increase the rate of organic matter degradation, as indicated by the significantly lower C:N. However, sea cucumbers reared on fully oxic sediments experienced stunted growth, while redox stratified oxic-anoxic sediments, which represent the natural habitat of *H. scabra*, supported a significantly higher biomass. It was hypothesised that the differing conditions of carbon oxidation and nitrogen cycling under the different redox regimes affected the quality and quantity of food resources available for deposit feeder growth with concomitant impacts on growth rate and biomass carrying capacity.

The supply of oxygen is one of the most important chemical influences on the sediment with profound effects on the microbial and chemical properties in sediments and therefore on the nutritional environment of deposit feeders (McCall and Tevesz, 1982; Lopez and Levinton, 1987). This chapter aims to advance observations detailed in Chapter 2 by characterising the diversity, structure and predicted function of the microbial communities present in the sediment of *H. scabra* culture tanks subjected to contrasting redox regimes (oxic and oxic-anoxic) using high-throughput 454-pyrosequencing of barcoded 16S ribosomal ribonucleic acid (rRNA) genes. It is hypothesised that increasing the availability of oxygen, via the percolation of oxygenated water, will increase the diversity and functional potential of microbial communities for the bioremediation of aquaculture wastes. Accurate evaluation of microbial communities is essential for understanding global biogeochemical processes and can help to guide bioremediation treatments (Sharon *et al.*, 2015). Consequently, the environmental drivers behind changes in the microbial community are evaluated and the taxonomic, metabolic and predicted functional role of bacteria in aquaculture waste bioremediation are characterised.

3.2 Materials and methods

3.2.1 Study site

The study was conducted at HIK Abalone Farm (Pty) Ltd in Hermanus, on the southwest coast of South Africa (34°26'04.35"S; 19°13'12.51"E) between 12th September and 5th December 2012, approximately three months after the experiment in Chapter 2 was terminated.

3.2.2 Experimental animals

Experimental animals were imported from a commercial hatchery in Madagascar in November 2011, quarantined and acclimated to the experimental system (Chapter 2, Section 2.2.1).

3.2.3 Experimental design

This study was designed to test the effect of sediment redox regime on the growth of *H. scabra* and the diversity, structure and predicted functional role of the microbial communities identified from sediment cores sectioned at three depths (0, 2 and 4 cm). As in Chapter 2, the two experimental treatments comprised a manipulated sediment system designed to maintain the sediment in the culture tanks under a fully oxic redox regime, hereafter referred to as the 'oxic' treatment and naturally redox-stratified sediments, hereafter referred to as the 'oxic-anoxic' treatment (Table 3.1). The two experimental treatments were

allocated to one of three tanks using a randomised block design of two tanks distributed in three blocks with one replicate per block.

Table 3.1. Description of the two experimental treatments

Treatment no.	Tank structure	Aeration	Redox regime
1	Plenum	Airlift pump	Oxic
2	No plenum	Airstone	Oxic-anoxic

3.2.4 Experimental system and rearing conditions

Six polyethylene tanks (455 × 328 × 175 mm) with calcium carbonate sediment were supplied with recirculating, heated (29.13 ± 0.12 °C) seawater (Chapter 2, Section 0). The aeration and tank design used to create the two contrasting redox regimes (oxic and oxic-anoxic) was as described in Chapter 2, Section 0, Figure 2.2. The type of feed, the feeding, and the siphoning regimes employed were also as described in Chapter 2, Section 0; however, the daily feed rations ranged from one to four percent of the total tank biomass per day. The feed rations were adjusted on a daily basis, equitably across treatments, based on observations of sediment quality. Green epiphytic algae and cyanobacteria (*Oscillatoria* sp.) were removed on a monthly basis, separated and dried at 50 °C for 48 h and tanks walls were manually scrubbed to remove biofilm. Experimental tanks were subject to a natural photoperiod which increased from 11.45:12.15 L:D (0647 to 1832 hours, sunrise to sunset) to 14.20:09.40 L:D (0524 to 1947 hours, sunrise to sunset) as day length increased with the onset of summer.

3.2.5 Growth of Holothuria scabra

Prior to stocking into experimental tanks the sea cucumbers (n = 18) were gut evacuated and weighed (Chapter 2, Section 2.2.1). Animals with a mean weight of 14.98 ± 0.41 g (mean ± SE) were allocated randomly to six groups of three individuals per group. Each individual was re-weighed every 28 d over the 84 d experimental period. Wet weight data were used to calculate growth rate (g d^{-1}; Equation 2, Chapter 2, Section 2.2.5).

3.2.6 Water quality, sediment quality, and environmental variables

Water quality parameters including temperature (0.01 ^{o}C), pH (0.01 pH units), dissolved oxygen (0.01 µg L^{-1}), total ammonia nitrogen (TAN; 0.01 µg L^{-1}) and nitrate (NO_3^-; ± 0.01 mg L^{-1}) were recorded weekly (Chapter 2, Section 2.2.4). In addition, light (0.01 lux) readings (aerial) were taken using a portable light meter (LX-107, Lutron Electronic

Enterprise Co. Ltd, Taipei, Taiwan) positioned 10 cm directly above the tank outflow on a weekly basis at 1000 hours.

At the end of the trial on day 84, the sediment redox potential (0.01 mV) was measured (Chapter 2, Section 2.2.4). Composite samples of the sediment surface layers (upper 2-3 mm) were collected from all replicate tanks per treatment for determination of sediment quality parameters. Chlorophyll *a* (0.01 µg g^{-1}) and phaeopigment (0.01 µg g^{-1}) concentrations were measured using a variation of the spectrophotometric method described by Lorenzen (1967). The extraction step was carried out overnight at four degrees centigrade with 100 % acetone, with the addition of distilled water to return the fluid concentration to 90 % acetone before the first spectrophotometric step. Absorbance of one millilitre of the supernatant was read at 665 and 750 nm before and after acidification with 40 µl of 10 % HCl against a 90 % acetone blank. The sediment samples were dried at 50 °C for 48 h and weighed. Chlorophyll *a* and phaeopigment concentrations were calculated according to Equation 9 and Equation 10 respectively:

Equation 9 Chlorophyll *a* µg g^{-1} = [(AK(665o-665a)v)/Vl]

Equation 10 Phaeopigment µg g^{-1} = [(AK((R*665a)-665o)v)/Vl]

where:

A = 11.0 µg, the inverse extinction coefficient for chlorophyll *a* in 90 % acetone;

R = 1.7, maximum absorbance ratio for 665o/665a without phaeopigment;

K = 2.43;

665o and 665a are the absorbances before and after acidification respectively;

v = volume of acetone used in the extraction (mL);

V = dry sediment weight (g); and

l = cuvette path length (cm).

The remainder of the composite samples were dried to a constant weight at 50 °C for 48 h and organic carbon and total nitrogen content per replicate samples were analysed prior to, and after, carbonate removal (Chapter 2, Section 0).

3.2.7 Deoxyribonucleic acid extraction

Sediment samples were collected from each replicate tank using a coring device (one centimetre internal diameter) and sectioned at 2.0 cm intervals (i.e. from the surface of the sediment) and at depths of 2.0 and 4.0 cm. Genomic deoxyribonucleic acid (DNA) was extracted from approximately 250 mg of sediment using a DNA isolation kit (PowerSoil™,

MoBio, Solana Beach, USA), following manufacturer's instructions, yielding purified genomic DNA for use in polymerase chain reaction (PCR) amplification.

3.2.8 Polymerase chain reaction amplification of the variable region 4-5 of the 16S ribosomal ribonucleic acid gene

The 16S ribosomal ribonucleic acid (rRNA) gene was amplified using fusion primers, consisting of sequencer specific nucleotides, multiplex identifier tag and template-specific nucleotides with the template-specific sequences within the forward and reverse primers respectively. The primer pair E517F (5'-CAGCAGCCGCGGTAA-3') and E969-984 (5'-GTAAGGTTCYTCGCGT-3') was selected for amplification of variable regions four and five of the bacterial 16S rRNA gene, as it has been shown to have high taxonomic coverage (Wang and Qian, 2009). Multiplex identifier (MID) tags were used in barcoding the amplicons from each sample to facilitate the assignment of each sequence generated to the correct sample identification.

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene fragments from each sample was carried out as follows: a 25 µl PCR mixture consisting of ~5 ng of the extracted genomic DNA, 1X PCR buffer (containing $MgCl_2$), 300 µM dNTPs, 10.0 µM of each primer set and 0.5 µl KAPA HiFi HotStart DNA Polymerase (KAPA Biosystems) were subjected to initial enzyme activation and DNA denaturation at 98 °C for five minutes followed by cycling parameters of 98 °C for 45 s, 45 °C for 30 s, 72 °C for one minute (for five cycles) and 98 °C for 45 s, 50 °C for 30 s and 72 °C for one minute (20 cycles). A final extension was done at 72 °C for five minutes. The resultant ~540 nt PCR products were gel purified using the AMPure® XP (Beckman Coulter, Ireland) and the double-stranded DNA (dsDNA) concentration was determined using PicoGreen® (Invitrogen, Germany) on a Thermo Scientific NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, USA). The respective amplicons generated were pooled in equal amounts and subjected to emulsion PCR before sequencing.

3.2.9 Pyrosequencing and sequence analysis

Amplicons were sequenced using the GS Titanium Sequencing chemistry (454 Life Sciences, Roche, USA). Reads were de-multiplexed and pre-processed using the automated Roche GS Run Processor pipeline to remove adapter sequences and low quality reads. After de-multiplexing, flowgram data (SFF-files) for each sample were processed using the QIIME Denoiser (Reeder and Knight, 2010) according to the QIIME standard protocol (Caporaso *et al.*, 2010b). Individual sequences were parsed into sample-specific libraries and screened for reads less than 200 base pairs. Chimera detection was completed using Chimera Slayer (Haas

et al., 2011) in the QIIME 1.8.0 software package. Only sequences flagged as non-chimeras by *de novo* and reference-based (Greengenes database, August 2013 release; DeSantis *et al.*, 2006) methods were retained for further analyses. Sequences with 97 % similarity were clustered into operational taxonomic units (OTUs) using UCLUST (Edgar, 2010). Taxonomic assignment of the resulting reads was performed using PyNAST (Caporaso *et al.*, 2010a) to align sequences against the Greengenes core reference set (August 2013 release) to genus level where possible. The OTU tables produced by the quality filtering containing the taxonomic assignment of the OTUs were used for all downstream analyses.

3.2.10 Statistical and bioinformatics analysis

Sea cucumber growth and environmental metadata

The light readings and water quality data were averaged across the 84 day period to provide a mean value per replicate tank. The mean wet weight of individual *H. scabra* per replicate tank was averaged and the mean value per tank was used for further statistical analysis. Growth and environmental data were tested for homogeneity of variance and for the normal distribution of the residuals using Levene's (Levene, 1960) and Shapiro Wilk's (Shapiro and Wilk, 1965) tests respectively. All data that met the test assumptions were analysed using a Student t-test at alpha < 0.05. Results are expressed as mean ± standard error. Statistical analyses were performed using Statistica version 12.

Microbial community composition

Alpha diversity

Alpha diversity measures were calculated to provide information about the diversity within individual samples. Sequences were normalized to the minimum number of reads per sample (1,264) and alpha diversity parameters were computed using QIIME 1.8.0 (Caporaso *et al.*, 2010b). Richness estimators included the total number of OTUs observed in a sample (S_{obs}) and Chao1 (Chao, 1984), and diversity indices included Shannon (Shannon, 1948) and Simpson (Simpson, 1949) diversity. Evenness was calculated using the equitability metric defined in QIIME as: (Shannon entropy) / $\log_2$ (S_{obs}). Mean diversity indices were tested for normality using Shapiro Wilk's test and homogeneity of variance and for the normal distribution of the residuals using Levene's test. Data that met the test assumptions were compared across experimental treatments using mixed-model analysis of variance (ANOVA) to test the effects of redox regime and sediment depth on the alpha diversity measures. Redox regime was included as a fixed factor and sediment depth included as a covariate. Significant differences between treatments were identified by Tukey honest significant difference (HSD) post-hoc tests. Results were expressed as mean ± standard error and differences were

considered significant at alpha < 0.05. Statistical analyses were performed using Statistica version 12.

Taxonomic groups with statistical differences

As the Proteobacteria had the highest sequence abundance, representing 41.12 ± 1.07 % (n = 15) of the total number of sequences, the Proteobacteria sub-classes of Alpha-, Beta-, Delta-, and Gammaproteobacteria were included in all phylum-level analyses. As the relative abundance data at the phylum level did not meet the test assumptions for ANOVA, non-parametric Kruskal-Wallis tests were used to identify bacterial phyla that were significantly different between experimental treatments. The analysis was performed in RStudio (version 0.98.1091) using a script (KW.R) written by U. Ijaz available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html. Data were log transformed and differences were considered significant at alpha = 0.01.

Beta diversity measures

Beta diversity analysis was performed on taxonomic data at the OTU level to explore the similarity of the microbial community composition between samples. Non-metric multidimensional scaling (NMDS; Borg and Groenen, 1997) based on Bray-Curtis dissimilarity (Bray and Curtis, 1957), was used to represent dissimilarities between treatments and ellipses were added to group the samples by redox regime. The analysis was performed in RStudio (version 0.98.1091) using a script (NMDS.R) written by U. Ijaz available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html.

Linking community analyses to environmental variables

To examine how biotic and abiotic factors were related to overall bacterial community composition, a script (NMDS_bioenv.R) written by U. Ijaz available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html, was used to plot significant taxa and environmental variables on an NMDS plot. The script uses the bio.env () and bv.step () functions to find the best set of environmental parameters and taxa respectively, by examining all the possible subsets of variables that, in combination, correlate most strongly with community dissimilarities (Clarke and Ainsworth, 1993). Similarity matrices for taxonomic and environmental data were computed based on Bray-Curtis similarity and Spearman rank correlation coefficients were calculated between the two matrices. The Spearman rank ρ values indicate the rank correlation between the matrix of bacterial community composition and the similarity matrices from the environmental variables. Only significant ρ values are shown. The best subset of taxa and environmental variables were identified by Mantel tests, performed by the BVSTEP and BIOENV functions respectively, and subjected to a permutation test to determine significance. The vectors of the best-

correlated environmental and biological variables were overlaid on the NMDS plot. Finally, permutational multivariate analysis of variance (PERMANOVA) was used to quantitatively evaluate the contribution of environmental metadata to the microbial community structure using the 'ADONIS' function in the vegan package (Oksanen *et al.*, 2016). The analyses were performed in RStudio (version 0.98.1091).

Microbial biomarker discovery and visualization

Taxonomic biomarkers were identified by linear discriminant analysis effect size (LEfSe; Segata *et al.*, 2011) from samples taken at three different depths in the oxic and oxic-anoxic sediments. This method is designed to analyse large data sets using robust statistical tests (Kruskal-Wallis) that incorporate biological consistency (Wilcoxon rank-sum tests), to predict taxonomic biomarkers based on effect-size estimation (Segata *et al.*, 2011). Differentially abundant and biologically relevant taxonomic biomarkers (from phylum to genus level) were determined by linear discriminant analysis (LDA) with an effect size threshold of 5.0 (on a $\log_{10}$ scale; Segata *et al.*, 2011). To further elucidate the functional diversity and metabolic role of the bacterial communities in the oxic and oxic-anoxic treatments, the 86 taxonomic biomarkers identified at an LDA score greater than 5.0 were collapsed to reflect only the highest level of taxonomic resolution. This reduced the number of biomarkers to 24 and 16 in the oxic and oxic-anoxic sediments, respectively. These biomarkers were classified according to their type of dissimilatory metabolism, oxygen-related ecophysiology and putative functional role based on available literature (Appendix A).

Functional gene prediction

To gain further insight into the predicted metabolic functions of bacteria that were differentially enriched in the oxic and oxic-anoxic treatments, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) v0.9.0 was applied (Langille *et al.*, 2013). The de-multiplexed and quality-filtered reads were re-processed by picking closed-reference OTUs based on the Greengenes database (August 2013 release). During closed-reference OTU picking, approximately 35 % of the sequences were excluded. The closed-reference OTUs were assigned to Greengenes clades containing the most genomes for that particular clade from genus level up. The gene counts of predicted genes were reconstructed based on Greengenes phylogeny and assigned Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology (KO) copy numbers (Kanehisa *et al.*, 2012). Each KO entry represents a manually defined group corresponding to a node of the KEGG pathway map or module, which consists of orthologous genes in all the genomes deposited in the KEGG database (Kanehisa *et al.*, 2012). The relative abundance of each gene (KO) was then estimated for each sample by multiplying each OTU abundance by each predicted functional

trait abundance. Inferred relative gene abundances were subsequently grouped into the six main categories and their respective functional categories defined by the BRITE functional hierarchy, which represents hierarchical classifications of known genes and proteins, diseases and drugs, compounds and reactions, and organisms and cells.

To evaluate the accuracy of the metagenome predictions, weighted nearest sequenced taxon index (weighted NSTI) scores were calculated (Langille *et al.*, 2013). The NSTI scores refer to the average branch length that separates OTUs in the sample from the reference genome, weighted by the abundance of the OTU in the sample; thus low NSTI scores refer to shorter branch lengths and indicate a more accurate prediction (Langille *et al.*, 2013). The relative gene counts were analysed in the graphical software package 'statistical analysis of taxonomic and functional properties' (STAMP; Parks *et al.* (2014)). To simplify analysis any non-microbial categories, for example 'organismal systems' and 'human diseases', were excluded from further analysis. To identify metabolic pathways that were significantly different between oxic-anoxic and oxic treatments, two-sided Welch's t-tests were used to compare the gene counts at levels two and three of the BRITE hierarchies with a Bonferroni multiple test correction to control for false discovery rate. Only pathways or modules with a significantly different mean proportion of gene counts between the two redox regimes are presented (alpha = 0.05). The data are presented as extended error bar plots showing the mean proportion (%) and the difference in the mean proportion of gene counts between the oxic and oxic-anoxic treatments with 95 % confidence intervals.

3.3 Results

3.3.1 Water and sediment quality

The water temperature in oxic-anoxic treatments (29.81 ± 0.01 °C) was significantly higher than in the oxic treatments (29.13 ± 0.12 °C; Student's t-test, t = 5.84, p = 0.004; Table 3.2). Similarly, dissolved oxygen concentrations were significantly higher in oxic treatment tanks fitted with an airlift pump and plenum (7.89 ± 0.06 mg L^{-1}) than in the oxic-anoxic treatments with no plenum and an air stone (7.49 ± 0.11 mg L^{-1}; Student's t-test, t = -3.15, p = 0.035). These differences in temperature and dissolved oxygen concentration may be due to differences in aeration and water movement between treatments (Chapter 2, Section 0, Figure 2.2).

There was a significant difference in the mean sediment redox potential of the oxic and oxic-anoxic treatments (Student's t-test; t = -13.93, p = 0.0002; Table 3.2). The stratified oxic-anoxic sediment had a negative reading of -188.42 ± 11.52 mV, indicating predominantly reduced conditions, while the sediment in the oxic treatments was positive at

33.50 ± 11.00 mV, indicating predominantly oxic conditions. Over the duration of the experiment the sediment redox regime in the oxic treatments produced more than twice the biomass of the cyanobacteria *Oscillatoria sp.* on the sediment surface and in floating colonies (221.61 ± 34.95 g dry weight compared with 99.66 ± 2.72 g dry weight in the oxic-anoxic treatments, Student's t-test, t = -3.48, p = 0.025).

Table 3.2. Mean (± standard error) values for the environmental parameters recorded over the 84 d experimental period in sea cucumber tanks subjected to oxic and oxic-anoxic redox regimes. Significant differences ($p < 0.05$) are indicated by an asterisk (*).

Parameter	Oxic-anoxic			Oxic			t-value	p
Light (Lux)	1 406.67	±	119.21	1 465.67	±	217.13	-0.24	0.8234
Temperature (°C)	29.81	±	0.01	29.13	±	0.12	5.84	0.0043*
pH	8.39	±	0.01	8.33	±	0.02	2.30	0.0831
Dissolved oxygen (mg L^{-1})	7.49	±	0.11	7.89	±	0.06	-3.15	0.0347*
Dissolved oxygen (%)	116.00	±	1.64	121.33	±	1.54	-2.37	0.0770
Ammonia (µg L^{-1})	17.79	±	2.33	19.15	±	5.17	-0.24	0.8225
Nitrite (µg L^{-1})	15.48	±	1.13	15.61	±	1.02	-0.08	0.9383
Chlorophyll *a* (µg g^{-1})	2.05	±	0.75	2.43	±	0.57	-0.41	0.7038
Phaeopigment (µg g^{-1})	0.21	±	0.28	0.39	±	0.09	-0.61	0.5738
Green macroalgae (dry g $tank^{-1}$)	12.74	±	3.24	7.19	±	2.81	1.29	0.2655
Cyanobacteria (dry g $tank^{-1}$)	99.66	±	2.72	221.61	±	34.95	-3.48	0.0254*
Redox potential (mV)	-188.42	±	11.52	33.50	±	11.00	-13.93	0.0002*
Organic carbon (%)	1.59	±	0.32	1.58	±	0.13	0.03	0.9781
Total nitrogen (%)	0.06	±	0.02	0.08	±	0.01	-0.74	0.4982
C:N (%)	26.03	±	2.32	21.19	±	3.69	1.04	0.3562

3.3.2 **Holothuria scabra** ***survival and growth***

Survival of *H. scabra* was 100 % in all treatments. The mean wet weight (± standard error) was similar in both treatments at the start of the trial (7.57 ± 0.27 g, Student's t-test; t = -2.03, p = 0.11). After 84 d there was no significant difference in mean growth rate of *H. scabra* reared on sediments with different redox regimes (t-test; t = 1.24, p = 0.28; Figure 3.1a).

Sea cucumbers in the oxic-anoxic treatments achieved a final mean density of 1028.50 ± 117.46 g m^{-2}, while the density of sea cucumbers reared on fully oxic sediments decreased from 943.76 ± 56.84 g m^{-2} on day 56 to 837.96 ± 99.70 g m^{-2} on day 84 (t-test; t = 1.24, p = 0.28; Figure 3.1b).

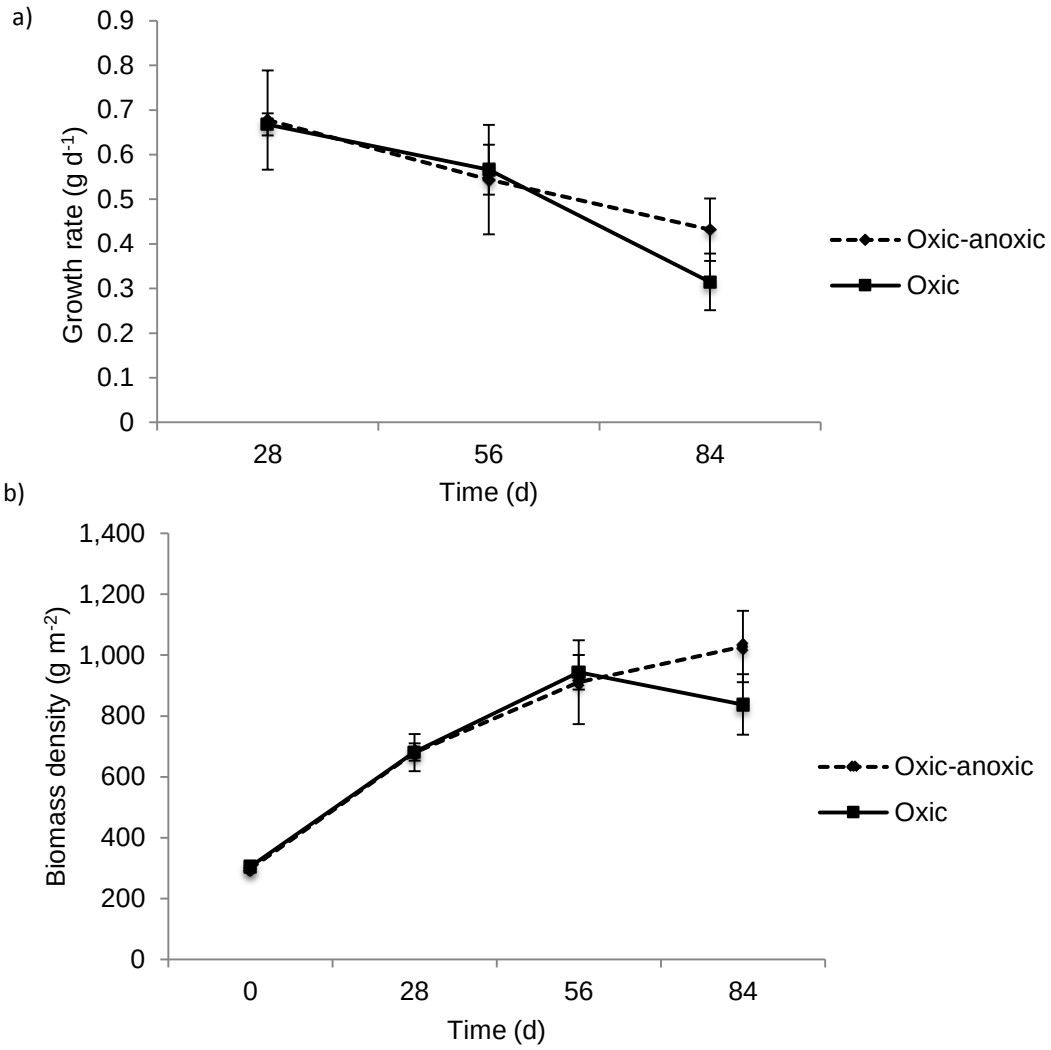

Figure 3.1. a) The mean (± standard error) growth rate of *Holothuria scabra* (n = 4) reared in tanks with a stratified oxic-anoxic and fully oxic sand sediment; b) the mean (± standard error) biomass density of *Holothuria scabra* (n = 4) reared in tanks with a stratified oxic-anoxic sand sediment

3.3.3 Sequencing and quality control

A total number of 72,675 PCR amplicons spanning the V4-5 hypervariable region of the 16S rRNA gene were generated by pyrosequencing triplicate samples from three sediment depths subject to two different redox regimes (n = 18). Due to a low abundance of reads, three samples (oxic-anoxic_0 cm_replicate_a, oxic_4 cm_replicate_a, and oxic_4 cm_replicate_b) were removed from further analysis. Subsequent to quality control, primer trimming, size exclusion, and removal of OTUs classified as unassigned bacteria and archaea, a total of 47,573 optimised reads in the 15 samples remained.

3.3.4 Species richness, diversity and evenness of bacterial communities

Rarefaction analysis was used to provide an indication of sampling saturation and compare bacterial richness between the different sediment samples, both between and within treatments. For rarefaction analysis, the data were subsampled to the minimum number of

sequences in the 15 samples (n = 1,264). This demonstrated that oxic treatments did not plateau indicating that the sediments were not sampled to saturation (Figure 3.2). Conversely, the depth of sequencing was sufficient for the oxic-anoxic treatments as the rarefaction curves for all samples started to reach the curvilinear or plateau phase.

All of the richness estimators and diversity indices were significantly higher in the fully oxic sediment treatments (mixed model ANOVA; $p > 0.05$; Table 3.3). The Chao 1 index, indicating the number of rare OTUs and the observed number of OTUs (sobs) were significantly higher in the oxic treatments (mixed model ANOVA; $F_{(1, 12)} = 39.20$, $p < 0.001$ and $F_{(1, 12)} = 48.05$, $p < 0.001$ respectively). The Shannon diversity index, which combines species richness and evenness, was significantly higher in the oxic treatments (mixed model ANOVA; $F_{(1, 12)} = 50.43$, $p < 0.001$). Similarly, Simpson's dominance index and evenness were significantly higher in oxic treatments compared to the oxic-anoxic treatments (mixed model ANOVA; $F_{(1, 12)} = 22.65$, $p < 0.001$ and $F_{(1, 12)} = 27.45$, $p < 0.001$ respectively). Taken together, these results indicate that the sediments maintained under a fully oxic redox regime harboured more diverse and stable bacterial communities compared to the stratified oxic-anoxic sediments.

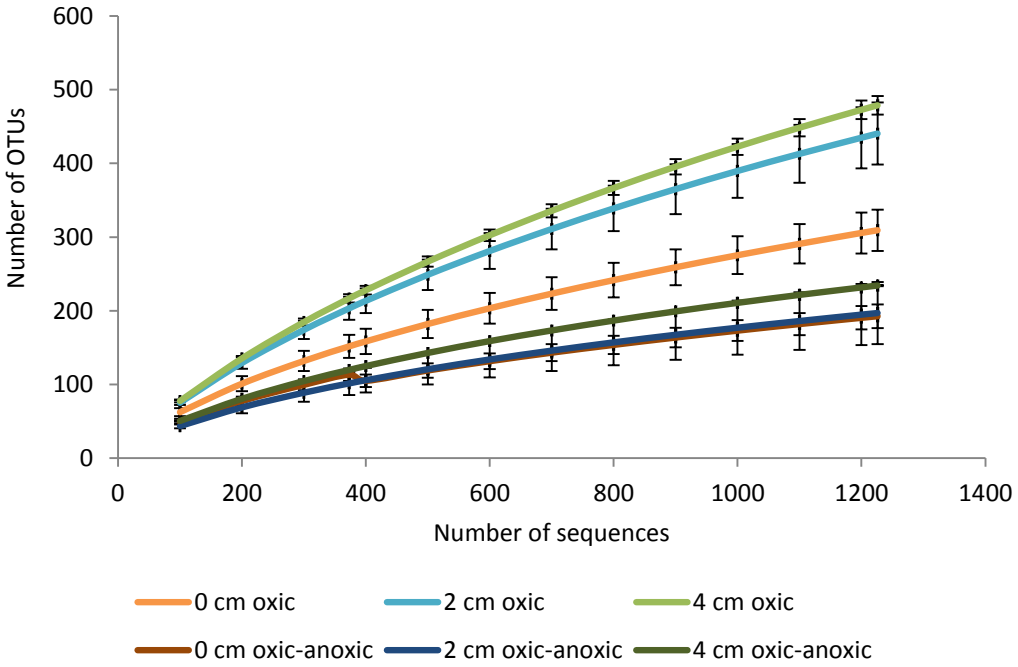

Figure 3.2. Rarefaction curves of bacterial 16S rRNA gene sequences recovered from the three depths (0, 2 and 4 cm) of sediment taken from *Holothuria scabra* culture tanks subjected to a fully oxic and a stratified oxic-anoxic redox regime. Each line represents the mean of treatment replicates. Error bars are standard error of the mean (n = 3 except for treatments 'oxic-anoxic 0 cm' and 'oxic 4 cm' where replicates were excluded due to low read abundance). OTU is operational taxonomic unit.

Table 3.3. Mean (± standard error) values for alpha diversity measures computed in QIIME for bacterial communities present at three different depths in the sediment of sea cucumber culture tanks subjected to contrasting oxic and oxic-anoxic redox regimes. Different superscript letters within the same row indicate significant differences (mixed-model ANOVA; $p < 0.05$). OTUs = operational taxonomic units. ns = not significant.

	Oxic-anoxic			Oxic				
	0 cm	2 cm	4 cm	0 cm	2 cm	4 cm	Redox regime	Depth
No. of OTUs	254.55 ± 32.00^{a}	247.77 ± 14.86^{a}	268.73 ± 38.04^{a}	357.13 ± 11.49^{a}	472.40 ± 14.76^{b}	565.70 ^{b}	p<0.001	ns
Chao 1	380.98 ± 49.46^{a}	393.97 ± 39.60^{a}	410.91 ± 65.97^{a}	542.84 ± 25.10^{ab}	707.23 ± 27.18^{bc}	857.68 ^{c}	p<0.001	ns
Simpson	0.96 ± 0.01^{a}	0.96 ± 0.01^{a}	0.97 ± 0.01^{ab}	0.97 ± 0.00^{ab}	0.99 ± 0.00^{b}	0.99 ^{ab}	p<0.001	ns
Shannon	5.73 ± 0.39^{ab}	5.70 ± 0.13^{a}	6.01 ± 0.06^{ab}	6.43 ± 0.11^{b}	7.59 ± 0.10^{c}	7.94 ^{c}	p<0.001	ns
Evenness	0.71 ± 0.03^{a}	0.72 ± 0.03^{a}	0.75 ± 0.03^{ab}	0.78 ± 0.02^{ab}	0.87 ± 0.02^{b}	0.88 ^{ab}	p<0.001	ns

3.3.5 Relative abundance of prominent taxa

Taxonomic analysis of all the samples detected 20 unique phyla, 21 candidate divisions, and two phyla proposed by the Greengenes database ([Caldithrix] and [Thermi]). Bacteroidetes had the highest sequence abundance representing 27.83 ± 2.04 % (n = 15) of the total number of sequences, followed by Gammaproteobacteria (20.92 ± 2.52 %), Deltaproteobacteria (13.74 ± 2.16 %), Planctomycetes (5.25 ± 1.52 %), Fusobacteria (5.02 ± 1.56 %), Epsilonproteobacteria (4.53 ± 1.42 %) and Cyanobacteria (4.52 ± 1.79 %; Figure 3.3). Prior to quality control, unclassified bacteria represented 2.61 % of the total number of sequences, which were removed to facilitate downstream analyses. Analysis of the taxonomic groups detected in all of the sediment samples combined showed that the phylum Nitrospira, the candidate divisions AncK6, GAL15, SBR1093, TM7 and the Proteobacteria sub-class TA18 were only present in oxic sediments. Similarly, phylogenetic groups from the phyla Thermotogae, Fibrobacteres, [Thermi] and the candidate divisions KSB3 and LCP-89 were only present in the redox stratified oxic-anoxic sediments.

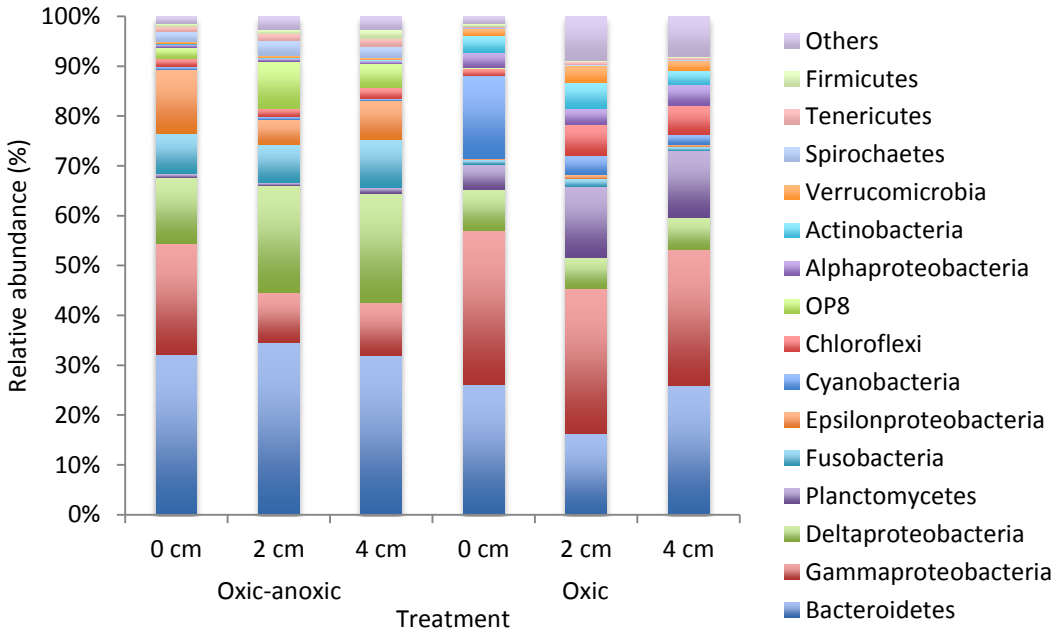

Figure 3.3. The relative abundance of the bacterial reads classified at phylum level (including Proteobacteria sub-classes) from the different sediment redox regimes and depths. Each bar represents the mean of treatment replicates (n = 3).

3.3.6 Phylum-level taxa with statistical differences between treatments

The sediment redox regime led to significant differences in the relative abundance of eight phyla, five candidate divisions and four of the Proteobacteria classes between the oxic-anoxic and oxic treatments. There was a significantly higher number of sequences classified

within the phyla Actinobacteria, Cyanobacteria, Nitrospirae, Planctomycetes, Verrucomicrobia, the Alpha-, and Gammaproteobacteria sub-classes and the candidate division TM7 in the fully oxic sediments, while the number of sequences within the Chlorbi, Fusobacteria, Spirochaetes, the Delta-, and Epsilonproteobacteria sub-classes and the candidate divisions H-178, KSB3, OP8 and SAR406 were significantly higher in the oxic-anoxic sediments ($p > 0.01$; Figure 3.4).

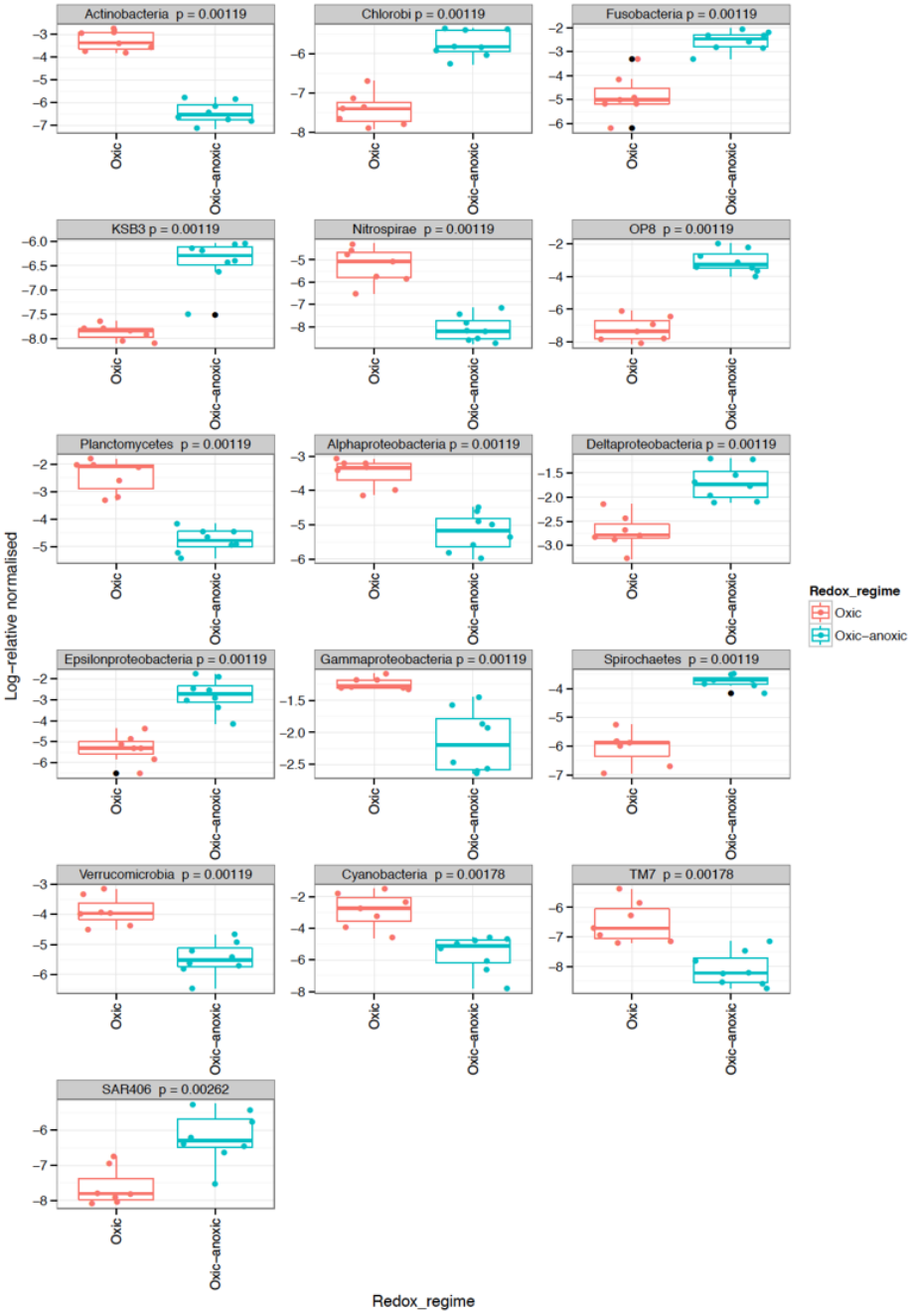

Figure 3.4. Bacterial phyla with significantly different relative abundances between the oxic and oxic-anoxic redox regimes. Data are presented as log normalised relative abundances.

3.3.7 Comparison of bacterial community composition between treatments

Non-metric multi-dimensional scaling analysis performed on the OTU tables revealed that bacterial communities within sediments subject to the two different redox regimes were distinct, with the oxic and oxic-anoxic communities clustering together into two clear groups (Figure 3.5).

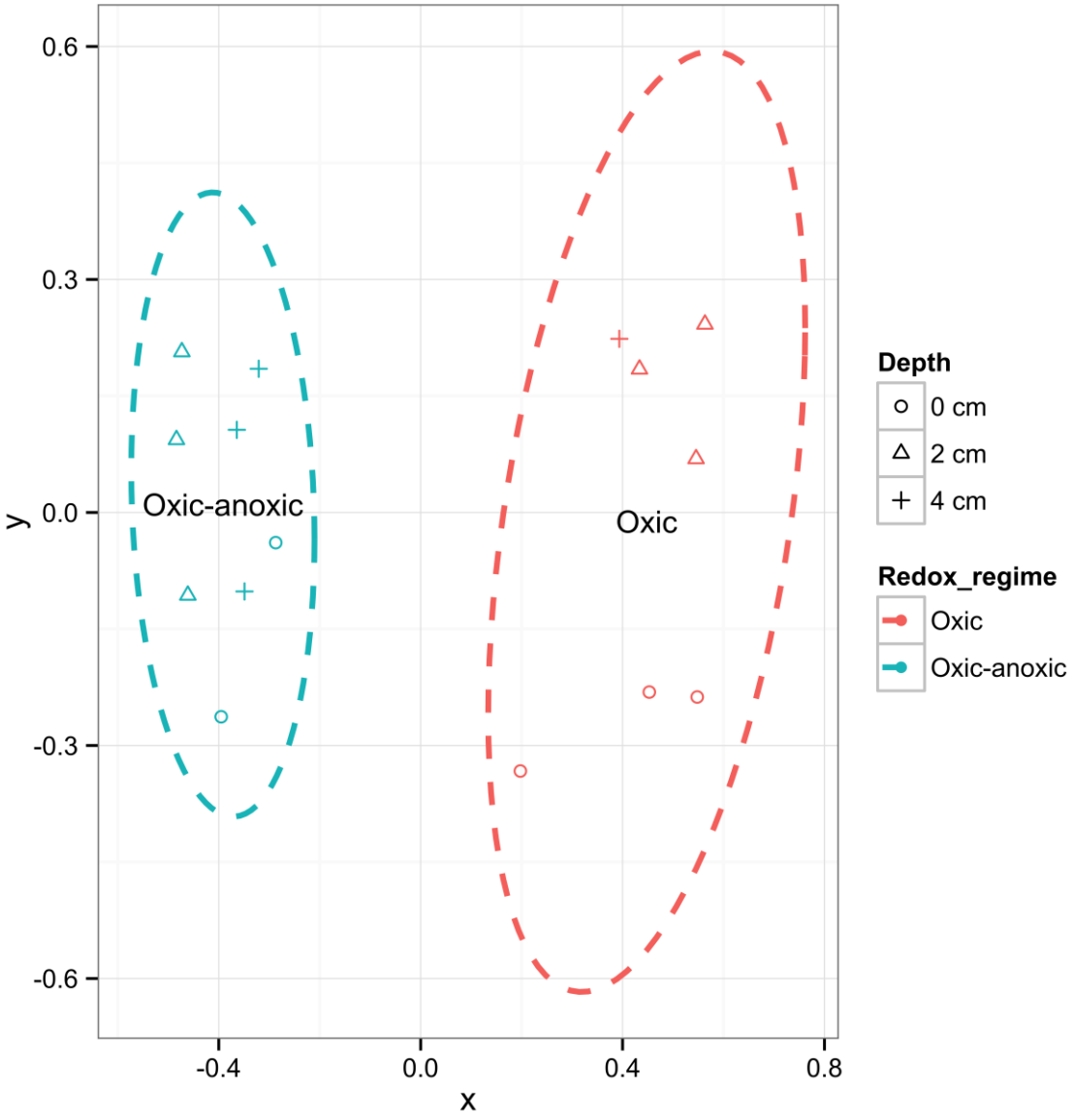

Figure 3.5. Non-metric multidimensional scaling (NMDS) ordination of bacterial community structure at the different sampling depths in the oxic and oxic-anoxic treatments.

3.3.8 Environmental factors driving bacterial community composition

The best subset of environmental parameters with the highest rank correlation with bacterial community dissimilarities was obtained with a combination of light, dissolved

oxygen saturation, nitrite, chlorophyll *a*, redox potential and total nitrogen (ρ = 0.83; Figure 3.6). The sediment redox potential and oxygen saturation of the water column were associated with differences in the bacterial community structure in the oxic and oxic-anoxic treatments by permutation tests (Table 3.4 and Figure 3.6).

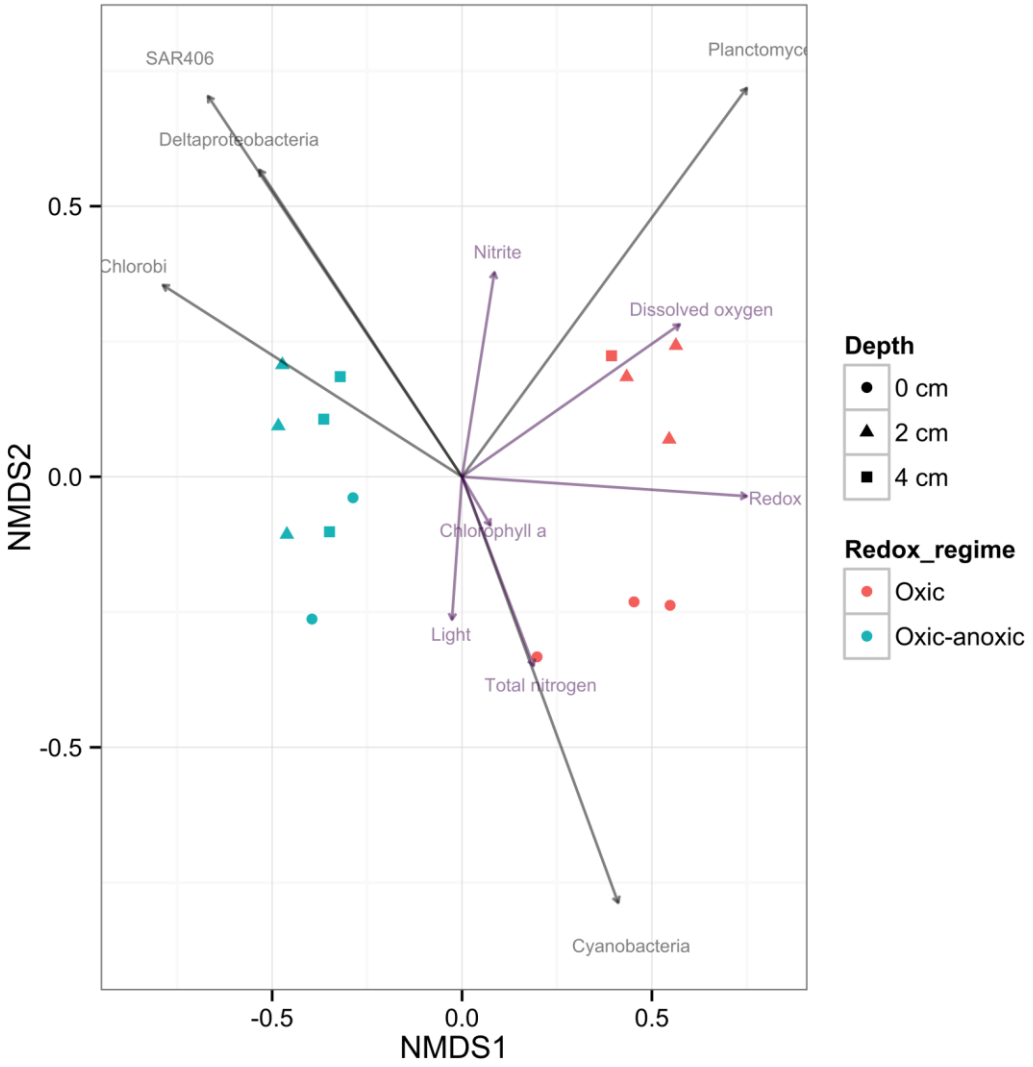

Figure 3.6. Non-metric multidimensional scaling (NMDS) ordination of the correlation between the bacterial community composition and the best sub-set of taxa and environmental parameters with maximum correlation with community dissimilarities plotted as vectors.

Environmental variables associated with primary production, including light, chlorophyll *a* and total nitrogen content of the sediment, were important in shaping the microbial communities in the surface (0 cm) sediment in the oxic treatments. The best sub-set of taxa identified by the BV STEP function was Chlorobi, Cyanobacteria, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Planctomycetes, SBR1093, Tenericutes and Thermotogae with a rank correlation of 0.94, which were all significantly correlated with community

dissimilarities with the exception of Lentisphaerae (Table 3.5). Quantitative evaluation of the contribution of the environmental parameters to patterns in bacterial community structure identified sediment redox potential as the only significant driver behind differences in bacterial community structure between treatments (Table 3.6).

Table 3.4. The best sub-set of environmental variables found to have maximum rank correlation with bacterial community structure determined by Mantel tests performed by the BIOENV function and their significance level determined by permutation tests. The r^2 is the squared correlation coefficient; Pr(>r) is the significance level (or P-value) determined after 999 random permutations of the data. Significance codes denote the level of significance: 0-0.001 '***'; 0.001-0.01 '**' ; 0.01-0.05 '*'.

Environmental variable	NMDS1	NMDS2	r^2	Pr(>r)	Significance level
Light	-0.09955	-0.99503	0.1162	0.474	
Dissolved oxygen	0.89743	0.44115	0.6721	0.001	***
Nitrite	0.22044	0.97540	0.2473	0.193	
Chlorophyll *a*	0.63988	-0.76848	0.0225	0.859	
Redox	0.99885	-0.04795	0.9287	0.001	***
Total nitrogen	0.47450	-0.88025	0.2598	0.181	

Table 3.5. The best subset of phyla found to have maximum rank correlation with bacterial community structure determined by Mantel tests performed by the BVSTEP function and their significance level determined by permutation tests. The r^2 is the squared correlation coefficient; Pr(>r) is the significance level (or P-value) determined after 999 random permutations of the data. Significance codes denote the level of significance: 0-0.001 '***'; 0.001-0.01 '**' ; 0.01-0.05 '*'.

Phylum	NMDS1	NMDS2	r^2	Pr(>r)	Significance level
Chlorobi	-0.91191	0.41039	0.6202	0.005	**
Cyanobacteria	0.46303	-0.88635	0.6556	0.002	**
Lentisphaerae	-0.93531	0.35382	0.2925	0.132	
OP3	0.53150	0.84706	0.7780	0.001	***
Planctomycetes	0.72213	0.69176	0.8961	0.001	***
Deltaproteobacteria	-0.68522	0.72833	0.5039	0.009	**
SAR406	-0.68927	0.72451	0.7830	0.001	***

Table 3.6. Quantitative contribution of environmental parameters to bacterial community structure determined by permutational multivariate analysis of variance (PERMANOVA) using distance matrices (ADONIS; $p < 0.05$)

Environmental variable	df	SS	Mean squares	F model	R^2	p
Redox potential	1	0.33417	0.33417	15.2182	0.58837	0.001
Dissolved oxygen	1	0.00712	0.00712	0.3243	0.01254	0.784
Light	1	0.02019	0.02019	0.9193	0.03554	0.411
Temperature	1	0.00205	0.00205	0.0933	0.00361	0.971
pH	1	0.00680	0.00680	0.3099	0.01198	0.819
Residuals	9	0.19763	0.02196		0.34796	
Total	14	0.56796			1.00000	

3.3.9 Microbial biomarker discovery and visualization

There was a total of 86 taxa distinguishing the two groups with logarithmic LDA scores greater than 5.0. A total of 50 taxonomic biomarkers, from phylum to genus level, were identified as being consistently different, both statistically and biologically, in the oxic treatments compared to 36 taxonomic biomarkers in oxic-anoxic treatments. The 50 bacterial lineages enriched in the sediment in the oxic treatments were classified within the phyla Actinobacteria, Bacteroidetes, Caldithrix, Chloroflexi, Cyanobacteria, Nitrospirae, Planctomycetes, Verrucomicrobia; the Alpha-, Delta- and Gammaproteobacteria classes and the candidate division TM6. In the redox-stratified sediments in the oxic-anoxic treatments, there were 16 bacterial lineages classified within eight phyla including Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Fusobacteria, Planctomycetes, Spirochaetes, Tenericutes, the Delta-, and Episilonproteobacteria subclasses and the three candidate divisions H-178, KSB3, and OP8 (Figure 3.7). Among the 86 taxonomic biomarkers identified by LEfSE, spanning a total of 14 phyla and four candidate divisions, only biomarker taxa within the phyla Chloroflexi, Plantomycetes, and the Deltaproteobacteria class were identified in both treatments.

Classification of the biomarker taxa according to their oxygen-related ecophysiology elucidated clear differences in the composition of bacterial communities based on their relationship with molecular oxygen. The majority of biomarker taxa identified in oxic-anoxic treatments were strict or obligate anaerobes, while all of the biomarkers in the sediments maintained under fully oxic conditions by the circulation of oxygenated water were obligate or facultative aerobic bacteria (Appendix A; Tables A1.11 and A1.2). Classification of the biomarker taxa, based on their type of dissimilatory metabolism, revealed stark differences in the metabolic capacity and putative functional roles of the microbial communities under different redox regimes. Biomarker taxa that were significantly enriched in the oxic treatments represented a broad spectrum of dissimilatory types of metabolism including heterotrophic taxa with aerobic and anaerobic respiratory and fermentative metabolisms; chemolithotrophic bacteria; methylotrophic and phototrophic bacteria performing both oxygenic and anoxygenic photosynthesis (Appendix A; Table A1.1). In contrast, all of the biomarker taxa enriched in the oxic-anoxic treatments were classified as heterotrophic bacteria (Appendix A; Table A1.2).

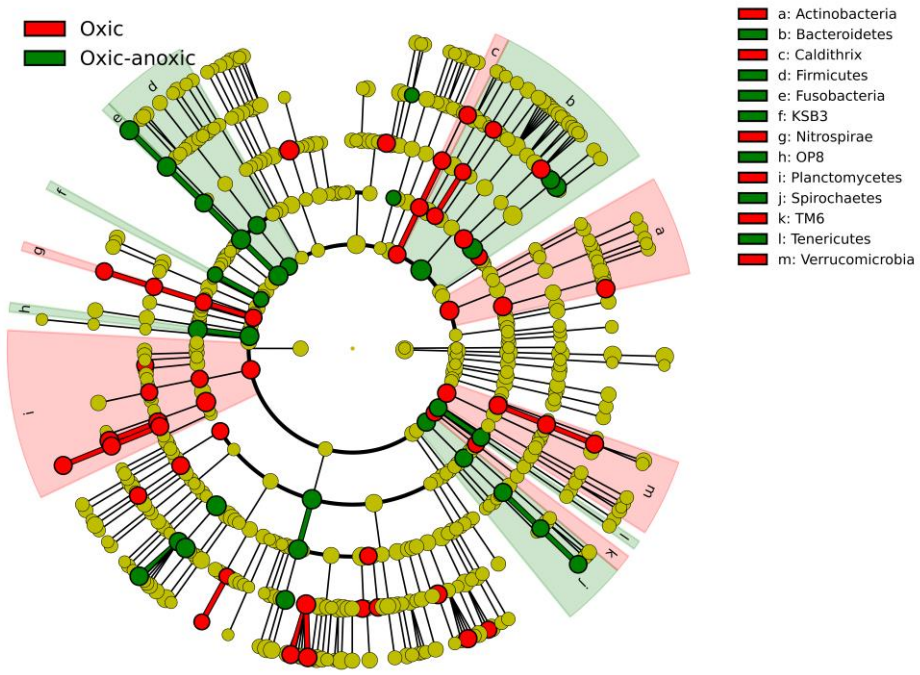

Figure 3.7. The phylogenetic distribution of microbial lineages associated with the two different sediment redox regimes (oxic-anoxic and oxic). Lineages with linear discriminant analysis (LDA) values of 5.0 or higher determined by effect size measurements (LEfSe) are displayed. The six rings of the cladogram stand for domain (innermost), phylum, class, order, family and genus. Enlarged circles in dark green and red are differentially abundant taxa identified as taxonomic biomarkers in the two different redox regime treatments (red = oxic-anoxic sediment, green = oxic sediment). Light green labels indicate taxa that were not over represented in either treatment. Labels are shown at the phylum level only.

3.3.10 Predicted metagenome

The metagenome predictions using PICRUSt had NSTI scores ranging from 0.11 to 0.21 with an overall mean of 0.16 ± 0.01 (Langille *et al.*, 2013). Redox regime had an effect on the accuracy of the predictions, with significantly lower NSTI scores and higher sequence similarity to bacterial genomes in the database for samples taken from the redox-stratified sediments in the oxic-anoxic treatment (mixed model ANOVA; $F_{(1,\ 12)} = 60.33$, $p > 0.001$; Table 3.7). The lower accuracy of the predictions of the metagenomes of bacteria in the oxic sediments may be due to higher bacterial diversity and increased abundance of bacteria that do not yet have sequenced representatives.

Table 3.7. Accuracy of the predicted metagenomes for samples evaluated by nearest sequenced taxon identify (NSTI) score and sequence identity (calculated as 1-NSTI score). Data are presented as means ± SE.

Redox regime	Sediment depth	NSTI score	Sequence identity (%)
Oxic-anoxic	0 cm	0.14 ± 0.01	0.86 ± 0.01
Oxic-anoxic	2 cm	0.14 ± 0.00	0.86 ± 0.00
Oxic-anoxic	4 cm	0.13 ± 0.01	0.87 ± 0.01
Oxic	0 cm	0.20 ± 0.01	0.80 ± 0.01
Oxic	2 cm	0.18 ± 0.00	0.82 ± 0.00
Oxic	4 cm	0.18 ±	0.82 ±

A total of 236 pathways were identified at the third-tier level of functional categories defined by the BRITE hierarchy. At the two-tier level, there were significant differences in the predicted bacterial metagenome in the sediments subject to differing redox regimes. At the top level of pathway modules, genes involved with metabolism were significantly enriched in the fully oxic sediment. At the functional subcategory level, three pathways within metabolism, (xenobiotics biodegradation and metabolism, metabolism of terpenoids and polyketides, and metabolism of other amino acids) were enriched in oxic treatments. At the functional subcategory (level two), the oxic-anoxic treatments were significantly enriched with genes involved in cell growth and death (cellular processes); signal transduction, translation, replication and repair (genetic information processing), signal transduction (environmental information processing, glycan biosynthesis and metabolism, and nucleotide metabolism (metabolism; Figure 3.8).

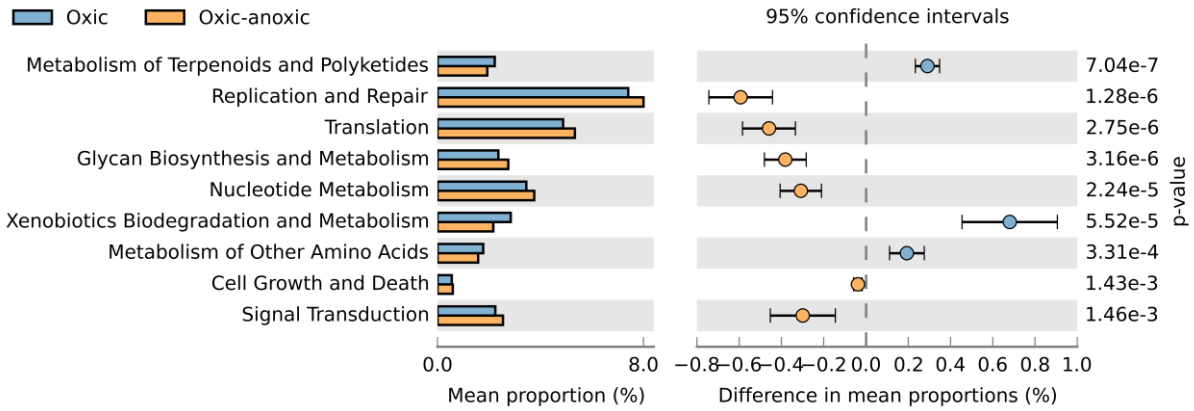

Figure 3.8. The mean proportion (%) and the difference in the mean proportion of gene counts at level two of the BRITE functional hierarchy between oxic-anoxic and oxic treatments with 95 % confidence intervals.

The higher resolution analysis of the predicted functional capacity of bacterial communities present within the sediments subjected to contrasting redox regimes, followed a

similar trend with a predominance of orthologous genes involved in metabolism and degradation pathways enriched in the oxic sediments. Of the 28 functional pathways that had gene counts with a significantly higher abundance in the oxic sediments, five included genes involved in the biodegradation of xenobiotics including fluorobenzoate, chlorocyclohexane and chlorobenzene, polycyclic aromatic hydrocarbons, naphthalene, aminobenzoate and the degradation of terpenoids and polyketides including geraniol, limonene, and pinene (Figure 3.9). In contrast, the bacterial communities in the redox-stratified sediments had significantly higher abundance of genes involved in purine and pyrimidine (nucleic acid metabolism), nitrogen metabolism, carbon fixation pathways and a variety of functional pathways categorised within genetic information processing and cellular processes.

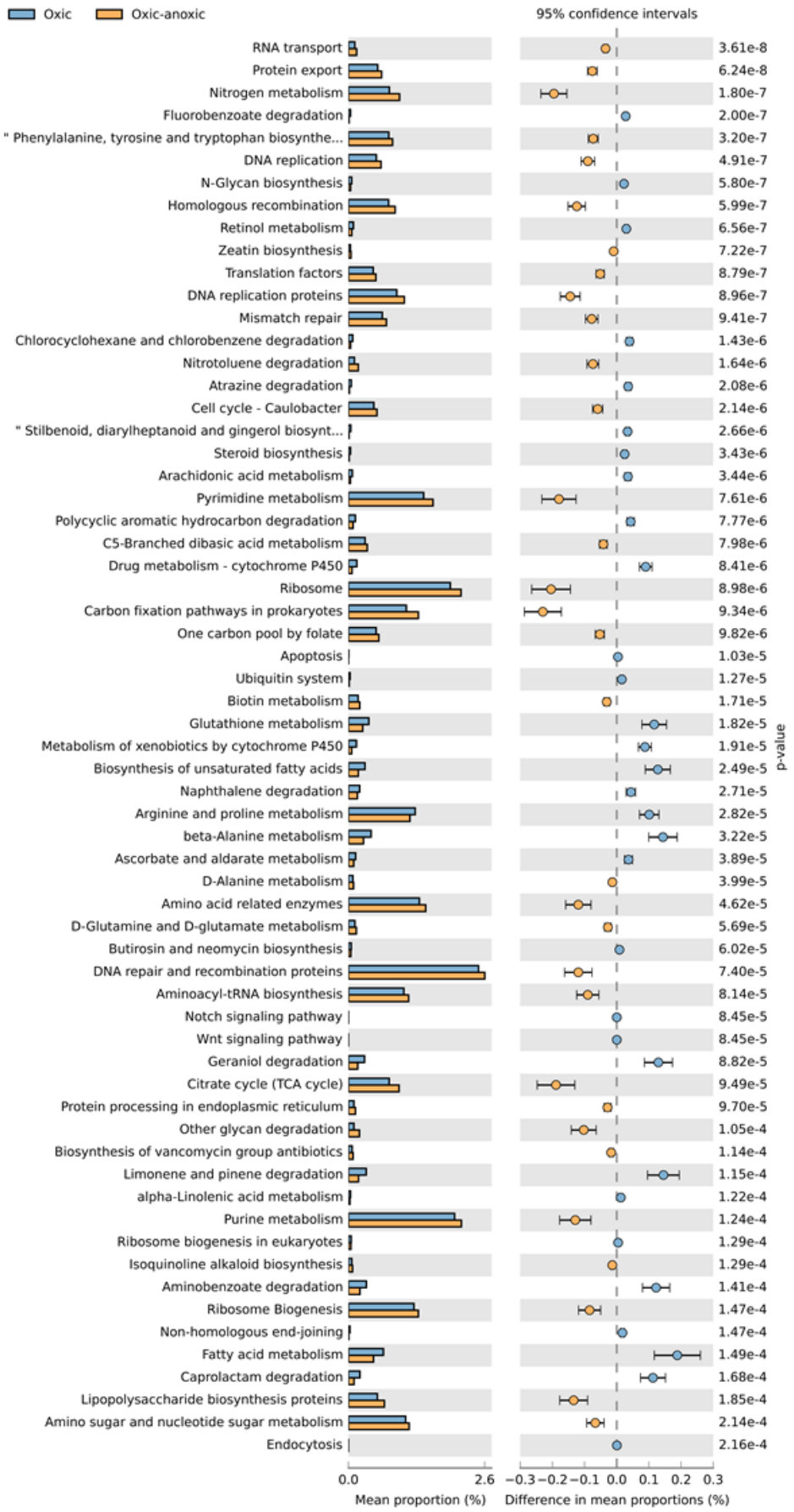

Figure 3.9. The mean proportion (%) and the difference in the mean proportion of gene counts at level three of the BRITE functional hierarchy between oxic-anoxic and oxic treatments with 95 % confidence intervals.

3.4 Discussion

Analysis of 16S rRNA gene clone sequences from the manipulated sediment systems, describing fully oxic and redox-stratified conditions, showed that the microbial communities were highly differentiated. Correlation of the environmental variables with the taxonomic data confirmed that redox potential was the principal driver of shifts in bacterial community composition. Although redox regime was the principal environmental variable associated with differences in the diversity, taxonomic composition and predicted functional capacity of bacterial communities, there were no significant differences in growth rate or biomass density of *H. scabra* in contrast to previous results. In Chapter 2 and in Robinson *et al.* (2015), the redox-stratified sediments resulted in significantly higher overall growth rates and final *H. scabra* biomass densities of 626.89 ± 35.44 g m^{-2} after 81 d, compared to only 454.84 ± 14.30 g m^{-2} for sea cucumbers cultured on fully oxic sediments. In the current study, the final sea cucumber biomass densities were not significantly different; however, overall they were higher than the densities achieved in Chapter 2. The biomass of sea cucumbers in the oxic-anoxic treatments increased throughout the trial to achieve a final mean density of 1,028.50 ± 117.46 g m^{-2}, whereas the sea cucumber density on fully oxic sediments decreased in the final month, from 944.76 ± 56.84 g m^{-2} on day 56 to 837.96 ± 99.70 g m^{-2} on day 84.

The differences in growth rate and final biomass densities between the chapters can largely be attributed to seasonal differences between the respective growth trials (Wolkenhauer *et al.*, 2013). The growth trial in Chapter 2 commenced at the end of the austral summer on March 20th and ran into mid-winter before terminating on June 9th. In contrast, the current trial was conducted at the start of the austral summer from September 12th to December 5th 2012. Over this summer period the day length was longer, ranging from 11.45 to 14.20 hours of daylight as the season progressed, compared to 10 hours in the previous growth trial (Chapter 2, Section 2.2.1). The longer day length and stronger irradiation resulted in significantly higher ambient temperatures in the biosecure facility and in the heated re-circulating seawater system. In this study, the mean seawater temperature recorded weekly during the 84 day trial was 29.49 ± 0.09 °C, almost four degrees centigrade higher than the temperatures recorded in the previous study, which ranged from 25.18 to 26.40 °C with an overall mean of 25.71 ± 0.05 °C.

Temperature is one of the key environmental variables affecting the activity, metabolism and growth rate of marine invertebrates, including *H. scabra* (Brockington and Clarke, 2001; Wolkenhauer and Skewes, 2008). The higher seawater temperatures recorded in the sea cucumber tanks were positively correlated with the higher overall mean growth rates of 0.53 ± 0.04 g d^{-1} observed in this study, more than double the growth rate of sea cucumbers

in the study reported in Chapter 2. In the previous chapter, significantly higher growth rates of 0.20 ± 0.02 g d^{-1} were achieved on redox-stratified sediments, compared to only 0.12 ± 0.01 g d^{-1} on the fully oxic sediment. Nevertheless, the shape of the growth curves between the two trials followed the same pattern, although the density limitation of growth was not yet fully apparent in this study. Unfortunately, the experiment had to be terminated earlier than planned after only three months as laboratory space and staff were booked to undertake DNA extraction and sequencing in the second week of December before the seasonal closure over the Christmas and New Year period. Had the growth trial been continued for a further 28 days, then it is likely that the growth rate of sea cucumbers reared on the fully oxic substrate would have plateaued as in Chapter 2, possibly resulting in significant differences between treatments.

The active circulation of oxygenated seawater through the sediment in the oxic treatments led to the establishment of a microbial community characterised by high diversity, richness and evenness, with an enrichment of metabolic and functional potential for bioremediation, compared with communities present in the predominately anaerobic sediment of the oxic-anoxic treatments. Molecular oxygen is one of the most important reactants in biogeochemical cycles; consequently, the redox conditions in marine sediments has a significant impact on bacterial communities that mediate organic matter mineralisation and biogeochemical cycles (van der Zaan *et al.*, 2009; Liu *et al.*, 2013). The presence or absence of oxygen is a major factor determining changes in bacterial community structure based on oxygen-related ecophysiology, since bacteria vary in their requirement and response to molecular oxygen, based on their type of dissimilatory metabolism (Lüdemann *et al.*, 2000; Durbin and Teske, 2011; Gao *et al.*, 2011; Rusch and Gaidos, 2013). Bacteria classified as obligate aerobes and microaerophiles require oxygen to grow as their methods of energy production and respiration depend on the transfer of electrons to oxygen, which is the final electron acceptor in electron transport-linked oxidative phosphorylation (Fenchel *et al.*, 2012). Where the sediment was maintained under fully oxic conditions due to the continual circulation of oxygenated water, all of the bacterial biomarker taxa were classified as obligate or facultative aerobic bacteria. In the redox-stratified sediments, molecular diffusion of oxygen would have been the main transport mechanism, limiting the depth of oxygen penetration to a few millimetres below which microbial activity would have depleted high-energy electron acceptors such as oxygen and nitrate leading to predominately anaerobic conditions (Cai and Sayles, 1996; Meijer and Avnimelech, 1999). Here, the majority of biomarker taxa were classified as strict or obligate anaerobes, which can only grow in the absence of oxygen (Lodish *et al.*, 2000).

The significantly higher relative abundance, richness, diversity and evenness of the bacterial communities present in the fully oxic sediment systems may be due to the predominance of bacteria with an aerobic respiratory metabolism. Competition between bacteria is based on the kinetics of substrate uptake, as well as the efficiency with which the substrate is coupled to growth (Fenchel *et al.*, 2012). Due to the higher efficiency of carbon incorporation during aerobic respiration, which is on average 50 % compared to approximately 20 % in fermentation, aerobic respiration produces a significantly higher quantity of microbial biomass (Fenchel *et al.*, 2012). The effectiveness of aerobic respiration is therefore manifested not only by enhanced bacterial growth rates but also amplified growth yields since aerobes can multiply up to four times faster than anaerobes (Fenchel *et al.*, 2012). The type of dissimilatory metabolism employed by bacteria has a large impact on the growth rate of bacteria and their dominance in the community, hence the increased diversity in oxic treatments may also be due to the dominance of aerobic bacteria which exhibit a high taxonomic diversity due to adaptations based on energy metabolism, including specialisations based on the substrate they can metabolise and the macromolecules they can hydrolyse (Fenchel *et al.*, 2012). Furthermore, the higher evenness in oxic treatments suggests that the bacterial communities may be more functionally stable under high oxygen concentrations.

Advective transport of molecules is an important mechanism that shapes microbial communities (Fenchel *et al.*, 2012). Within redox-stratified sediments, bacteria with very different metabolic capacities are distributed according to redox potential and the spatial distribution of electron acceptors (Brune *et al.*, 2000). The differing transport regimes between oxic treatments (advective) and oxic-anoxic treatments (molecular diffusion) is likely to have affected microbial activity and metabolism in two ways: the active circulation of oxygenated water in oxic treatments and the rapid exchange of pore water with the overlying water column would have increased the rate of the supply of nutrients to bacteria, and the removal of waste products. The accumulation of metabolic waste products can inhibit metabolic pathways, thus their removal and re-distribution for assimilation by other species is an important process (Hunter *et al.*, 2006; Fenchel *et al.*, 2012).

Inferred functionality from the predicted metagenome analysed by LEfSE identified ‘metabolism’ as the only major pathway significantly enriched in oxic treatments. Enrichment in genes for metabolism is likely to be due to the metabolic response of obligate and facultative aerobic bacteria that predominated in oxic treatments in response to increased oxygen concentrations, since their rate of growth and metabolism is directly related to the amount of available oxygen (Costilow, 1981; Gerritse *et al.*, 1990). Within the sub-categories of metabolism, genes for xenobiotics degradation and metabolism were enriched in the oxic

sediment systems. Their increased biodegradation potential may reflect the high diversity of aerobic bacteria that possess a wide range of metabolic and enzymatic capabilities (Kristensen *et al.*, 1995).

A goal of microbial ecology research in the field of bioremediation is to understand the diversity, structure and functional role of bacterial communities, relevant to the design of effluent treatment systems (Brune and Bayer, 2012; Martinez-Porchas *et al.*, 2014). Successful bioremediation of aquaculture wastes involves the combined actions of a diverse consortium of heterotrophic, chemolithotrophic and phototrophic bacteria (Antony and Philip, 2006). Specifically, aquaculture bioremediation technologies aim to optimise: 1) carbon mineralization to minimize sludge accumulation; 2) nitrification rates to maintain a low ammonia concentration; 3) denitrification rates to eliminate excess nitrogen; 4) sulphide oxidation to reduce accumulation of H_2S; 5) primary productivity to stimulate productivity; and, 6) maintaining a diverse and stable community where undesirable species do not become dominant (Antony and Philip, 2006).

Classification of biomarker taxa according to their type of dissimilatory metabolism elucidated stark differences in the metabolic capacity and putative functional roles of the microbial communities under different redox regimes. In the oxic treatments, a high diversity of dissimilatory metabolisms was present, including taxa with heterotrophic metabolism, including aerobic and anaerobic respiration and fermentation; chemolithotrophic; methylotrophic and phototrophic metabolisms, performing both oxygenic and anoxygenic photosynthesis (Appendix A; Table A1.1). In contrast, biomarker taxa enriched in the oxic-anoxic treatments, exhibited limited functional diversity, representing only chemoorganotrophic (heterotrophic) bacteria (Appendix A; Table A1.2), lending support to the previous conclusion that redox-stratified sediments may exhibit limited bioremediation potential (Chapter 2 and Robinson *et al*., 2015).

Particulate organic wastes originating from waste feed and faeces comprise the bulk of aquaculture effluents in recirculating aquaculture systems. To minimize sludge accumulation, a broad spectrum of heterotrophic bacteria with a high enzymatic capacity and ability to multiply rapidly is essential to maximise rates of carbon mineralization to carbon dioxide (Antony and Philip, 2006). In the oxic treatments, there was a high diversity of heterotrophic bacteria utilising the full spectrum of carbon oxidation pathways including aerobic and anaerobic respiration and fermentation (Appendix A; Table A1.1). The majority of the biomarker taxa within the phyla Bacteroidetes, Planctomycetes, Verrucomicrobia, Delta-, and Gammaproteobacteria sub-classes, were classified as taxa with an aerobic respiratory metabolism, able to oxidise a range of complex polymeric carbon compounds (including

sugars, alcohols, organic acids, amino acids and carbohydrates), underscoring the enhanced capacity for organic matter degradation (Couradeau *et al.*, 2011).

Bioremediation of nitrogenous compounds relies on maximising chemolithotrophic processes that remove potentially toxic compounds (ammonia and nitrite) via nitrification, which is mediated in a step-wise process by ammonia-oxidising bacteria and nitrite oxidising bacteria. The phylum Nitrospirae, containing chemolithotrophic bacteria that oxidise nitrite to nitrate, had a significantly higher relative abundance in the oxic treatments (Figure 3.4) and was enriched to family level (*Nitrospiraceae*) in oxic treatments, present at 2 and 4 cm depths and not present in oxic-anoxic treatments. Similarly, ammonia-oxidising bacteria within the family *Nitrosomonadaceae* (Betaproteobacteria) were present in oxic treatments at the 2 and 4 cm depths, although not identified as a biomarker taxon, indicating that nitrification was probably occurring in the fully oxic sediment. Denitrifying bacteria are also considered to be important, particularly in re-circulating aquaculture systems where nitrate accumulates due to the high nitrification capacity of biofilters, for the permanent removal of nitrogen through the conversion of nitrate to dinitrogen gas (Ebeling *et al.*, 2006; van Rijn *et al.*, 2006). Due to the lack of resolution in identifying bacterial taxa to genus level, no biomarker taxa known to be involved in denitrification were identified; however, sequences assigned to the family *Pseudomonadaceae* (Gammaproteobacteria), which contains denitrifying and nitrogen fixing bacteria, were present in both oxic and oxic-anoxic treatments.

Additional chemolithotrophic biomarkers involved in biogeochemical cycles included an enrichment of oxidisers of ferrous iron (Acidomicrobiales) and the sulphur oxidising genus *Thiopilula* (Thiotrichales) in the oxic treatments (Jones *et al.*, 2015). The study of bacteria involved in sulphur cycling is important in aquaculture systems since unionised dissolved hydrogen sulphide (H_2S) is extremely toxic to many aquatic organisms, even at natural levels (Knezovich *et al.*, 1996). In marine sediment-based aquaculture systems it is particularly relevant since sulphate reduction can account for over 50 % or organic matter degradation in marine sediments, leading to the production of considerable quantities of H_2S (Muyzer and Stams, 2008). In shrimp pond aquaculture, phototrophic bacteria (notably purple and green sulphur bacteria within the families *Chromatiaceae* and *Chlorobiaceae*, respectively), are frequently mass cultured and applied to aquaculture ponds to bioremediate H_2S toxicity (Antony and Philip, 2006). These bacteria perform anoxygenic photosynthesis at low light intensities under anaerobic conditions, acquiring reducing electrons from H_2S at a lower energy cost than phototrophs that use H_2O (Antony and Philip, 2006). The oxic treatments contained two anoxygenic photosynthetic biomarker taxa, represented by purple sulphur within the family *Ectothiorhodospiraceae* (Gammaproteobacteria) and purple non-sulphur

bacteria, within the family *Rhodobacteracae (*Alphaproteobacteria) that are capable of bioremediating H_2S toxicity by oxidising H_2S to sulphur or thiosulfate. They are typically found in illuminated anoxic zones of sediments and intertidal microbial mats (Proctor, 1997).

In addition, phototrophic cyanobacteria performing oxygenic photosynthesis had a significantly higher relative abundance in the oxic treatments and were important in driving differences between the different communities (Figure 3.6). Cyanobacterial orders that predominated in the surface sediment layers in the oxic treatments included; 4C0d-2, *Oscillatoriophycideae* and *Synechococcophycideae*, which were identified as biomarkers in oxic treatments, contributing towards *in situ* primary production and increasing the availability of natural food resources, which is another important process in aquaculture bioremediation systems (Antony and Philip, 2006). Finally, successful aquaculture bioremediation technologies rely on the maintenance of a diverse and stable community where undesirable species do not become dominant (Antony and Philip, 2006). In oxic-anoxic treatments, taxonomic biomarkers included the class Mollicutes (phylum Tenericutes), which are commensals or parasites, occurring in a wide range of vertebrate, insect, and plant hosts (Brown *et al.*, 2010). Many are considered as significant pathogens and furthermore display resistance to penicillin (Brown *et al.*, 2010). In contrast, in the oxic treatments, bacteriolytic taxa were represented by the family *Nannocystaceae* (order Myxococcales) which are micropredators that may play a key role in suppressing unwanted organisms (Brown *et al.*, 2010).

Finally, in addition to environmental concerns over organic enrichment and the depletion of dissolved oxygen stemming from the discharge of suspended solid wastes, the potential toxic effects of chemicals used to control and treat disease outbreaks must also be considered (Jerbi *et al.*, 2011). The enrichment of functional pathways for xenobiotics degradation and metabolism in the aerobic sediment system demonstrates the potential to bioremediate a variety of chemical therapeutants used in intensive aquaculture, including antibiotics, anaesthetics and anti-parasitic agents. Additional benefits of an aerobic sediment-based treatment system may include the removal of pathogenic bacteria present in the discharge water.

In stark contrast to the high functional diversity present in the oxic treatments, all of the biomarkers enriched in the oxic-anoxic treatments were classified as chemoorganotrophic bacteria, obtaining both carbon and energy for biosynthetic reactions from organic compounds. In anoxic sediment layers, chemoorganotrophic mutualistic consortia of bacteria, with dissimilatory metabolisms based on fermentation and anaerobic respiration, mediate the decomposition of organic matter (Kristensen *et al.*, 1995; Fenchel *et al.*, 2012). In the oxic-

anoxic treatments, the majority of the biomarkers were predominantly classified as anaerobes, with metabolisms based on anaerobic respiration, fermentation and phototrophy (anoxygenic photosynthesis). Only two biomarker taxa were classified as obligate aerobes or microaerophiles; the genus SJA-88, within the proposed order Leptospirales (phylum Spirochaetes), which utilize long-chain fatty acids or long-chain fatty alcohols as carbon and energy sources (Gupta *et al.*, 2013); and the microaerophilic *Heliobacteraceae* which obtain energy from amino acids or the tricarboxylic acid cycle intermediates and reduce fumarate to succinate (Garrity *et al.*, 2005; Gupta *et al.*, 2013). Strictly anaerobic bacteria with a respiratory metabolism identified as biomarker taxa in the oxic-anoxic treatments included the class Mollicutes (phylum Tenericutes). The remainder of biomarker taxa identified in oxic-anoxic treatments with anaerobic respiratory metabolism were sulphate-reducing bacteria within the families *Desulfobacteracae* and the order Desulfobacterales. These dissimilatory sulphate-reducing bacteria are commonly found in anoxic marine sediments, utilising simple organic compounds such as long-chain fatty acids and alcohols as electron donors and carbon sources (Kuever *et al.*, 2005a). Most members of the family *Desulfobulbaceae* are incomplete oxidizers that form acetate as an end product, which is in turn utilised by *Desulfobacter* as the preferred general electron donor and carbon source who oxidise it completely to CO_2 (Kuever *et al.*, 2005a; Kuever *et al.*, 2005b). This example serves to illustrate how the concerted actions of a consortium of bacteria are necessary for the complete mineralisation of organic matter under anaerobic conditions. During carbon oxidation, these bacteria utilise oxidised sulphur compounds, including sulfate, sulfite and thiosulfate as terminal electron acceptors reducing them to H_2S, which is toxic to many organisms. However, a representative of the green sulphur bacteria, the Chlorobi clade OPB56, which performs anoxygenic photosynthesis and metabolizes small organic molecules, was also identified as a biomarker taxon in the oxic-anoxic treatments (Hiras *et al.*, 2016). Green sulphur bacteria are important drivers of oxidation of reduced sulphur compounds in stratified sulphide-containing environments receiving low irradiation, (Imhoff, 2001) and are important in remediating H_2S toxicity (Antony and Philip, 2006). Under the scenario of high rates of organic loading, it is plausible that zones of sulphate reduction may develop, leading to the production of H_2S; however, the presence of purple and green sulphur bacteria would bioremediate any potential H_2S toxicity that may negatively impact the sea cucumber health.

Although sulphate-reducing bacteria play an important role in the oxidation of organic carbon in marine sediments, they have no capacity to hydrolyse particulate organic matter, therefore other bacteria must also be present that are capable of performing these complex hydrolyses, thereby obtaining energy from the fermentation of hydrolytic products

(Jørgensen, 1977; Blackburn, 1988). The majority of bacteria identified as biomarker taxa in the oxic-anoxic treatments possessed a fermentative type of metabolism, which included the saccharolytic Bacteroidales and Spirochaetales, which ferment carbohydrates; cellulolytic Clostiridiales which ferment cellulose; the class Phycisphaerae and candidate phylum KSB3 which ferment sugars (Appendix A; Table A1.2). The only biomarker taxon with a fermentative metabolism identified to genus level was *Propionigenium* (Fusobacteria), which are well adapted to marine anoxic sediments since their energy metabolism is based on sodium ions as coupling ions in energy conservation, preferentially using dicarboxylic acids as substrates (Schink and Janssen, 2010).

In Chapter 2, it was hypothesised that the predominately anaerobic conditions of mineralisation in the naturally stratified sediment, provided a steady release of bioavailable food resources that supported the significantly higher sea cucumber biomass observed in the oxic-anoxic treatments in Robinson *et al.* (2015), a trend which was supported by the current study. Organic matter resulting from anaerobic bacterial processing in the predominately anoxic redox-stratified sediments may have been nutritionally valuable for deposit feeders (Schroeder, 1987). Anaerobic respiration produces considerable amounts of extracellular, low molecular weight organic compounds (van Soest, 1982) as complex polymeric organic molecules are stepwise split into water-soluble monomers, such as amino acids, monosaccharides, organic acids, and fatty acids (Kristensen *et al.*, 1995), which serve as substrates for fermentation. In fermentation, energy is conserved by substrate-level phosphorylation and the redox balance is achieved by the excretion of reduced substances such as fatty acids and organic acids, including lactic, formic, acetic, proprionic and butyric acids and H_2, produced as the end products of catabolism (Fenchel *et al.*, 2012). Fermentation products are energy-rich compounds due the presence of energy rich phosphate bonds or a molecule of coenzyme A and may be important to the nutrition of deposit feeders (Penry and Jumars, 1987; Plante *et al.*, 1990). The importance of essential long-chain polyunsaturated fatty acids and sterols for invertebrate growth and reproduction is well established (Brett and Muller-Navarra, 1997; Elert *et al.*, 2003). Low molecular weight organic compounds may be adsorbed to extracellular polymers or inorganic sediment particles and thus become available to deposit feeders by direct ingestion (Schroeder, 1987). Uptake of dissolved organic compounds may also occur across the epithelium (Pequigna, 1972; Ahearn and Townsley, 1975; Jangoux and Lawerence, 1982) or across the respiratory trees since aspidochirotid sea cucumbers that possess respiratory trees are nutritionally bipolar, possessing an ability to anally suspension feed (Jaeckle and Strathmann, 2013).

The high diversity of metabolisms and functional groups in the fully oxic sediment may be linked to the energetic costs of biosynthesis in relation to differences in oxygen concentration and redox potential. The availability of oxygen has a tremendous impact not only on the redox potential of the environment, but also on the energetic situation of the microorganisms (Reimers *et al.*, 2013). As biomolecular synthesis involves redox reactions, the oxidation state of the environment is a critical parameter influencing the amount of energy involved in the conversion of inorganic precursors to biomass, with very different energetic costs for bacteria between oxic and anoxic environments (McCollom and Amend, 2005). As aerobic respiration yields the most energy of any catabolic processes, the high redox state of oxic sediments imposes an energetic cost on the pathways available for biomass synthesis (McCollom and Amend, 2005; Fenchel *et al.*, 2012).

While elevated redox conditions may provide energy benefits to heterotrophic microbial communities that utilize the most energetically favourable metabolism, they may simultaneously penalise organisms that do not (Durbin and Teske, 2011; Reimers *et al.*, 2013). It is likely that autotrophic organisms have adapted to use the most energetically favourable carbon fixation pathway available given the thermodynamic constraints of the cost of biomass synthesis (McCollom and Amend, 2005). Organisms inhabiting reducing environments therefore may utilize pathways with lower energy expenditure (e.g. the acetyl-CoA or reductive TCA pathways), while organisms inhabiting oxidizing environments must utilize energetically more expensive pathways such as the Calvin cycle (McCollom and Amend, 2005). Autotrophy is an energetically demanding process, as an energy poor and highly oxidised form of carbon – CO_2 – must be reduced and assimilated into cell material, which requires adenosine triphosphate (ATP) and reducing power (Ebeling *et al.*, 2006). Consequently, the Calvin cycle is the most widely distributed autotrophic pathway in nature, present in cyanobacteria, purple bacteria and most chemolithotrophic bacteria (Yurkov and Beatty, 1998). This may explain why bacteria with autotrophic metabolisms were only identified as biomarker taxa in the oxic treatments.

3.5 Conclusion

Classification of taxonomic biomarkers according to their oxygen-related ecophysiology and dissimilatory metabolism permitted an exploration of the functional diversity of microbial communities underpinning organic matter mineralization and biogeochemical cycling. This study demonstrated that redox-stratified sediments supported predominately heterotrophic sediment microbial communities with metabolisms based on anaerobic respiration and fermentation, supporting the hypothesis posed in Chapter 2. This

provides further evidence that redox-stratified sediments are likely to provide a steady release of bioavailable food resources underpinning improved growth performance of sea cucumbers.

Analysis of the taxonomic composition and functional diversity of bacteria communities by 454-pyrosequencing demonstrated that the oxic treatments subjected to a fully oxic redox regime, contained many of the requisite functional groups for successful bioremediation of aquaculture wastes. This study demonstrates support for the hypothesis that low-cost, *in situ* sediment manipulation by the percolation of oxygenated seawater, is capable of increasing the relative abundance, diversity, and functional potential of microbial communities for aquaculture waste bioremediation. This approach should markedly improve our understanding of the potential to integrate epibenthic deposit feeders into sediment-based bioremediation systems.

Chapter 4. Effect of resource quality on growth of *Holothuria scabra* during aquaculture waste bioremediation

4.1 Introduction

Microorganisms are central to all aquaculture bioremediation technologies. Historically, aquaculture bioremediation technologies have evolved from the exploitation of autotrophic pathways, including photoautotrophic and chemolithoautotrophic microorganisms, to fully heterotrophic systems (Figure 1.1; Ebeling *et al.*, 2006). The transition to heterotrophic systems, where the emphasis is on the re-use and recycling of feed residues within the culture system, is particularly relevant in present day aquaculture where feed, space, and energy are limiting (Chávez-Crooker and Obreque-Contreras, 2010). Since sediments are primarily net heterotrophic systems, the recycling of nutrients *in situ* may provide a viable means for the intensive culture of deposit feeding sea cucumbers. As microbial assemblages are the basis of the heterotrophic food web and the link to higher trophic levels, it is of great interest to exploit these linkages with the aim of increasing the overall energy transfer efficiency (Schroeder, 1987). Furthermore, research conducted to date (Chapter 2, 3 and Robinson *et al.*, 2015), demonstrated that redox-stratified sediments supported higher biomass and faster growth rates of *Holothuria scabra*, indicating that heterotrophic systems are more favourable for deposit feeder growth.

Heterotrophs differ fundamentally from autotrophs due to their requirement for an organic source of carbon for biosynthesis and energy generation. Heterotrophic bacteria assimilate organic carbon (C) and nitrogen (N) in a stoichiometric balance based on the carbon to nitrogen ratio (C:N) of the bacterial cytoplasm (Herbert, 1967; Goldman *et al.*, 1987). From a thermodynamic perspective, heterotrophic bacteria preferentially utilise reduced inorganic forms of nitrogen such as ammonium (NH_4^+; Church, 2008). Furthermore, since NH_4^+ assimilation in marine bacteria occurs via the glutamine synthetase pathway, which requires four atoms of carbon for every atom of nitrogen assimilated, the uptake of NH_4^+ is fundamentally dependent on the availability of carbon substrates (Fenchel and Blackburn, 1979; Church, 2008). Since carbon and nitrogen are incorporated into bacterial biomass tissue at a fixed rate, the C:N of organic substrates is an important parameter determining the degree of nitrogen regeneration (Tezuka, 1990). For bacteria with a C:N of 5:1 and an average growth efficiency of 50 % under aerobic conditions, the threshold between net release and net immobilisation is 10:1 (Rittmann and McCarty, 2001; Azim *et al.*, 2008). Increasing C:N to greater than 10:1 provides sufficient carbon for heterotrophic bacteria to

assimilate NH_4^+ into biomass, thus mediating a shift from net NH_4^+ release (ammonification) to net immobilisation (assimilation) (Avnimelech, 1999; Ebeling *et al.*, 2006; Azim *et al.*, 2008; Avnimelech, 2014).

Particulate organic wastes originating from land-based aquaculture, primarily comprising waste food and faeces, are generally deficient in organic carbon, with an average C:N of 7:1; thus, there is a net release of NH_4^+ during decomposition (Avnimelech, 1999; Schneider *et al.*, 2006; Mirzoyan *et al.*, 2012; Castine, 2013). Particulate organic waste recovered from mechanical filtration in recirculation aquaculture systems (RAS) have previously been used as substrates producing heterotrophic bacteria and deposit feeding macrofauna, including polychaete worms and sea cucumbers (Schneider *et al.*, 2006; Schneider *et al.*, 2007a; Schneider *et al.*, 2007b; Palmer, 2010; Brown *et al.*, 2011; MacDonald *et al.*, 2013). The production of secondary organisms on aquaculture waste can provide a direct means of assimilating a proportion of the effluent nitrogen (Erler *et al.*, 2004); however, detritivores such as sea cucumbers are generally predicted to have poor nitrogen retention compared to organisms in other trophic groups (Schneider *et al.*, 2005). It has been hypothesized that stimulation of bacterial processing of nitrogen during the production of secondary organisms on RAS effluents may be more important than direct assimilation (Erler *et al.*, 2004). Schneider *et al.* (2006) demonstrated that the addition of labile organic carbon sources, such as molasses, could increase the production of heterotrophic bacterial biomass and increase the conversion of inorganic nitrogenous wastes to bacterial biomass. Stimulation of heterotrophic bacteria through manipulating C:N may therefore offer an indirect means of increasing nitrogen retention in macrofauna reared on aquaculture effluents.

In sediment-based systems integrating deposit feeding sea cucumbers, control of inorganic nitrogen through carbon addition may be particularly relevant due to the need to counteract additional sources of NH_4^+. Other sources of NH_4^+ that may contribute to the build-up of inorganic nitrogen in the system include: i) net efflux of NH_4^+ from the sediment (Hargreaves, 1998); ii) excretion from holothurians (Uthicke and Klumpp, 1997); and, iii) decomposition from labile sources of aquaculture waste and commercial feeds (Avnimelech, 1999). Carbon supplementation to promote heterotrophic assimilation of NH_4^+ may offer an indirect means of safely retaining nitrogen in the system by immobilising NH_4^+ into microbial biomass that can be upcycled into high value secondary biomass.

The threshold defining the balance between net immobilisation and net release of nitrogen is dependent upon; the physiological state of the bacterial populations; the biochemical composition of the substrate; and the reduction-oxidation (redox) potential

(Goldman *et al.*, 1987; Tezuka, 1990; Fenchel *et al.*, 2012). As the application of C:N manipulation to sediment-based systems, where bacterial growth efficiencies are generally lower (Fenchel *et al.*, 2012), is novel, there is a need to test different carbon sources to determine their efficacy. This chapter aims to compare a range of carbon sources of different biochemical composition and degradation rates on the growth of *H. scabra* reared on particulate organic waste from an intensive abalone RAS. It is hypothesised that increasing the C:N of aquaculture waste from 5:1 to 20:1 will increase the growth rate and biomass density of *H. scabra* reared on redox-stratified sediments and reduce the concentration of NH_4^+.

4.2 Methods

4.2.1 Study site

The study was conducted in the purpose built, bio-secure, heated, RAS described in Chapter 2, Section 2.2.1, between October 8th 2013 and January 28th 2014.

4.2.2 Experimental animals

Experimental animals were imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam) on September 5th 2013 and quarantined in a bio-secure facility for six weeks in accordance with South African importation and scientific investigations licenses. Following the quarantine period and prior to experimentation, the animals were held in the RAS in tanks filled with 10 cm of calcium carbonate sand and were fed a 34 % protein commercial abalone weaning diet (Abfeed®-S34, 1.0 mm sugar grain pellet; Marifeed Pty Ltd, South Africa).

4.2.3 Experimental design

The experiment was designed to test a range of carbon sources with different biochemical composition on *H. scabra* growth in sediment-based systems receiving particulate organic aquaculture waste. Three different carbon sources, namely glucose, starch and cellulose were tested in conjunction with aquaculture waste at a C:N of 20:1 and a fourth treatment receiving aquaculture waste only (mass C:N of 5:1) was included as a control (Table 4.1).

The average C:N of the dried particulate aquaculture waste was 5.21 ± 0.55 (Section 4.2.5). For treatments amended with their respective carbon sources, the overall carbon to nitrogen ratio was increased from 5:1 to 20:1 (Table 4.1). The quantity of carbon necessary to increase the ratio to 20:1 was calculated by taking into account the proportion of carbon in the molecular composition of each compound (Table 4.2). Carbon additions were standardised

between treatments based on a daily addition of 200 mmol C m^{-2} d^{-1}, which is within the upper range tolerated by benthic animals under eutrophic conditions of 100-400 mmol C m^{-2} d^{-1} (Lehtoranta *et al.*, 2009). At a carbon to nitrogen ratio of 20:1, this equates to 2.4 g m^{-2} d^{-1} of carbon and 0.12 g m^{-2} d^{-1} of nitrogen, which is between a 'mid' and 'high' ration of 100 and 150 mg m^{-2} d^{-1} of nitrogen respectively for deposit feeders (Alongi and Hanson, 1985; Tenore and Chesney, 1985).

Table 4.1. Description of experimental treatments including daily additions of particulate aquaculture waste and the different carbon sources standardised to 200 mmol C m^{-2} d^{-1}.

Carbon source	Aquaculture waste wet weight (g)	Dry weight carbon source (g)	Carbon to nitrogen ratio (C:N)
None	2.41	N/A	5:1
Glucose	2.41	0.75	20:1
Starch	2.41	0.68	20:1
Cellulose	2.41	0.68	20:1

Table 4.2. Details of the tested carbon sources that were sourced from Merck Millipore, South Africa.

	Glucose	Starch	Cellulose
Product name	D (+) glucose, anhydrous	Starch	Cellulose microcrystalline
Chemical formula	$(C_6H_{12}O_6)n$	$(C_6H_{10}O_5)n$	$(C_6H_{10}O_5)n$
Molecular weight	180.18	162.16	162.16
Carbon content (%)	39.99	44.44	44.44
Compound	Monosaccharide	Polysaccharide	Polysaccharide
Solubility in water	Soluble	50 g/L at 90 °C	Insoluble (20 °C)
Catalogue number	346351	1012520	102331
*First-order decomposition rate constant	1.1500	0.8000	0.0495
**Half-life (d)	0.6	5.0	14.0

*First-order decomposition rate constant is the percentage of a given compound that degrades each day

**Half-life is the time it takes to reach 50 % of the complete degradation of a given substrate concentration

4.2.4 Carbon sources

The carbon sources tested include two soluble sources: D (+) glucose (anhydrous) and starch and one insoluble form, microcrystalline cellulose (Merck Millipore, South Africa; Table 4.2). The different carbon sources tested were chosen to represent a range of different first-order decomposition rate constants, i.e. the percentage of a given compound that degrades each day, alternatively described as the half-life, based on their biochemical

composition. The carbon sources tested ranged in lability from glucose, a relatively labile carbon source with high decomposition rate constant and short half-life, to cellulose, a more refractory complex polysaccharide which has a lower first-order decomposition rate constant and a half-life of approximately 14 days (Table 4.2).

4.2.5 Aquaculture waste collection

The organic carbon and total nitrogen content of the pre-weighed and dried (105 °C, 24 h) replicate samples (n=3) of abalone waste, comprising mainly uneaten feed and faeces from the South African abalone *Haliotis midae*, collected from the RAS sand filter over five days prior to the experiment and analysed on a CHN elemental analyser. During the experiment, aquaculture waste was collected every morning at 09:00 from the backwash of a sand filter in a recirculating abalone grow-out system at HIK Abalone Farm (Figure 4.1).

Figure 4.1. The land-based abalone *Haliotis midae* recirculating aquaculture system (RAS) at HIK Abalone Farm Pty (Ltd) in Hermanus, South Africa, a) the sand filter used to collect particulate organic waste from the daily wash; b) the South African abalone reared on the top-feeder plate; c) the mesh baskets used to rear *H. midae* intensively in land-based tanks; and d) the commercial feed with a pellet size of 10 x 10 x 1.2 mm that was fed to the abalone (Abfeed®-S34, Marifeed Pty Ltd, South Africa). Photos courtesy of Matthew Naylor.

The total system volume was approximately 32,000 L, which held a maximum of 800 kg of adult abalone (50-60 and 90-110 g.abalone^{-1}) reared on vertical plates in mesh

baskets in canvas tanks (total tank volume 21,000 L, Yearsley *et al.*, 2009). The abalone were fed daily to satiation with a 34 % protein commercial abalone weaning diet (S34 Abfeed®, 10 x 10 x 1.2 mm pellet; Marifeed Pty Ltd, South Africa). The water leaving the production tanks drained through a 500 L sand filter filled with BS8:16 silica filter media for removal of particulate organic waste (1-2 mm), prior to foam fractionation and biofiltration. To counteract decreases in pH from nitrification activity in the biofilter, the system was dosed regularly with calcium hydroxide to buffer the pH. The sand filter was fitted with a Jetco 5-port valve to change the direction of water flow and permit backwashing by re-directing water back up through the sand bed to flush out the dirt, which normally ran to waste. The particulate organic matter (~1000-2000 µm) that was retained by the sand filter was collected every morning. A 50 mm flexihose pipe was connected from the outflow of the sand filter to a 100 L conical fibreglass tank to collect the waste during the first 30 s of back washing. After allowing the waste to settle for one hour, the supernatant was siphoned off and the waste collected by draining the tank. The particulate waste was then concentrated by centrifuging in 50 mL tubes on a bench-top centrifuge (compact centrifuge Z 206 A, Hermle Labortechnik, Germany) at 10,000 *g* for 10 min.

4.2.6 Experimental system and rearing conditions

The four experimental treatments were allocated to one of sixteen tanks using a randomised block design of four blocks with each treatment represented in each block. As such, there were four replicates per treatment and each replicate consisted of one tank containing four sea cucumbers. The 16 polyethylene tanks (455 × 328 × 175 mm) were filled with calcium carbonate 'builders' sand' sourced from a commercial dune quarry (SSB Mining, Macassar, South Africa) sieved to a fine particle size of 125-250 µm. Tanks were supplied with recirculating heated (29.18 ± 0.26 °C) seawater and aeration as described in Section 2.2.1. The tank outflows, however, were adapted to enable the water from the tanks to run to waste, exiting the RAS via a chlorination tank with a three hour retention time. This arrangement was made to prevent the soluble carbon sources from entering the biological filter, which may have encouraged the proliferation of heterotrophic bacteria that can rapidly overgrow slow-growing nitrifying bacteria and affect performance of the RAS (Ebeling *et al.*, 2006).

Aeration was supplied continuously, except during feeding when the air and water supplies were interrupted for 45 minutes. Additions of aquaculture waste and the respective carbon sources were made to all experimental tanks on a daily basis at 16:00 hours. All tanks received 2.41 g of concentrated aquaculture waste on a wet weight basis per day. The

experimental treatments designed to test the effects of different carbon sources received, on a dry weight basis, 0.68 g of starch and cellulose and 0.75 g of glucose per day respectively (Table 4.1). Prior to feeding, the particulate abalone waste was mixed with ambient seawater from the RAS into a wet slurry. Similarly, the carbon sources were prepared by dissolving the soluble carbon sources (glucose and starch) in beakers of ambient seawater while the insoluble cellulose was mixed in beakers to facilitate homogenous distribution of the carbon source in the tank. Prior to additions of aquaculture waste and carbon, the aeration and seawater supply to tanks was interrupted by shutting off the main valves and 25 mm polyvinylchloride end-caps were placed over the tank standpipes and maintained in position for one hour to prevent the waste and carbon sources being washed out of the tanks.

The frequency of tank cleaning had to be increased from previous trials, from once per month to every two weeks, due to the rapid growth of floating colonies of cyanobacteria (*Oscillatoria* sp.), particularly in the treatments amended with starch (Figure 4.2). Green epiphytic algae and *Oscillatoria* sp. were removed and tank walls were scrubbed to remove the biofilm. Experimental tanks were subject to natural light with a photoperiod that increased from 12.41:11.19 L:D (06:10 hours to 18:51 hours, sunrise to sunset) to 13.53:10.07 L:D (05:59 hours to 19:52 hours, sunrise to sunset) as day length increased during the austral summer season.

Figure 4.2. a) Higher production of floating colonies of *Oscillatoria* sp. in the treatments amended with starch compared to b) other treatments , illustrated here as ‘none’ (waste only).

4.2.7 Water quality, sediment quality, and environmental variables

Light (0.01 lux) readings were recorded on a weekly basis (Chapter 3, Section 3.2.6). Water quality parameters including salinity (0.01 g L^{-1}), temperature (0.01 ^{o}C), pH (0.01 pH units), dissolved oxygen (0.01 mg L^{-1}) and total ammonia nitrogen (TAN; 0.01 mg L^{-1}) were recorded weekly (Chapter 2, Section 0). Nitrate concentration (NO_3^--N; ± 0.01 mg L^{-1}) was also measured weekly using a commercially available test kit (Merck Nitrate Test Kit, 109713, Merck, South Africa) and colour absorbance was read using a spectrophotometer (Prim Light, Secomam, 30319 Ales, France).

Sediment quality parameters were monitored monthly to coincide with monthly assessments of sea cucumber growth. Sediment reduction-oxidation (redox) potential (0.01 mV) was measured (Chapter 2, Section 2.2.4) and composite samples of the sediment surface layers (upper 2-3 mm) were collected from all replicate tanks to determine sediment characteristics. Chlorophyll *a* (0.01 µg g^{-1}) and phaeopigment (0.01 µg g^{-1}) concentrations were measured using the spectrophotometric method described in Chapter 3, Section 3.2.6. The organic content measured as particulate organic carbon (0.01 %) and total nitrogen (0.01 %) was determined on an elemental analyser after removal of carbonates by fuming with HCl. Total sediment carbohydrates (0.01 µg g^{-1}) were measured using the phenol-sulphuric acid method (Underwood *et al.*, 1995). The absorbance of the supernatant was measured at 485 nm quantified against a glucose standard and converted into the concentration of total carbohydrates using the coefficients derived from standard curves.

4.2.8 Growth of **Holothuria scabra**

At the start of the experiment and before treatments were randomly assigned to the experimental tanks, juvenile sea cucumbers (4.08 ± 0.06 g) were gut evacuated, allocated to tanks to ensure homogenous size distribution among tanks, photo-identified and weighed (Chapter 2, Section 2.21). Each individual was re-weighed every 28 d over the 112 d experimental period. Wet weight data were used to calculate biomass density (g m^{-2}) and growth rate (g d^{-1};Equation 2; Chapter 2, Section 2.2.5).

4.2.9 Statistical analyses

For all parameters measured, the data recorded per replicate tank were averaged and the mean value per tank was used for statistical analysis. The light readings and water quality data collected on a weekly basis were averaged to provide a mean value per month to give five time intervals for repeated measures analysis of variance (repeated measures ANOVA) and ensure consistency with the sediment quality and sea cucumber weight data (g). Units of

pH were transformed prior to averaging using the antilog function in Microsoft Excel. Results are expressed as mean ± standard error.

Growth and environmental (light, water and sediment quality) data were tested for homogeneity of variance and for the normal distribution of the residuals using Levene's (Levene, 1960) and Shapiro Wilk's (Shapiro and Wilk, 1965) tests respectively. Initial weight data did not meet the assumptions of homogeneity of variance, therefore a non-parametric Kruskal-Wallis one-way ANOVA was used to test for significant differences between treatment medians. Data that met the test assumptions were analysed using repeated measures analysis of variance (repeated measures ANOVA) with treatment (carbon source) as the main factor and sampling time (month) as the repeated measure. Tukey's post-hoc honest significant difference (HSD) tests were used to compare differences among means. Multiple regression analysis was used to identify significant categorical predictors of sea cucumber biomass density. Differences were considered significant at alpha < 0.05. All statistical analyses were performed using Statistica version 13.

4.3 Results

4.3.1 Water quality, sediment quality and environmental variables

There were no significant differences in light, salinity, temperature, dissolved oxygen concentration, total ammonia or nitrate between treatments over the course of the experiment (repeated measures ANOVA; $p > 0.05$). Salinity was maintained at a constant 35 g L^{-1} and dissolved oxygen concentration varied between 6.05 and 8.95 mg L^{-1} (7.26 ± 0.07 mg L^{-1}). The mean water temperature increased over the course of the experiment with the onset of the austral summer from a mean temperature of 25.49 ± 0.14 °C recorded at the start of the experiment, which increased to a mean of 32.30 ± 0.34 °C recorded during the final month of the experiment in January 2014. The pH was significantly different between treatments over the 112 days experiment (repeated measures ANOVA; $F_{(12,\ 48)} = 2.79$, $p = 0.006$). The treatment amended with soluble starch had the highest overall pH between month 1 and month 3 which reached a peak of 9.12 ± 0.05 in month 3. The overall trend in pH in all treatments was an increase over the course of the experiment with all values measured in the final month returning to values measured at the start of the experiment (Figure 4.3).

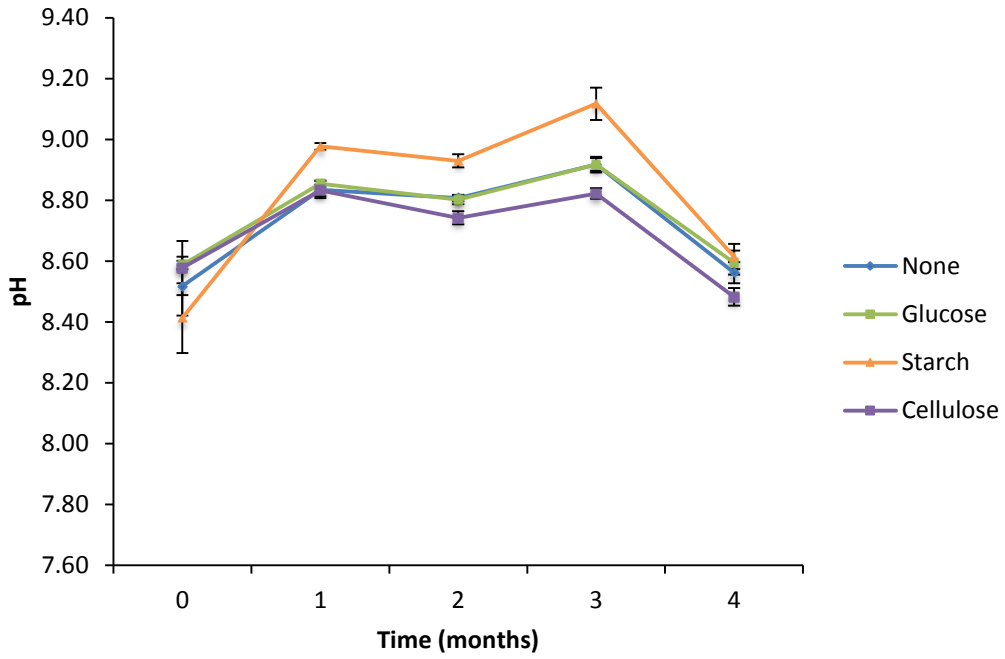

Figure 4.3. The mean (± standard error) pH in the water column of sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

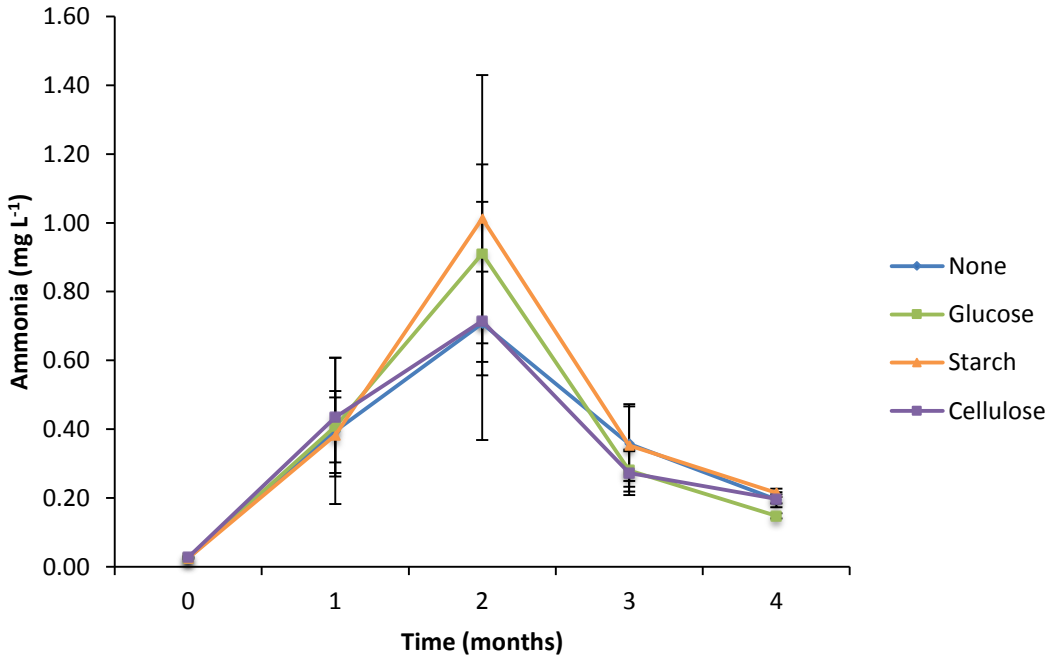

Figure 4.4. The mean (± standard error) concentration of total ammonia-nitrogen (TAN) in the water column of sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

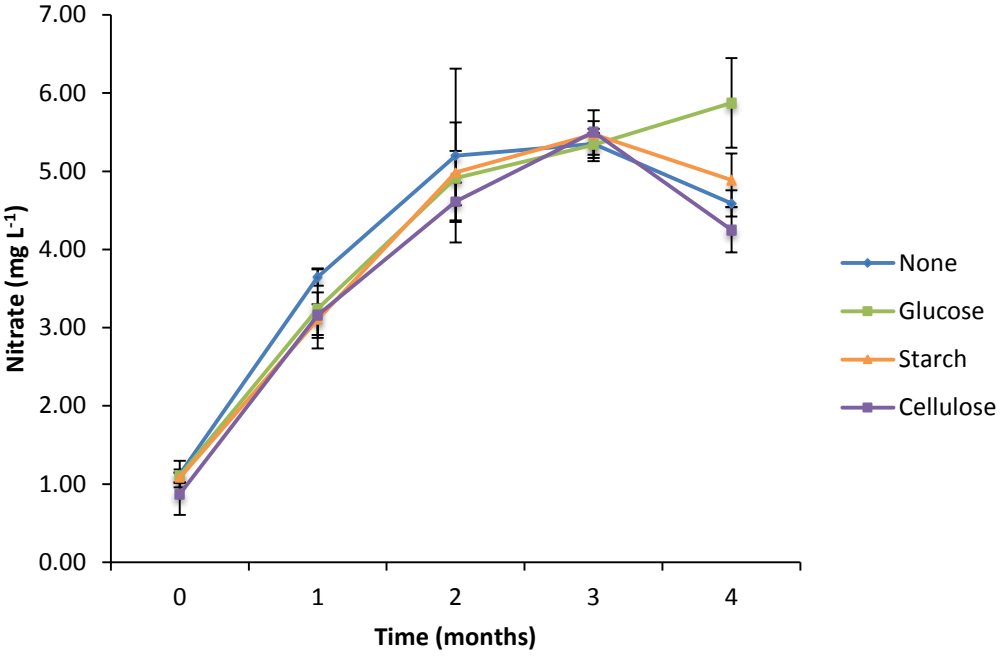

Figure 4.5. The mean (± standard error) concentration of nitrate in the water column of sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

Ammonia concentration increased in all treatments from 0.026 ± 0.001 mg L^{-1} at the start of the experiment to a peak of 0.84 ± 0.15 mg L^{-1} in month 2 and then decreased during the remaining two months to a mean of 0.19 ± 0.01 mg L^{-1} (Figure 4.4). Nitrate levels increased significantly over the experiment from 1.05 ± 0.08 mg L^{-1} on day 0 to 4.90 ± 0.23 mg L^{-1} in the final month (repeated measures ANOVA; $F_{(4, 48)} = 84.45$, $p < 0.001$), although there were no significant difference between treatments (3.92 ± 0.20 mg L^{-1}; Figure 4.5).

4.3.2 Sediment characteristics

The type of carbon source had a significant effect on sediment redox potential (repeated measures ANOVA, $F_{(12, 48)} = 4.76$, $p < 0.001$; Figure 4.6). At the start of the experiment there was no significant difference between treatments with a positive reading of 173.06 ± 5.99 mV indicating fully oxic conditions. The redox potential decreased over time in all treatments; however, there was a marked difference related to the lability of the carbon sources. Starch and cellulose, the more refractory carbon sources, had the greatest impact on redox potential. The sharpest decrease in redox potential was in the cellulose amended treatment where the redox potential decreased to -179.75 ± 18.93 mV in the final month, followed by the treatment receiving soluble starch, which decreased to -109.50 ± 6.14 mV. In the final month of the trial the redox potential in tanks receiving only particulate aquaculture

waste was only just negative at -22.00 ± 12.19 mV and was not significantly different to tanks amended with glucose, the most labile carbon source (-45.75 ± 8.47 mV).

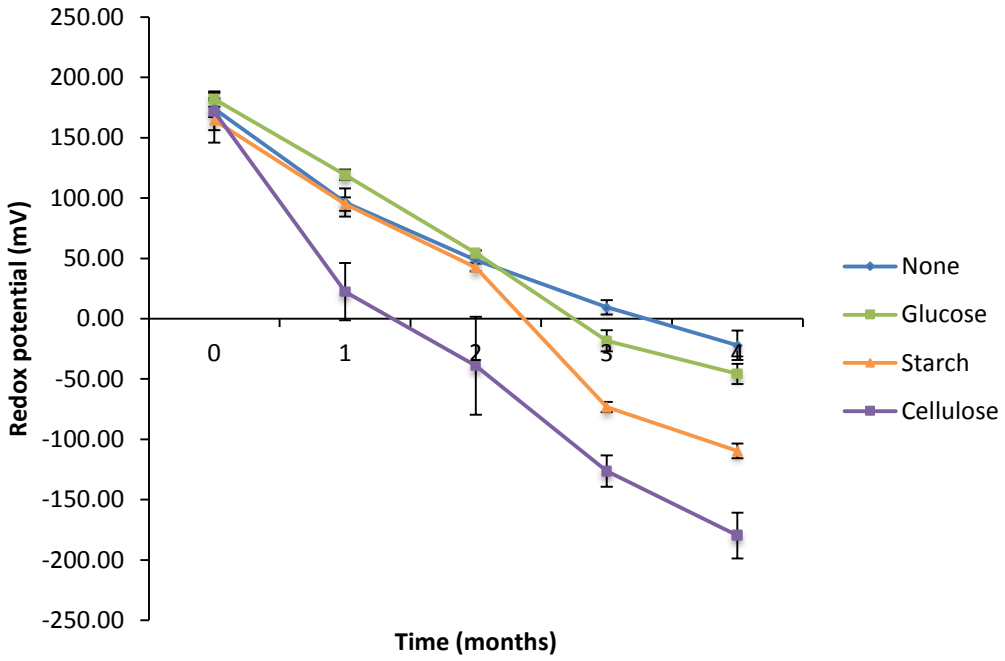

Figure 4.6. The mean (± standard error) reduction-oxidation (redox) potential at the base of the sediment (4 cm deep) in sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

There were no significant differences in the levels of organic carbon, total nitrogen, C:N, total carbohydrate concentration, chlorophyll *a* or phaeopigment between treatments over the course of the experiment (repeated measures ANOVA; $p > 0.05$; Table 4.3). Levels of organic carbon were initially low and increased in all treatments over time, with the highest levels of 0.20 ± 0.05 % recorded in the cellulose-amended treatments (Figure 4.7 and Table 4.3). Levels of total nitrogen were generally low (0.05 ± 0.00 %) with minor fluctuations between treatments over time (Figure 4.8 and Table 4.3). The carbon to nitrogen ratios of the surface sediments of the tanks amended with starch were relatively constant over the four month experiment with a mean of 7.41 ± 0.23 %. The C:N in the other three treatments fluctuated over time and between treatments with no clear trend, although there was a clear increase in the C:N in all of these treatments in the final month of the experiment (Figure 4.9).

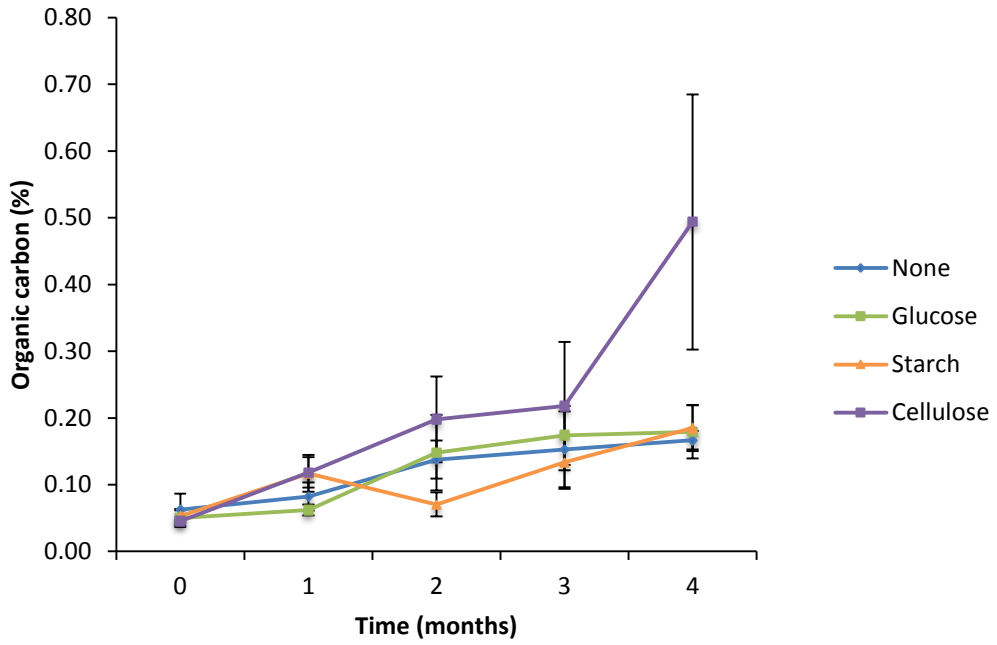

Figure 4.7. The mean (± standard error) organic carbon content measured in the surface sediment in sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

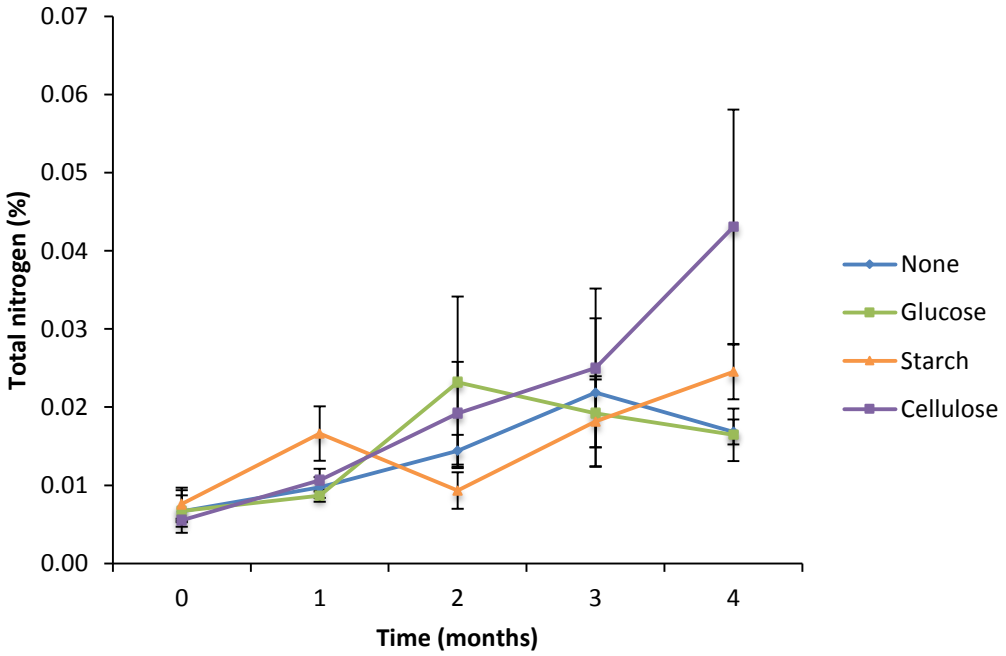

Figure 4.8. The mean (± standard error) total nitrogen content measured in the surface sediment in sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

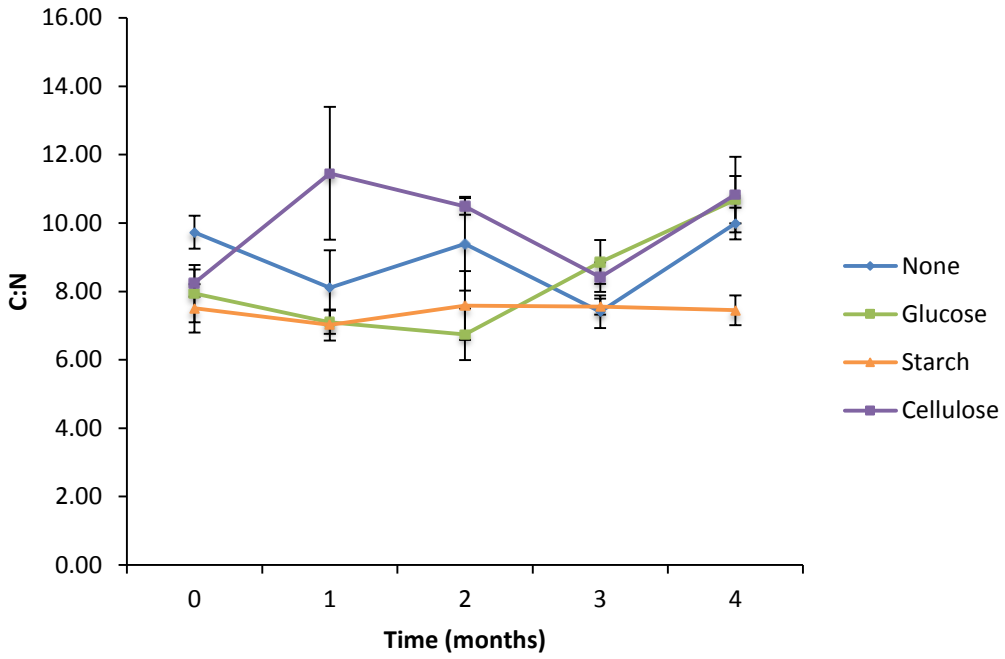

Figure 4.9. The mean (± standard error) carbon to nitrogen ratios (C:N) measured in the surface sediment in sea cumber tanks dosed with aquaculture waste only (none) or with aquaculture waste and various carbon sources (i.e. glucose, starch or cellulose) over the experimental period.

4.3.3 Growth and survival

There were no significant differences in mean biomass density between treatments at the start of the experiment (130.59 ± 1.92 g m^{-2}; Kruskal-Wallis, $H_{(3, 16)} = 1.53$, $p = 0.68$). Survival was 100% in all treatments. Over the course of the four month growth trial, the mean growth rate and biomass density of *H. scabra* reared in the starch treatment was significantly higher than sea cucumbers reared on the waste alone (repeated measures ANOVA, $F_{(12, 48)} = 2.49$, $p = 0.013$; Figure 4.10 and Figure 4.11). Sea cucumbers in the starch treatment reached a final biomass density of 1,011.46 ± 75.58 g m^{-2} at the end of the experiment compared to only 702.12 ± 35.93 g m^{-2} of *H. scabra* in the control tanks with no carbon supplementation. There was no significant difference in growth rate or biomass density between the three tested carbon sources.

Table 4.3. Mean (± standard error) water and sediment quality parameters recorded in sea cucumber tanks dosed with aquaculture waste (none) and aquaculture wasted combined with various carbon sources (glucose, starch and cellulose) during the four month experiment.

	None	Glucose	Starch	Cellulose	Overall
Water quality					
Light (lux)	139.25 ± 5.22	141.25 ± 5.22	141.25 ± 5.22	136.35 ± 5.41	139.53 ± 2.59
Temperature (℃)	29.07 ± 0.50	29.24 ± 0.53	29.21 ± 0.55	29.19 ± 0.51	29.18 ± 0.26
Dissolved oxygen (mg L^{-1})	7.28 ± 0.17	7.19 ± 0.12	7.47 ± 0.14	7.11 ± 0.14	7.26 ± 0.07
Ammonium (mg L^{-1})	0.34 ± 0.07	0.35 ± 0.09	0.40 ± 0.11	0.33 ± 0.09	0.35 ± 0.04
Nitrate (mg L^{-1})	3.98 ± 0.41	4.09 ± 0.43	3.91 ± 0.40	3.68 ± 0.38	3.92 ± 0.20
Sediment quality					
Total carbohydrate (µg g^{-1})	64.27 ± 28.08	49.90 ± 19.37	41.11 ± 21.96	96.08 ± 40.80	62.84 ± 14.30
Chlorophyll *a* (µg g^{-1})	0.86 ± 0.56	0.85 ± 0.37	1.29 ± 0.54	2.67 ± 1.19	1.42 ± 0.37
Phaeopigment (µg g^{-1})	1.08 ± 0.77	0.97 ± 0.49	3.04 ± 1.10	4.76 ± 2.15	2.46 ± 0.66
Organic carbon (%)	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	0.20 ± 0.05	0.14 ± 0.01
Nitrogen (%)	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Carbon to nitrogen ratio (C:N)	8.97 ± 0.44	8.43 ± 0.43	7.41 ± 0.23	9.84 ± 0.51	8.66 ± 0.23

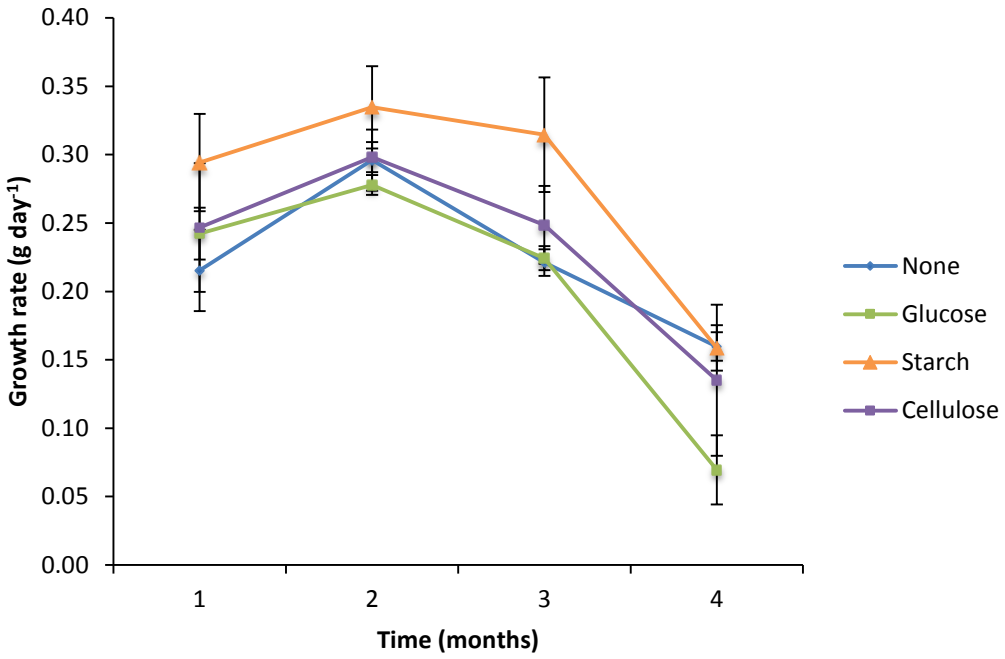

Figure 4.10. The mean (± standard error) growth rate per sampling period of *Holothuria scabra* (n=4) reared on particulate aquaculture waste (none) or this same waste in conjunction with different carbon sources (glucose, starch or cellulose).

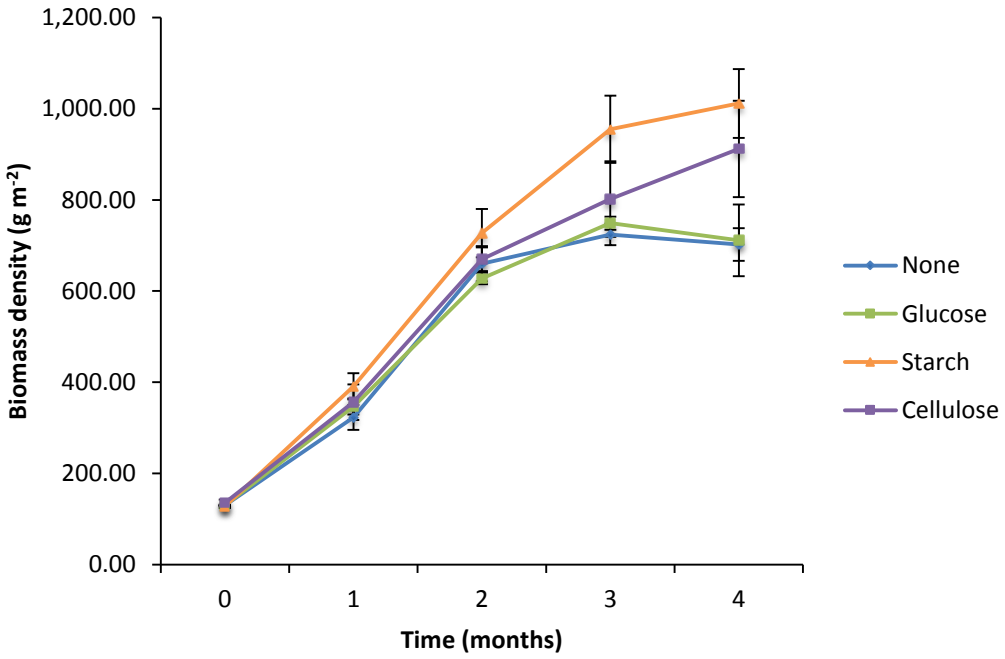

Figure 4.11. The mean (± standard error) cumulative biomass density of *Holothuria scabra* (n=4) reared on particulate aquaculture waste (none) or the same waste in conjunction with different carbon sources (glucose, starch or cellulose).

4.3.4 Multiple regression analysis

Sediment redox potential, light and nitrate were significant predictors of biomass density of *H. scabra* (multiple regression, $F_{(13, 60)} = 36.26$, $r^2 = 0.89$; $p < 0.001$). Light and nitrate showed a positive relationship to biomass density while the redox potential of the sediment had a negative relationship with sea cucumber density.

4.3.5 Economic analysis

A simple cost-benefit analysis was undertaken to determine whether there was a net financial gain from culturing *H. scabra* on particulate aquaculture waste, with or without carbon supplementation, compared to the formulated feed used previously (Chapters 2 and 3). To minimise the effects of seasonality and temperature, only growth data of *H. scabra* on redox-stratified sediments from Chapter 3 is used, since this study was run over a similar time (12th September to 5th December 2012). To allow for direct comparison between Chapters 3 and 4, only growth data for culture day 84 in the current study (31st December 2013) is used. The assumptions used in the cost-benefit analysis are based on current mid-market prices for feed ingredients and *H. scabra* (Purcell *et al.*, 2012a; Purcell *et al.*, 2012b; Purcell, 2014). To enable a direct comparison with the carbon sources used in this study, a comparison is made with food-grade carbon sources (Table 4.4); however, in practise, complex carbohydrates from agricultural waste streams should offer a more cost-effective and sustainable alternative.

Production of *H. scabra* on particulate aquaculture waste yielded a revenue of US$ 14.13 m^{-2} which increased to US$ 19.67 m^{-2} when supplemented with starch (Table 4.5). The cost-benefit analysis indicated that only the starch treatment produced a net financial gain compared to culturing *H. scabra* on the formulated feed (Table 4.6). Despite the lower biomass yield (954.88 versus 1,015.01 g m^{-2}), replacement of formulated feed with aquaculture waste in conjunction with soluble starch, reduced the total feed cost from US$17,100 ha^{-1} to US$1,500 ha^{-1} and resulted in a 22.3 % increase in net value of sea cucumbers grown on starch amended waste compared with the formulated feed. In the absence of carbon supplementation, comparison of the economics of producing *H. scabra* on waste only, yielded a net value of US$141,287 ha^{-1} or 88.5 % of the value relative to the formulated feed treatment (Table 4.6).

Table 4.4. Assumptions in the cost-benefit analysis comparing the culture of *Holothuria scabra* on formulated feed (Abfeed®-S34, Marifeed Pty Ltd, South Africa) versus different carbon sources used to increase the carbon to nitrogen ratio of particulate aquaculture waste from 5:1 to 20:1.

Assumption	Unit
Feed costs	
*Abfeed diet	1,930 US$ tonne^{-1}
^Glucose	500 US$ tonne^{-1}
^Corn starch	635 US$ tonne^{-1}
^Cellulose	390 US$ tonne^{-1}
Marketing of H. scabra	
Processing yield (wet to dry weight)	8 %
Market value (dry weight)	300 US$ kg^{-1} (dry weight)
Market value (wet weight)	24 US$ kg^{-1} (wet weight)
Feeding regimes	
Total Abfeed added	0.887 kg m^{-2}
Total starch and cellulose added	0.383 kg m^{-2}
Total glucose added	0.422 kg m^{-2}

* = cost supplied to G. Robinson by Marifeed Pty Ltd (South Africa) based on ZAR 27.75 kg^{-1} (equivalent to US$1.93 based on currency rates as of 12/07/2016.)
^ = mean cost extracted from the first ten suppliers of food grade carbon sources listed on Alibaba.com

Table 4.5. Biomass gain (wet and dry weight) of *Holothuria scabra* reared for 84 days on five diet treatments. Each treatment apart from 'Abfeed' had a baseline input of waste RAS effluent ('none') which was supplemented by starch, cellulose or glucose. A formulated feed only treatment ('Abfeed'; Abfeed®-S34, Marifeed Pty Ltd, South Africa) was used as a control. Final biomass = biomass density (g m^{-2}) on day 84; biomass gain = final biomass density - initial biomass density; value gain = cash equivalent value m^{-2}; biomass gain relative to 'none' = net biomass gain through carbon supplementation; biomass yield relative to 'Abfeed' = net biomass yield compared with the formulated feed; value difference = cash equivalent yield compared with either 'none' or 'Abfeed' treatments.

Feed	Final biomass (g ww)	Biomass gain (g ww)	Biomass gain (g dw)	Value gain (US$)	Biomass yield relative to 'none' (g ww)	Biomass yield relative to 'none' (g dw)	Biomass yield relative to 'Abfeed' (g ww)	Biomass yield relative to 'Abfeed' (g dw)	Value difference relative to 'none' (US$)	Value difference relative to 'Abfeed' (US$)
Abfeed	1015.01	736.33	58.91	17.67	147.67	11.81	-	-	3.54	-
None	724.06	588.66	47.09	14.13	-	-	-147.67	-11.81	-	-3.54
Starch	954.88	819.48	65.56	19.67	230.82	18.47	83.15	6.65	5.54	2.00
Cellulose	801.93	666.53	53.32	16.00	77.87	6.23	-69.80	-5.58	1.87	-1.68
Glucose	748.86	613.46	49.08	14.72	24.8	1.98	-122.87	-9.83	0.60	-2.95

Table 4.6. Feed costs calculated for each diet treatment.

Feed	Cost/m^2 (US$)	Cost/ha (US$)	Cost/kg biomass gain ww (US$)	Net value/m^2 (value – feed costs) (US$)	Net value/ha (US$)	Net cost relative to Abfeed (US$)	Total value of product/ha (US$)	Net value/ha relative to Abfeed (US$)	Net financial return relative to Abfeed (%)
Abfeed	1.71	17,100	2.32	15.96	159,600.38	-	176,719.38	-	-
None	-	-	-	14.13	141,278.40	-0.38	141,278.40	-3,841.00	88.52
Starch	0.15	1,500	0.18	19.52	195,175.20	3.56	196,675.20	35,580.00	122.29
Cellulose	0.24	2,400	0.37	15.75	157,567.20	-3.76	159,967.20	-37,646.00	98.73
Glucose	0.21	2,100	0.29	14.51	145,130.40	-1.24	147,230.40	-12,417.00	90.93

4.4 Discussion

The present study demonstrated that *Holothuria scabra* can be reared successfully at high densities (> 700 g m^{-2}) solely on particulate aquaculture wastes from a marine recirculating aquaculture facility. The growth rates and final biomass densities of *H. scabra* fed on aquaculture waste plus starch as a carbon source were directly comparable to the final density (1,028.50 ± 117.46 g m^{-2}) achieved by rearing *H. scabra* on a commercial formulated feed under the same experimental conditions (Chapter 3). In this trial, the daily feed rations of particulate aquaculture waste equated to ~ 1 % of the total tank biomass (corrected on a dry weight basis), while the formulated feed rations used in the growth trial in Chapter 3 ranged from one to four percent of the total tank biomass (Chapter 3, Section 3.2.4). Furthermore, daily additions of aquaculture waste were standardised at 200 mmol C m^{-2} day^{-1} and were not adjusted with increasing biomass of *H. scabra* as in previous growth trials (Chapters 2 and 3). As the quantity of organic matter input is directly related to the carrying capacity of deposit feeder populations (Olafsson, 1986; Fenchel *et al.*, 2012), increasing the volume and frequency of waste additions would further increase the biomass density of *H. scabra* from the levels attained in this study.

The cost-benefit analysis indicated that particulate aquaculture waste supplemented with soluble starch, outperformed formulated feeds, yielding a net financial return of 122.29 % compared to the formulated feed (Abfeed®-S34, Marifeed Pty Ltd, South Africa) when supplemented with starch. The potential exists to further increase the economic and environmental sustainability of aquaculture waste remediation, by utilising cheaper complex carbon sources, such as bagasse or biochar, which are agricultural by-products. Furthermore, *H. scabra* fed on waste only yielded 88.5 % of the value relative to the formulated feed treatment, even at feed inputs that were up to four times lower. This demonstrates that potential to entirely replace formulated feeds with particulate organic waste in sea cucumber aquaculture. This would not only reduce pressure on wild resources but could increase the sustainability of the aquaculture industry by up-cycling nitrogen-rich effluent originating from land-based aquaculture into high value biomass (Brown *et al.*, 2011).

The majority of studies focusing on utilising waste from land-based recirculating aquaculture systems have focused on other detritivorous invertebrates such as polychaete worms (Erler *et al.*, 2004; Yearsley *et al.*, 2009; Palmer, 2010; Brown *et al.*, 2011; Palmer *et al.*, 2014). Brown *et al.* (2011) found no significant difference in the final mean density of *Alitta virens* (previously *Nereis virens* (1,865 g m^{-2})) fed on different proportions of waste (0-100 %) collected from a recirculating halibut (*Hippoglossus hippoglossus*) nursery compared to a commercial diet, underscoring the potential to culture deposit feeders at high density fed

solely on aquaculture wastes. In Australia, the use of polychaete-assisted sand filters is being pioneered as a means of treating particulate organic wastes from prawn farms (Palmer, 2010); however, the densities achieved were low ranging from 300 – 400 g m^{-2} compared to Brown *et al.* (2011). To date, few studies have examined the potential for sea cucumbers to bioremediate waste from intensive land-based aquaculture operations, with research thus far limited to temperate species such as *Apostichopus japonicus, Holothuria forskali* and *H. arguinensis* (Kang *et al.*, 2003; MacDonald *et al.*, 2013; Bossers, 2015). Some studies were limited to reporting survival, feed consumption, and changes in organic carbon and total nitrogen content to indicate bioremediation potential; data on growth rates were absent e.g. MacDonald *et al.* (2013). However, juvenile sea cucumbers have been integrated into land-based abalone re-circulating systems in China, Korea and New Zealand (Lin *et al.*, 1993; Kang *et al.*, 2003; Maxwell *et al.*, 2009) illustrating the potential for co-culture or integrated production of these invertebrate species.

The current study indicated that increasing the carbon to nitrogen ratio from 5:1 to 20:1 through carbon supplementation successfully increased the growth rate and biomass density of *H. scabra* reared on particulate organic waste. It is possible that increasing the carbon to nitrogen ratio improved the nutritional value of the waste by increasing the quantity of organic carbon. It is commonly thought that deposit feeders gain energy for maintenance from carbon while nitrogen is used predominantly for growth (Lopez and Levinton, 1987). Deposit feeders are assumed to metabolise organic carbon and nitrogen in a 17:1 molar ratio (Russell-Hunter, 1970); therefore, carbon addition may have improved growth rates by balancing the elemental stoichiometry of the food (Rice and Rhoads, 1989). An alternate explanation for the increased growth rate and biomass density is that *H. scabra* was able to directly assimilate the carbon sources or their degradation products. Mechanisms for the uptake of organic molecules such as amino acids and carbohydrates are thought to exist in tissues such as the respiratory trees, body wall or tentacles, thereby enabling holothurians to assimilate dissolved organic matter directly from the water column (Lawerence, 1982). All aspidochirotid holothurians, including *H. scabra,* possess respiratory trees, which are blind-ended evaginations of the posterior digestive system, that perform respiratory, excretory and nutritional roles (Jangoux and Lawerence, 1982; Jaeckle and Strathmann, 2013). The respiratory trees are the most active organ of the body with an ability to use all sugars, including monosaccharides (Fontaine and Chia, 1968), disaccharides (Krishnan and Krishnaswamy, 1970) and polysaccharides such as dextrin – a low molecular weight carbohydrate produced from the hydrolysis of starch (Jaeckle and Strathmann, 2013). Since soluble starch yielded a significantly higher final growth rate and biomass density compared

to controls, the anal uptake of starch breakdown products, as part of a nutritional biopolar feeding strategy and proximal cause for increased growth, cannot be ruled out.

Direct utilisation of soluble starch for enhanced growth may not have been limited to the sea cucumbers. Although there was no significant difference in either chlorophyll *a* or phaeopigment concentration, there were apparent differences (albeit qualitative) in the extent of phototroph biomass production between treatments. The tanks amended with soluble starch supported notably thicker layers of diatoms e.g. *Nitzschia* and *Navicula sp.* together with denser floating colonies of the cyanobacteria *Oscillatoria sp.* These visual observations were not quantified, however they are supported by photographic evidence (Figure 4.2) together with significantly higher pH values, which can provide a proxy for primary production. Carbohydrates such as glucose and polysaccharides can also be photo-oxidised with high quantum efficiencies in illuminated habitats leading to concomitant increases in microbial and algal productivity (Krishnan and Krishnaswamy, 1970). Furthermore, cyanobacteria that can exhibit mixotrophic growth, by concurrently operating respiratory and photosynthetic metabolisms, have been observed following the addition of labile organic carbon sources (Stuart *et al.*, 2016).

The quality and quantity of organic matter supply to the sediment is modulated by sunlight, temperature and nutrient availability (Huettel *et al.*, 2014). Multiple regression analysis indicated that light and nitrate were significant predictors of sea cucumber biomass density with a positive relationship. Cyanobacteria, which play important roles in the degradation and reuse of excess organic carbon, may have supplied additional carbon to heterotrophic sediment bacteria and sea cucumbers as extracellular polymeric substances from photosynthesis (Stuart *et al.*, 2016). These autochonthous sources of organic carbon supplied by primary production under strong irradiance may have also contributed to the significantly higher growth of sea cucumbers supplemented with starch. This link between increased photoautotroph and sea cucumber biomass highlights the importance of the microbial-deposit feeder loop in recycling nutrients back to primary producers, which further supply sources of labile carbon to fuel deposit feeder growth.

It was hypothesised that manipulation of carbon to nitrogen ratios may result in a change in the pathways of nitrogen cycling by mediating a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ by heterotrophic bacteria (Avnimelech, 1999). For aerobic heterotrophic bacteria, the removal of ammonia-nitrogen by incorporation in cell biomass is enhanced by the addition of carbon in the form of any carbohydrate (Ebeling *et al.*, 2006). It was therefore expected in these experiments that carbon supplementation would result in an overall decrease in total ammonia nitrogen (TAN) concentrations compared to the

control. In the present study, carbon supplementation did not have a significant effect on the TAN concentration between treatments, although the levels of TAN were generally low throughout the study averaging 0.35 ± 0.04 mg L^{-1}. This may have been due to assimilation by heterotrophic bacteria, which are responsible for a large fraction of NH_4^+ assimilation in coastal sediments (Blackburn, 1988; Kirchman, 2012); however, the trends in the ammonia and nitrate data seem to indicate that nitrification rather than assimilation was the dominant pathway for the conversion of NH_4^+ released from ammonification of the aquaculture waste (Wu *et al.*, 2013). The increasing nitrate concentration from the start of the experiment to month two is consistent with an increasing nitrification capacity with the establishment of a community of nitrifying bacteria in the predominately oxic sediment layers during the first two months of the experiment (Wu *et al.*, 2013). In autotrophic nitrifying bacteria, the majority of energy is used for ammonia oxidation; consequently, nitrifiers are typically slow to establish due to slow growth rates in comparison to heterotrophic bacteria (Ebeling *et al.*, 2006). The trends in the redox data support this hypothesis, since the redox potential of the sediment reflected conditions suitable for nitrification in the first two months of the experiment (Gerardi, 2002). The stabilization of nitrate concentrations between months two and three may be an indication that the nitrification capacity reached steady state with the onset of coupled nitrification-denitrification as the redox potential indicated suitable conditions for denitrification (+50 mv to -50 mV) in month two (Gerardi, 2002). The quantity of organic carbon (i.e. carbon loading) is one of the main controls on the denitrification efficiencies of sediments since this is a heterotrophic pathway of anaerobic nitrate respiration (Blackburn and Blackburn, 1992; Joye and Anderson, 2008). The decreasing concentrations of nitrate in the final month of the experiment are consistent with the conversion to dinitrogen gas or nitrous oxide by denitrifying bacteria under anoxic conditions indicated by the negative sediment oxidation potentials recorded at the end of the trial (Seitzinger, 1988; Blackburn and Blackburn, 1992).

The conditions under which the organic carbon source are degraded (oxic or anaerobic) will also have an effect on the decomposition rate since bacterial growth efficiencies are a direct function of oxygen availability (Goldman *et al.*, 1987; Tezuka, 1990; Fenchel *et al.*, 2012). In the present study, the C:N of the aquaculture waste was increased from 5:1 to 20:1 in the treatments supplemented with carbon. For heterotrophic bacteria (average C:N of 5:1), a substrate C:N of 20:1 represents the threshold for net removal or net regeneration of ammonium at a bacterial growth efficiency of 25 %. Under anaerobic conditions, such as redox-stratified sediments, bacterial growth efficiencies range from 5 – 30 % (Goldman *et al.*, 1987). It is possible that the C:N of 20:1 was not high enough to mediate a

shift in the pathways of nitrogen cycling to promote net assimilation of NH_4^+ due to lower growth efficiencies of bacteria under reducing conditions. Knowledge of the sediment redox potential and how it changes over time in response to waste and carbon addition is therefore a pre-requisite for determining the correct C:N to assure the assimilation of NH_4^+ into bacterial biomass.

The biochemical composition of substrate is an important factor in defining resource quality since the degradability is linked to its structural complexity. The carbon sources tested in this study differed widely in their biodegradability from glucose, a highly labile carbon source with a first-order degradation rate constant of 1.15, to cellulose, a complex structural polysaccharide with a slow decomposition rate constant of 0.05 (Reddy *et al.*, 1986; Avnimelech *et al.*, 1995). Highly labile substrates and readily biodegradable substrates such as glucose are effective in promoting heterotrophic bacterial growth under aerated conditions in biofloc; however, in this study, glucose had little impact on sea cucumber growth (Crab *et al.*, 2010). In contrast, the biomass density curve of sea cucumbers reared in tanks amended with cellulose did not show any signs of plateauing, indicating that the maximum carrying capacity of the system had not been reached. It is possible that had the experiment continued for a further two months the cellulose treatment may have outperformed the starch treatment. In a redox-stratified sediment-based system where heterotrophic bacteria have lower growth efficiencies, more complex polysaccharides may be a more suitable long-term substrate for heterotrophic growth. Cellulose is more resistant to hydrolysis as its β-1,4 bonds form rigid, ribbon-like chains with crystalline structures (Fenchel *et al.*, 2012). Since the slow hydrolysis of more stable polysaccharides, such as cellulose, demands an incorporation of nitrogen and phosphorous into microbial cell biomass (Schroeder, 1987), the use of a more complex compound may offer a more stable means of ensuring sufficient carbon is available to bacteria in the long-term. This is particularly relevant in microbial-deposit feeder aquaculture bioremediation systems, due to the need to balance the constant efflux of NH_4^+ issuing from the redox-stratified sediment, sea cucumber excretion and ammonification following waste addition. The addition of cellulose as a carbon source would also approximate the natural habitat of *H. scabra* that comprises seagrass beds in areas with high terrigenous input (Hamel *et al.*, 2001). Cheap agricultural sources of polysaccharides that warrant further investigation include cassava, grains and potato processing wastes as sources of starch and sugarcane bagasse, straw from rice or wheat production and maize cobs as a source of cellulose (Srinivasva, 1987).

Carbon addition is utilised in other aquaculture treatment technologies including biofloc, denitrifying reactors and treatment of saline sludge by anaerobic digestion (van Rijn

et al., 2006; Hamlin *et al.*, 2008; Avnimelech, 2014; Luo *et al.*, 2015). While these technologies utilise low cost carbon sources, they focus on the permanent removal of nitrogen (N_2 gas via denitrification) or generate additional solid wastes that require disposal (biofloc and anaerobic digestion), thereby further contributing to sustainability issues in land-based aquaculture. Carbon supplementation may offer a more sustainable alternative to retain nitrogen in the system by promoting the net immobilisation of NH_4^+ into single cell biomass that can be up-cycled into high value secondary biomass. *H. scabra* currently retails for ~ US$ 300 kg^{-1} (dry weight), has a strong market demand, is over-exploited in the wild, and aquaculture technologies are well developed (Purcell *et al.*, 2012a; Hamel *et al.*, 2013; Robinson, 2013; Purcell, 2014). The commercial production of *H. scabra* on particulate organic waste plus starch at densities of ~ 1 kg m^{-2} achieved in this study would generate a value gain of US$ 19.67 m^{-2} based a processing yield of 8 %. The potential for high density culture is therefore an attractive approach to closing the nitrogen loop, especially if joint waste streams from aquaculture and agriculture are combined.

4.5 Conclusion

This study has demonstrated that *H. scabra* can be reared at commercially viable densities (> 700 g m^{-2}) solely on particulate organic waste from a land-based RAS. Increasing the carbon to nitrogen ratio from 5:1 to 20:1, with soluble starch as the carbon source, yielded significantly higher final growth rates and biomass density compared to controls (1,011.46 ± 75.58 g m^{-2} versus 702.12 ± 35.93 g m^{-2}). The final densities achieved with aquaculture waste and soluble starch, are directly comparable to the densities of *H. scabra* (1,028.50 ± 117.46 g m^{-2}) reared on redox-stratified sediments with a 34 % protein formulated feed (Chapter 3). Furthermore, the simplified cost-benefit analysis indicated that supplementation of waste with soluble starch as a carbon source yielded a net financial return of 122.29 % compared to the formulated feed. Given the cost-benefits associated with this knowledge, this research has important commercial application for future development of integrated aquaculture production and bioremediation systems.

The results of this study did not support the hypothesis that increasing C:N would promote heterotrophic assimilation of NH_4^+ since there were no significant differences in NH_4^+ between treatments. The accumulation of nitrate over the first two months, was indicative of increasing nitrification activity, with coupled nitrification-denitrification accounting for the removal of nitrate as the sediment redox potential became negative during the final two months. The positive relationship between sea cucumber biomass density, nitrate and light and increased cyanobacterial biomass indicated that autochonthous sources of

organic matter supplied by primary production under strong irradiance may have also contributed to the significantly higher growth of sea cucumbers supplemented with starch. While this lends additional support to the concept of balancing the stoichiometry of nutrient inputs where nutrients are recycled into deposit feeder biomass under irradiated systems, future studies should be conducted under controlled conditions to minimise the influence of confounding factors such as light and primary production. Furthermore, more precise techniques, such as labelled stable isotope studies or benthic flux incubations are needed to fully elucidate the role of C:N control on nitrogen dynamics in sediment-based systems.

Chapter 5. Effect of carbon supplementation on benthic nitrogen cycling during aquaculture waste bioremediation incubations

5.1 Introduction

Intensive land-based aquaculture systems produce nitrogen-rich effluent where microbial nitrogen removal is limited by the supply of carbon (Castine, 2013). Carbon supplementation is consequently employed by a number of aquaculture wastewater treatment technologies to overcome this deficiency (Avnimelech, 1999; Schneider *et al.*, 2006; Hamlin *et al.*, 2008). In recirculating aquaculture systems (RAS), the addition of exogenous carbon is a pre-requisite for the successful operation of denitrifying filters that permanently remove dissolved inorganic nitrogenous wastes by conversion to dinitrogen gas (Roy *et al.*, 2010). Alternatively, in zero exchange biofloc systems, carbon to nitrogen ratios (C:N) are increased through the addition of labile carbon sources to promote ammonia assimilation from the water column by heterotrophic bacteria (Avnimelech, 1999; Crab *et al.*, 2012). The fundamental difference between these approaches is the ultimate fate of nitrogen within the system, i.e. removal versus retention. Technological advances are focused on the development of dissimilatory processes to permanently remove nitrogen from the system as N_2 gas, while ecological-based systems, such as biofloc, aim to promote assimilation into biomass and thus re-use and re-cycle nitrogen within the culture system.

The stoichiometric approach taken in C:N control in biofloc systems, recognises that carbon and nitrogen cycles are coupled; therefore, the relative elemental abundances control the rate of nutrient cycling and energy flow within the system (Dodds *et al.*, 2004; Ebeling *et al.*, 2006). The potential for C:N manipulation in sediment-based aquaculture effluent treatment systems containing deposit feeders was demonstrated in Chapter 4, wherein the addition of soluble starch to aquaculture waste significantly improved sea cucumber growth rate and biomass density. Furthermore, redox-stratified sediments that harboured heterotrophic microbial communities supported higher biomass and faster growth rates of *Holothuria scabra*, indicating that predominately reducing conditions are more favourable for deposit feeder growth than fully oxic systems (Chapter 2 and 3; Appendix A, Table A1.2). Since reducing conditions favour anaerobic respiratory and fermentative pathways, organic carbon supplementation may stimulate anaerobic bacterial metabolism by increasing the availability of potential electron donors and/or substrates for fermentation, in addition to increasing heterotrophic assimilation of NH_4^+ (Fenchel *et al.*, 2012).

Essentially, substrate C:N affects the amount of nitrogen released during mineralisation, thus above a threshold of 20:1, there will be no net nitrogen release (Blackburn, 1986; Cook *et al.*, 2007). In Chapter 4, it was hypothesised that C:N manipulation may alter the pathways of nitrogen cycling by mediating a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ by heterotrophic bacteria; however, the effect of carbon supplementation on nitrogen cycling was not clearly elucidated. It was concluded that an improved understanding of how C:N manipulation influences benthic nitrogen cycling was necessary in order to improve nutrient assimilation and incorporation into secondary biomass. To further investigate the effect of carbon supplementation on pathways of nitrogen cycling, a coupled biogeochemical-molecular approach was used. Incubation experiments were conducted to quantify benthic fluxes, while sediment microbial communities were examined using 16S rRNA gene sequencing. The study aimed to test the hypothesis that increasing the C:N of particulate aquaculture waste from 5:1 to 20:1 via carbon supplementation would promote the assimilation of NH_4^+ by heterotrophic bacteria.

5.2 Materials and methods

5.2.1 Study site

The study was conducted in the purpose built bio-secure heated recirculating aquaculture system (RAS) described in Chapter 2, Section 2.2.1. The experiment was conducted over a fifteen day period from January 30th (day minus one) to February 14th (day 14).

5.2.2 Experimental animals

Experimental animals were imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam) on September 5th 2013, quarantined and acclimated to the experimental system (Chapter 4, Section 4.2.2).

5.2.3 Experimental design

The experiment was designed to test the effect of increasing the C:N of particulate aquaculture waste from 5:1 to 20:1 by supplementing with carbon on microbial community structure and biogeochemical cycling. The three experimental treatments were randomly allocated to 15 incubation chambers with five replicates per treatment (Table 5.1). The 'initial' treatment was included to ensure that there were no significant differences between treatments prior to the start of the experiment. The 'no carbon' treatment (-C) with C:N of 5:1 received aquaculture waste only (26.8 mg day^{-1} wet weight). The 'carbon' treatment (+C)

received aquaculture waste (26.8 mg day^{-1} wet weight) and carbon in the form of soluble starch (7.5 mg day^{-1} dry weight) increasing the C:N to 20:1 from day zero (Table 5.1).

Table 5.1. Description of the experimental treatments. Ticks (✓) indicate the addition of aquaculture waste, a carbon source or the presence of sea cucumbers respectively; crosses (x) indicate no addition of aquaculture waste, carbon source or the absence of sea cucumbers respectively from day zero.

Treatment	Treatment code	No of replicates	Aquaculture waste	Sea cucumber	Carbon source	C:N
Initial	I	5	x	x	x	n/a
No carbon	-C	5	✓	✓	x	5:1
Carbon	+C	5	✓	✓	✓	20:1

5.2.4 Experimental system and rearing conditions

Sediment incubation chambers were established by transferring unsieved $CaCO_3$ builder's sand sourced from a commercial dune quarry (SSB Mining Pty Ltd, city) into 25 cm long and 8.4 cm internal diameter glass tubes (Plexiglas®, Maizey, Cape Town, South Africa) sealed with a polyvinyl chloride (PVC) end cap to a depth of 7.5 cm. The incubation chambers were connected by 4.0 mm air tubing and 4.0 mm variflow valves to a manifold (50 mm polyvinyl chloride pipe; Figure 5.1) receiving seawater directly from the RAS biofilter (Chapter 4, Section 4.2.6).

The flow rate to the cores was set at 50 mL min^{-1}. As the total water volume in the cores was 0.817 L, this was equivalent to 16.34 exchanges h^{-1}. The outflows of the chambers were routed into a main drainage channel and allowed to flow to waste to prevent soluble carbon sources from entering the RAS (Chapter 4, Section 4.2.6). No aeration was provided; however, each chamber was fitted with an engine driven magnetic stirring rod, positioned 15 cm above the sediment surface. Stirring rates were just below that which caused sediment re-suspension. Water was mixed continuously throughout the experiment except during incubations when the stirrers were disconnected. The sediment was allowed to condition and stabilize into redox-stratified layers in the chambers for 14 days prior to commencement of the experiment (Figure 5.1d)

The experimental area was shaded from direct sunlight by hanging shade cloth curtains alongside the stands on which the chambers were placed and whitewashing the clear polycarbonate panels that formed the roof of the experimental system. Light intensity was measured during daylight incubations using a portable light meter (LX-107, Lutron Electronic Enterprise Co. Ltd, Taipei, Taiwan) positioned 10 cm above each chamber. In addition, a pendant temperature/light data logger (Hobo, UA-002-64, Onset, USA) was placed in an additional chamber, located in the centre of the experimental treatments to automatically log

light intensity and water temperature throughout the experiment. The mean (hours) natural photoperiod in hours was 13.34:10.26 (L:D).

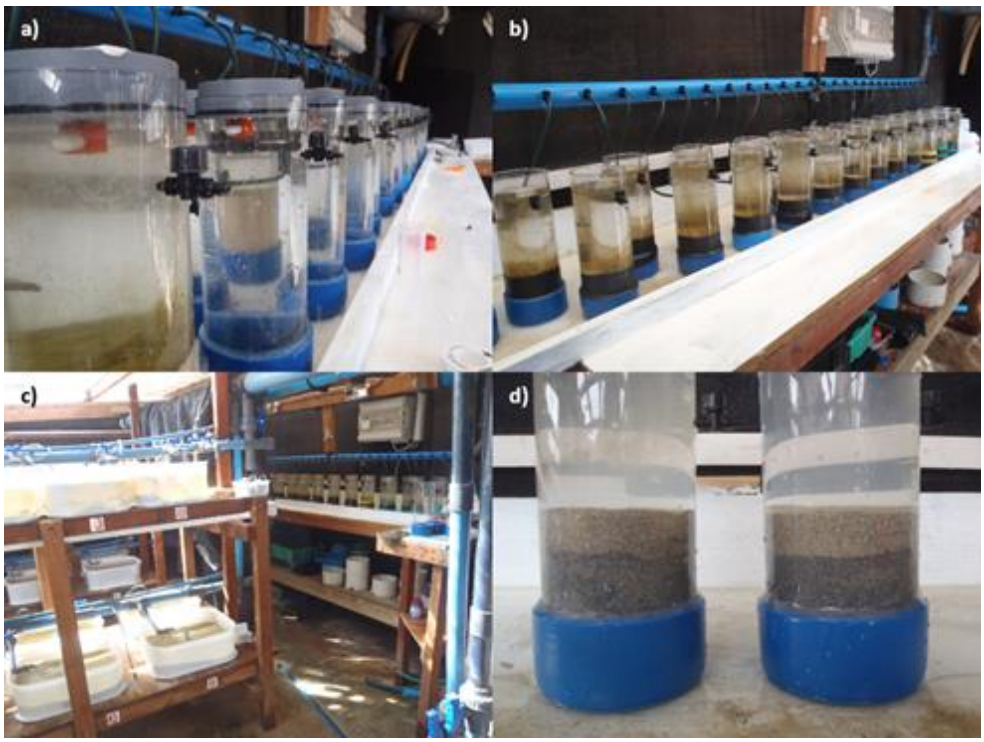

Figure 5.1. The experimental system indicating: a) incubation chamber lids fitted with magnetic stirring rods; b) incubation chambers connected to the 50 mm manifold; c) the differences in light intensity between the experimental tanks used in previous chapters (top row of tanks on left-hand side) and the incubation chambers that were shaded from direct sunlight; and, d) development of redox-stratification in the sediments during the 14 day conditioning period.

5.2.5 Aquaculture waste and carbon additions

The aquaculture waste, comprising mainly uneaten abalone (*Haliotis midae*) feed and faeces, was used as feed for the sea cucumbers and was collected daily from the backwash of a sand filter in a recirculating abalone grow-out system (Chapter 4, Section 4.2.5). Prior to the experiment, samples of particulate aquaculture waste were collected over five days and sent for laboratory analysis of organic carbon and total nitrogen content (Chapter 4, Section 4.2.5). The average C:N of the aquaculture waste was 5.21:1. Soluble starch (Merck Millipore, Pretoria, South Africa) was used as an additional carbon source, and this starch on its own had an average C:N of 20:1. Additions of aquaculture with carbon (+C) or without carbon (-C) also commenced on day zero (Figure 5.2b). The aquaculture waste was mixed into a wet slurry and added to the incubation chambers every day at 16:00 from day zero to day 14 at a concentration of 400 mmol C m^{-2} d^{-1}.

5.2.6 Experimental timeline

Baseline data were collected prior to the start of the experiment (i.e. day -1), where samples were collected from all fifteen of the incubation chambers containing sediment and to which no aquaculture waste was added and in which no sea cucumbers were present. The 15 chambers were incubated under light and dark conditions on day -1. After monitoring of fluxes during dark and light incubations on day -1, all replicates from the 'initial' treatment were sacrificed on day zero for sub-coring for analysis of sediment characteristics and no further data were collected from these treatments (Figure 5.2b).

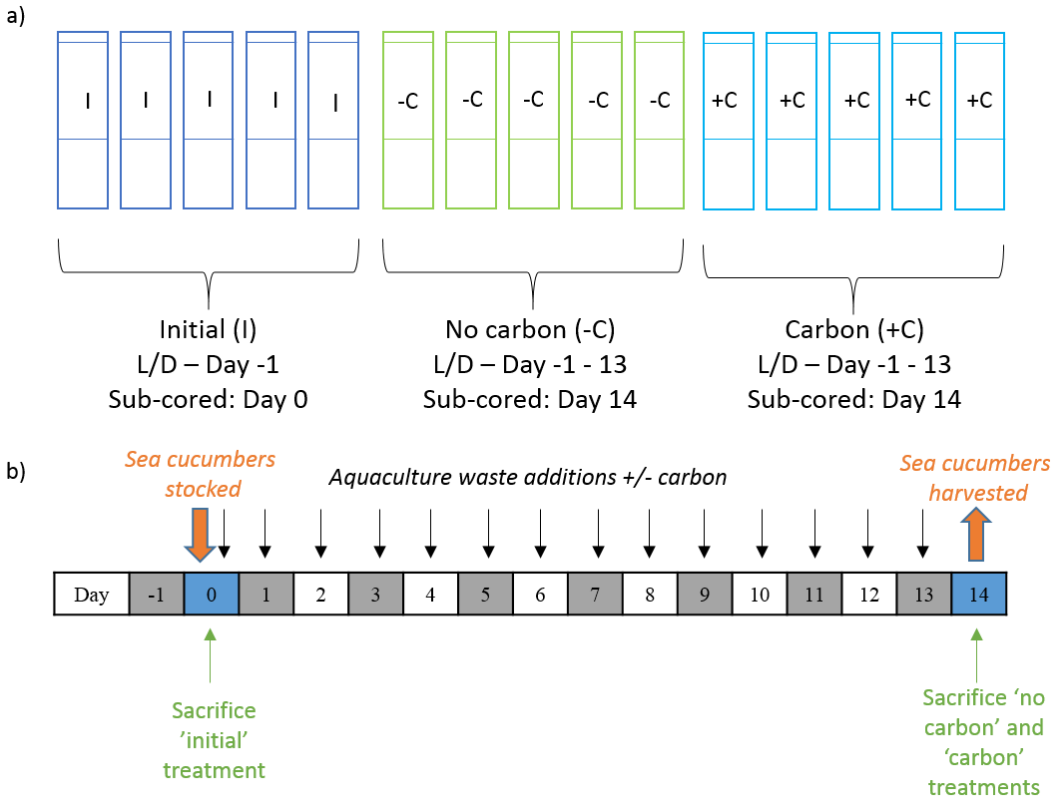

Figure 5.2. a) Overview of the experimental treatments, and b) timeline indicating the days when experimental treatments were sacrificed for sub-coring (green); the period when addition of aquaculture waste, with carbon (+C) or without carbon (-C) were made (days 0-14); the days when sea cucumbers were stocked and harvested (orange); and the days when light (L) and dark (D) incubations were conducted (indicated by grey shading).

5.2.7 Growth of Holothuria scabra

Experimental animals (n=30) previously acclimated in the RAS were suspended in mesh containers to evacuate their guts for 24 h prior to weighing and photo-identification (Chapter 2, Section 0). Three juvenile *H. scabra* with a mean (± standard error) weight of 1.91 ± 0.07 g were added to each of 10 cores (equivalent to a density of 1,034.00 ± 12.73 g m^{-2}) on day zero (Figure 5.2b). At the end of the experiment (day 14), the sea

cucumbers were removed, gut-evacuated for 24 h, and each individual was reweighed as above. Wet weight data were used to calculated growth rate (g d^{-1}; Equation 2 Chapter 2, Section 2.2.5).

5.2.8 Benthic flux incubations

Benthic flux incubations were conducted on day minus one for all treatments (I, -C and +C) and on alternate days from day one to day 13 for the -C and +C treatments, after sacrifice of the initial (I) treatment (Figure 5.2b). Light incubations were conducted during daylight hours, commencing approximately two hours after sunrise at 08:00 (local time) and dark incubations were conducted approximately two hours after sunset at 22:00 (local time). When data were collected from the chambers, the water flow to each chamber was interrupted, the stirrers were disconnected and chambers were uncapped by removing the rubber bung (20 mm internal diameter). A portable optical meter (YSI ProODO, YSI Pty Ltd, USA) was inserted through the sampling port to measure temperature (0.01 °C) and dissolved oxygen (DO) concentrations (0.01 mg L^{-1}) once the readings had stabilised. The pH (0.01 pH units) was measured electro-chemically using a portable meter (Eutech Instruments pH 6+ portable meter, Singapore).

Water alkalinity and nutrient concentration (ammonia, nitrate/nitrite, nitrite and phosphate) were recorded at the start and end of each light/dark incubation period. To do this, samples were withdrawn using a 50 mL acid washed plastic syringe connected to the outflow of the incubation chamber by 4.0 mm tubing and filtered (Whatman® glass microfiber filters grade GF/C, Sigma Aldrich, Johannesburg, South Africa) into 15 mL screw-capped polycarbonate vials. All nutrient samples were immediately frozen at –20 °C and alkalinity samples were kept cold at 4 °C. The N_2 samples were taken on three sampling occasions (days one, seven and 13) during dark incubations, as bubbles may form during daylight hours which interfere with estimation of N_2:Ar and may overestimate N_2 production (Eyre *et al.*, 2002). To minimise the introduction of bubbles, N_2 samples were collected by allowing the water to flow, by gravity, from the outflow of the incubation chamber, directly into 7 mL gas-tight glass vials with glass stoppers filled to overflowing. The N_2 samples were poisoned with 20 µl of 5 % $HgCl_2$ and stored submerged at 20 °C. The N_2 samples were collected in duplicate or triplicate, thus the final values represent the mean value calculated for each replicate.

After withdrawal of all water samples, replacement water was allowed to gravity feed into the chamber directly from the manifold by opening the 4.0 mm inflow value and the cores were re-capped by replacing the bungs to maintain constant Ar concentrations. All

materials used for sample collection (seven millilitre glass vials, 15 mL screw-capped test tubes, polycarbonate syringe filter holders, 50 mL syringes and 4.0 mm tubing) were acid washed with dilute hydrochloric acid, rinsed three times with distilled water and air dried prior to use. Total oxygen exchange was measured in five randomly selected cores, one from each treatment to ensure that the oxygen concentration did not decrease by more than 20 %. Incubation times were kept short, ranging from 68 to 146 min with an average duration of 104 min, to prevent oxygen depletion and ensure that flux rates were linear (Burford and Longmore, 2001; Glud, 2008).

5.2.9 Nutrient analyses

Dissolved nitrate and nitrite (NO_x) were determined colourimetrically by flow injection analysis (QuikChem® 8500 Automated Ion Analyzer, Hach Company, U.S.A.) and a commercially available test kit (QuickChem® method 31-107-04-1-E for the determination of nitrate and nitrite in seawater). All other nutrient samples were analysed manually. Ammonium and dissolved inorganic phosphate were determined using the methods of Grasshoff (1976) and Grasshoff *et al.* (1999) respectively and nitrite (NO_2^-) was determined according to the method of Bendscheider and Robinson (1952).

5.2.10 Gas analyses

Alkalinity and total dissolved CO_2 concentrations were determined by Gran (1952) potentiometric titration according to the method of Edmond (1970) using an automated titrator system (876 Dosimat plus, Metrohm, USA). Total alkalinity was calculated according to the method of Snoeyink and Jenkins (1980). CO_2 concentrations were calculated from alkalinity and pH using the equations given in Almgren *et al.* (1983). Changes in pH and alkalinity were used to calculate dissolved inorganic carbon (DIC) fluxes.

Dinitrogen gas (N_2) was determined from N_2:Ar measured using membrane inlet mass spectrometry (MIMS) with O_2 removal. Measurement of direct N_2 fluxes using this technique represents the net benthic flux of N_2 resulting from a combination of processes that produce N_2, such as denitrification and anammox, and processes that consume N_2 such as nitrogen fixation (Ferguson and Eyre, 2007). As such, the N_2 flux is the net result of processes operating over a longer timeframe than the incubation, although the technique can respond to more rapid changes (Ferguson and Eyre, 2007).

Nutrient and gas fluxes across the sediment-water interface during light and dark incubations were calculated using initial and final concentration data according to Equation 11. Net flux rates, representing the net result of 13.57 h of dark fluxes and 10.43 h of light fluxes were calculated according to Equation 12 (Veuger *et al.*, 2007). Gross primary

production was calculated according to Equation 13, where light O_2 fluxes represent net primary production and dark fluxes represent respiration. Remineralisation ratios were calculated according to Equation 14 according to Eyre *et al.* (2013).

Equation 11 $$Flux = \frac{(C_n - C_0) \times V}{A \times t} \times 10\,000$$

where:

Flux = flux (μmol m^{-2} h^{-1})

C_0 = concentration at time zero (μmol L^{-1})

C_n = concentration at time n (μmol L^{-1})

t = incubation time (h)

A = area of sediment surface (cm^2)

V = volume of water in core (L)

Equation 12 Net flux rates = ((hourly dark rates x hours of darkness) + (hourly light rates x hours of daylight))/24 h

Equation 13 Gross benthic oxygen production = light O_2 flux − dark O_2 flux

Equation 14 $$\text{Remineralisation ratio} = \frac{\text{Dark } O_2 \text{ flux}}{N_2 + NH_4^+ + NO_x}$$

5.2.11 Sediment sectioning

On day zero and 14, three sub-cores (internal diameter 30 mm) were taken of the initial and experimental (-C and +C) chambers respectively. Each sub-core was sectioned into the following five depth intervals: 0.0 - 0.5, 0.5 - 1.0, 1.0 - 2.0, 2.0 - 4.0 and 4.0 - 6.0 cm for analysis of sediment characteristics. One set of sub-cores were dried at 50 °C for 24 h for analysis of total organic carbon and total nitrogen; the second set were frozen in sealed vials in black bags for spectrophotometric analysis of chlorophyll *a*, phaeopigments and total carbohydrates. Two sets of samples were prepared from the third sub-core: sediment samples were frozen in two millilitre Eppendorf tubes for subsequent deoxyribonucleic acid (DNA) extraction and sequencing. The remaining sediment was added to 15 mL vials filled with 0.2 μm filtered, one percent buffered paraformaldeyde and refrigerated for determination of bacterial abundance by flow cytometry.

Organic carbon (0.01 %) and total nitrogen (0.01 %) were determined on an elemental analyser after removal of carbonates by fuming (Chapter 2, Section 2.2.7). The concentration of chlorophyll *a* (0.01 μg g^{-1}) and phaeopigments (0.01 μg g^{-1}) in the sediment was determined by extracting two gram wet subsamples in five millilitres of 100 % acetone

overnight at five degrees Celsius. The extract was centrifuged at 3,000 rpm (1,200 x *g*) for 10 minutes and the supernatant analysed spectrophotometrically at 665 and 750 nm before and after adding two drops of 2 M HCl according to Lorenzen (1967). Total sediment carbohydrates (0.01 µg g^{-1}) were measured on defrosted samples (Chapter 4, Section 4.3.2).

5.2.12 Flow cytometry

Aliquots of preserved samples were prepared in duplicate by staining with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at four degrees Celsius in the dark (Marie *et al.*, 1999). Bacterial abundance was analysed with a FACSCalibur flow cytometer (BD Biosciences, Singapore), fitted with a 488 nm, 15 mW laser, using the FL1 detector (λ = 530 nm). TruCount beads (BD Biosciences, Singapore) were used as an internal standard. All cytometric data were logged and analysed using Cell Quest (Becton-Dickinson) using *Escherichia coli* cells as a reference. Cell abundance was converted to cells/gram of dry sediment.

5.2.13 Deoxyribonucleic acid extraction and importation

Genomic DNA was extracted from approximately 250 mg of substrate samples using a DNA isolation kit (ZR Soil Microbe DNA MiniPrep, Zymo Research, USA) yielding purified genomic DNA for use in polymerase chain reaction (PCR) amplification. Genomic DNA was stored in sealed, labelled Eppendorf tubes at -20 °C prior to being couriered from the Republic of South Africa to the United Kingdom. In order to comply with regulations of the Animal Health Act 1981 issued by the Department of Environment, Food and Rural Affairs, the samples were accompanied by a general import license (IMP/GEN/2008/03) for the importation of animal and poultry products, including DNA, from all non-EU countries.

5.2.14 Polymerase chain reaction amplification and sequencing of the variable region 4 of the 16S ribosomal ribonucleic acid gene

Library preparation was performed using a modified version of the MiSeq WetLab protocol (Kozich *et al.*, 2013). One microliter of template DNA was arrayed into 96-well plate format with 17 µl of Accuprime Pfx Supermix (Thermofisher, UK), leaving two wells on each plate open for controls. Two microliters of reconstituted indexed primers at 100 µM were added to the samples to barcode them for sample identification. In order to identify any contaminating operational taxonomic units (OTUs), four control samples were included in the sequencing run. The negative control consisted of one microliter of PCR grade dH_2O and the positive control was one microliter of mock community (HM-278S, BEI Resources, Manassas, USA) at a 1:3 dilution. The primer pair 515F/806R was used to amplify the V4 region of the 16S rRNA gene. Polymerase chain reaction (PCR) was performed using the

following conditions: initial enzyme activation and DNA denaturation proceeded at 95 °C for two min followed by cycling parameters of 95 °C for 20 s, 55 °C for 15 s, 72 °C for five min for 30 cycles. A final extension was done at 72 °C for ten minutes. Amplification of the PCR products was checked on a subset of 12 samples using gel electrophoresis on a one percent agarose gel prior to library quality control. Samples from all wells were pooled and libraries were subjected to quality control including quantification using a KAPA Biosystems Q-PCR kit, obtaining a bioanalyzer trace using the Agilent Technologies HS DNA kit and normalization using the Invitrogen SequalPrep Plate Normalization Kit (Thermofisher, UK). Amplicons were sequenced on an Illumina MiSeq platform by NU-OMICS (Northumbria University, UK).

5.2.15 Processing of raw sequence data

The raw fastq files were processed using Mothur (version 1.37.0) based on the Schloss MiSeq SOP (Kozich *et al.,* 2013) briefly described herein. Raw forward and reverse sequence reads were merged to create contigs prior to quality filtering. The sequence reads were trimmed using a sliding window of five base pairs (bp) with an average window quality threshold (Q) of 22 or greater. Sequences containing an ambiguous (N) base, >8 homopolymers or had a sequence length <275 bp were discarded. Quality-filtered sequences were aligned using a custom alignment created for the variable four (V4) region of the 16S rRNA gene using the latest version of the Silva database (version 123; July 2015 release). The reads were screened to include only overlapping regions (based on alignment positions), pre-clustered (number of differences = 1) and checked for chimeras using the UCHIME algorithm (Edgar *et al.,* 2011).

Since the benthic flux data indicated that nitrogen fixation appeared to be an important process, ‘Archaea’ and ‘Chloroplast’ were not specified in the remove.lineage command, since specification of ‘Chloropast’ results in the removal of cyanobacteria, which in addition to some Archaea, contain a number of genera that perform nitrogen fixation. Furthermore, the primer pair 515F/806R used is suitable for the dual amplification of bacterial and archaeal genes (Caporaso *et al.,* 2012). Taxa classified as ‘Mitochondria’, ‘Eukaryota’ or ‘unknown’ were specified during the remove.lineage command. The count.groups command was used to determine the minimum number of reads per sample for normalisation. In order to standardize sequencing effort, all samples were subsampled to 550 (the minimum number of sequences per sample) using the sub.sample command, to ensure that all replicate samples from the experimental treatments (+C and –C) were retained. The subsampled OTU table (shared file) and assigned consensus taxonomy (cons.taxonomy.file) were used in downstream analyses,

including alpha and beta diversity, taxonomic composition and metagenome predictions of the microbial communities.

5.2.16 Statistical analyses and bioinformatics

Sea cucumber growth and environmental metadata

Environmental (light, temperature and salinity) and flux rate data for nutrients (NH_4^+, NO_2^-, NO_x and PO_4^{3-}), and gases (DO, DIC and N_2 – night only) collected on day minus one during light and dark incubations were averaged to provide a mean value per replicate chamber for each diurnal period respectively. The data were tested for homogeneity of variance and for the normal distribution of the residuals using Levene (Levene, 1960) and Shapiro Wilk (Shapiro and Wilk, 1965) tests. One-way analysis of variance (ANOVA) was used to test whether there were any significant differences in the environmental, nutrient and gas flux data between the initial (I), +C and -C treatments on day minus one, prior to the start of the experiment.

The light, water quality and flux rate data for nutrients and gases described above collected during light and dark incubations conducted on alternate days (days 1-13) over the course of the experiment were averaged to provide a mean value for each replicate incubation chamber. It was not possible to conduct daytime incubations on day nine due to slight hypoxia in the cores, therefore light incubation data represent a mean of six values (days one, three, five, seven, 11 and 13), while the mean dark incubation data were calculated from the full set of seven incubations. The mean temperature, salinity; mean light, dark and net fluxes of nutrients and gas fluxes; mean remineralisation ratios and mean gross primary production measured during the experimental period (days 1-13) were analysed using a Student t-test at alpha < 0.05. Sediment characteristics, including organic carbon, total nitrogen, C:N, phaeopigment concentration and bacterial cell abundance sub-sampled from the sediments subsectioned on day 14 were compared using mixed-model ANOVA with treatment (+C and -C) and sediment depth as fixed factors. When a significant effect was observed, post hoc comparisons of means were conducted with a Tukey's honest significant difference (HSD) test. The mean wet weight of individual *H. scabra* per replicate incubation chamber was averaged and the mean value used for calculation of growth rate (g d^{-1};Equation 2, Chapter 2, Section 2.2.5) and biomass density (g m^{-2}). Differences in growth rate and biomass density on day 14 between treatments (+C and -C) were analysed by Student t-test at alpha < 0.05. Data are presented as mean ± standard error unless otherwise stated. All statistical analyses were performed in Statistica v.13.

5.2.17 Alpha diversity

Alpha (within-sample) diversity metrics for the number of OTUs (observed), richness (Chao 1), abundance-coverage estimator (ACE) and diversity (Shannon, Simpson and Inverse Simpson) were calculated and visualised in the phyloseq package in R (McMurdie and Holmes, 2013). The above diversity metrics were generated by the summary.single command by subsampling to the lowest number of reads per sample (n=550) and compared across treatments and sediment depths using mixed model ANOVA (Section 5.2.16).

5.2.18 Beta diversity

Patterns in bacterial community structure between treatments and sediment depths were visualized using principal coordinates analysis (PCoA) based on a Bray–Curtis dissimilarity matrix calculated from the OTU table in R. In addition, a non-parametric multivariate analysis of variance (PERMANOVA; Anderson, 2001) (PERMANOVA; Anderson, 2001) was performed on the community distance matrix based on a Bray–Curtis dissimilarity index to test the null hypothesis that there was no difference in the structure of microbial communities between treatments (I versus -C versus +C) and sediment depth using the 'ADONIS' function of the vegan package in R (Oksanen *et al.*, 2016).

5.2.19 Correlation with environmental and benthic flux data

Mantel correlation tests were performed on dissimilarity matrices of the community and environmental data to provide an indication of how well microbial community data corresponded to the environmental data (Oksanen *et al.*, 2016). The environmental distance matrix was calculated as Euclidean distances computed from a metadata table containing all of the data describing light, water quality, sediment characteristics and net flux rates for gases and nutrients. The significance of correlation coefficients was assessed using a permutation procedure. In addition, the correlation between environmental data and the sediment microbial communities was determined using the 'envfit' function of the 'vegan' package in R (Oksanen *et al.*, 2016). Since none of the environmental characteristics were significantly correlated with the microbial community data, the environmental data was not plotted as vectors on the PCoA ordination.

5.2.20 Prediction of the metabolic capacities of bacterial communities

The Tax4Fun package in R was used to predict the metabolic capacities of the microbial communities from the 16S rRNA sequences (Aßhauer *et al.*, 2015). This software package was selected for metagenomic functional prediction since it is compatible with OTUs classified at 97 % similarity against the SILVA databases. Furthermore, Tax4Fun is reported

to outperform similar packages such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved states (PICRUSt; Aßhauer *et al.*, 2015). Tax4Fun maps the taxonomic profile of the OTUs to metabolic reference profiles based on the Kyoto Encyclopedia for Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2012). The fctProfiling option was set to TRUE (default) in order to predict the metabolic capacities of the metagenomes based on pre-computed KEGG Ortholog reference profiles (Aßhauer *et al.*, 2015). Only KEGG Pathways within 'nitrogen metabolism' were retained for analysis. The KEGG pathway map 00910 for nitrogen metabolism and associated information was used to extract the KEGG ortholog reference numbers involved in the six full characterized reactions listed under 'nitrogen metabolism' (Table 5.2 and Figure 5.3; Kanehisa *et al.*, 2012). The process of anaerobic oxidation of ammonia (anammox) was not included as although this process is recognized in the KEGG database, it has not yet been assigned its own module or reference profiles.

Table 5.2. Overview of the pathways modules and reference profiles within nitrogen metabolism used to calculate the predicted relative abundance of genes within each pathway. All data was extracted from the Kyoto Encyclopaedia for Genes and Genomes (KEGG) database www.genome.jp/kegg/ (Kanehisa *et al.*, 2012).

Pathway	**Overview**	**Module**	**KEGG Ortholog reference profile (KO)**
Nitrogen fixation	Nitrogen => ammonia	M00175	K02588 + K02586 + K02591 - K00531
Nitrification	Ammonia => nitrite	M00528	K10944+K10945+K10946 K10535
Denitrification	Nitrate => nitrogen	M00529	(K00370+K00371+K00374+K00373, K02567+K02568) (K00368,K15864) (K04561+K02305,K15877) K00376
Dissimilatory nitrate reduction	Nitrate => ammonia	M00530	(K00370+K00371+K00374+K00373, K02567+K02568) (K00362+K00363,K03385+K15876)
Assimilatory nitrate reduction	Nitrate => ammonia	M00531	(K00367,K10534,K00372-K00360) (K00366,K17877)
Complete nitrification (comammox)	Ammonia => nitrate	M00804	K10944+K10945+K10946 K10535 K00370+K00371

The relative abundance (%) of functional genes predicted from the 16S rRNA sequences within each ortholog reference profile were summed to provide a mean value for each pathway module for each replicate sample from all sediment depths sampled in all treatments (n=45). The relative abundance of functional genes in the initial and experiment treatments was illustrated by graphically plotting vertical depth profiles and analysed statistically in the same mixed-model ANOVA design (Section 5.2.16).

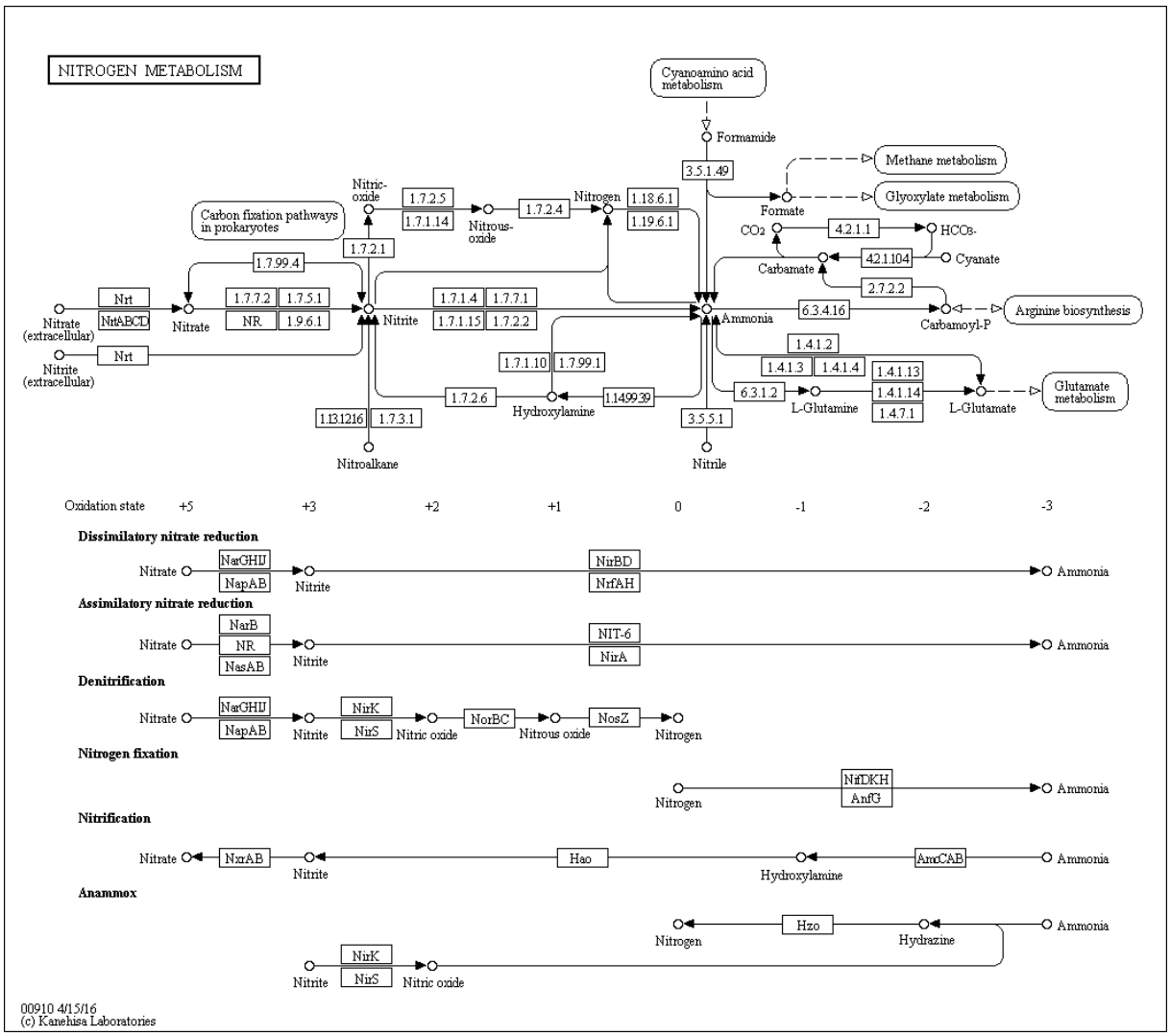

Figure 5.3. Nitrogen metabolism pathway map 00910 downloaded from the Kyoto Encyclopaedia for Genes and Genomes (KEGG) database. In the upper part of the diagram, the numbers in the boxes are Enzyme Commission (EC numbers) for enzymes and the chemical reactions they catalyse. In the lower part of the diagram, the enzyme numbers are replaced by the codes for the gene that codes for each enzyme. Arrows indicate the direction and pathway of the reactions: arrows pointing to the right indicate reduction reactions and arrows pointing to the left indicate oxidation reactions. The circles indicate the different forms of nitrogen.

5.3 Results

5.3.1 Environmental variables

Prior to the start of the experiment, on day minus one, there was no significant difference in light intensity between the initial and experimental chambers (mean 132.08 ± 9.63 lux; one-way ANOVA; $F_{(2, 9)} = 0.21$, $p = 0.82$). Throughout the experimental period (day 0 to day 14) light intensity ranged from 0 to 678.1 lux (Figure 5.4), reaching a peak of 850.4 lux on day one. During daytime incubations, there was no significant difference in light intensity between experimental treatments, with a mean of 151.60 ± 13.40 lux and 155.43 ± 13.41 lux in the +C and -C treatments respectively (Student's t-test; $t = -0.52$, $p = 0.84$).

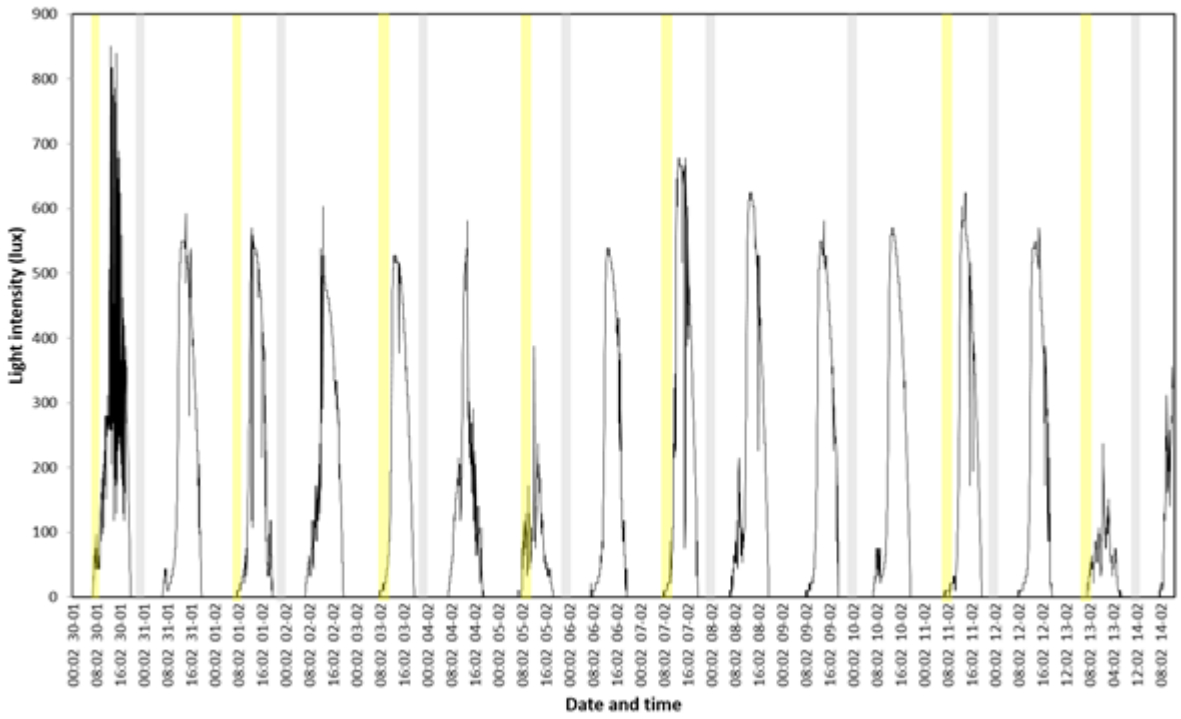

Figure 5.4. Light intensity recorded over the full duration of the experiment from day minus one on January 30th to day 14 on February 14th 2014. The time periods when light and dark incubations were conducted are illustrated by yellow and grey bars respectively.

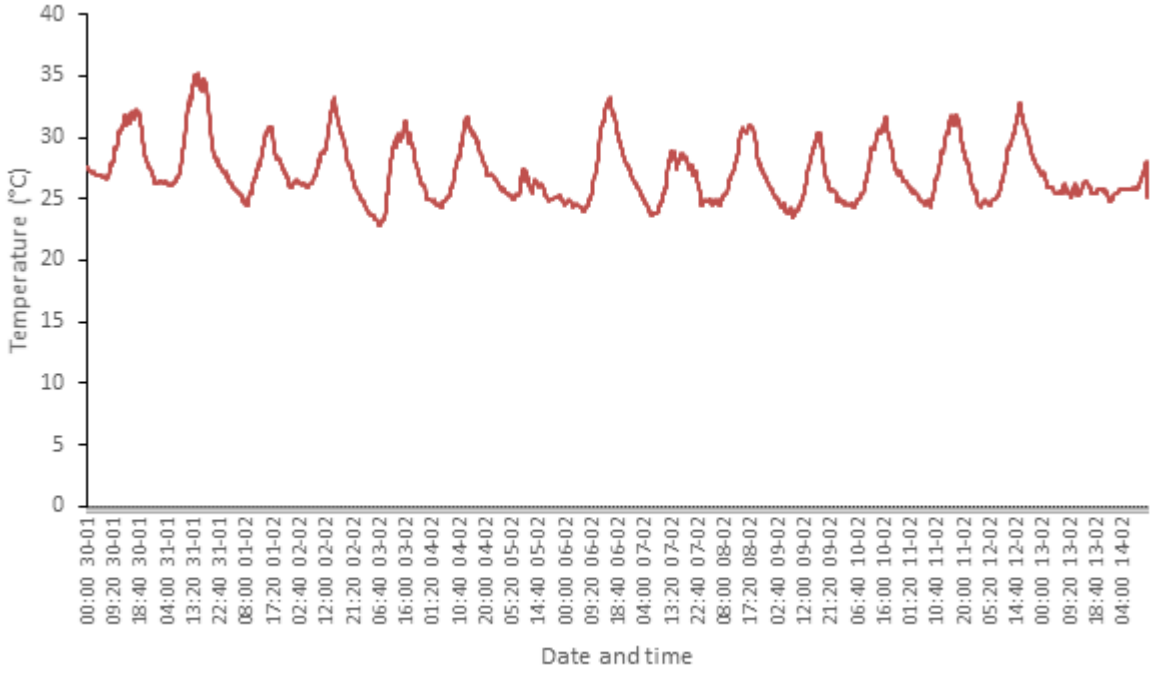

Figure 5.5. Ambient room temperature recorded over the duration of the experiment from day one on January 30th to day 14 on February 14th 2014.

On day minus one, there was no significant difference in water temperature measured at the start of the light and dark incubations between the initial and experimental chambers (one-way ANOVA; $p > 0.05$). Throughout the experiment, the ambient room temperature showed diel fluctuations, ranging from a minimum of 22.81 °C at night to a maximum of 35.22 °C during the day with a mean of 27.20 °C (Figure 5.5 and Figure 5.6b). There was considerable variation in the mean temperature between experimental treatments at the start of the light and dark incubations, but there were no significant differences between these treatments (Student's t-test; $t = -1.44$, $p = 0.15$). There was no significant difference in salinity between treatments on day minus one or between treatments during the experiment (one-way ANOVA; $p > 0.05$ and Student's test; $p > 0.05$; Figure 5.6a).

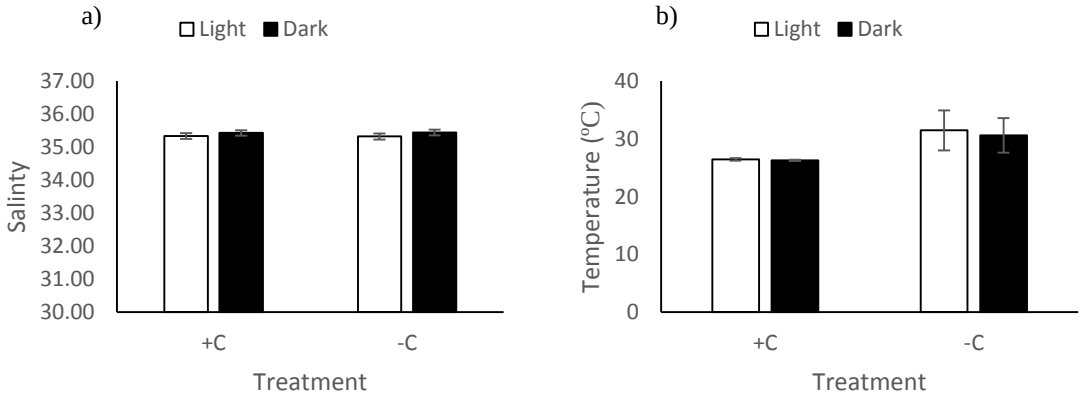

Figure 5.6. Mean (± SE) a) salinity and b) temperature of the water in experimental treatments in benthic incubation chambers containing sea cucumbers and subject to either aquaculture waste with (+C) or without (-C) the addition of carbon, incubated under light and dark conditions between day 1 and day 13.

5.3.2 **Holothuria scabra** *growth and survival*

Survival of *H. scabra* was 100 % in the treatments amended with carbon; however, one of the replicate cores from the -C treatment had to be terminated on the morning of day nine following a period of water column hypoxia during the night. This was caused by one of the sea cucumbers getting stuck in the 4.0 mm outflow value, preventing water exchange, resulting in mortality of all sea cucumbers in this chamber, and reducing overall survival to 80 %.

There was no significant difference in the mean wet weight of *H. scabra* at the start of the experiment on day zero (Student's t-test; $t = -0.52$, $p = 0.62$). There was also no significant difference in the mean wet weight of *H. scabra* when the trial was terminated on day 14 (Student's t-test; $t = -0.95$, $p = 0.37$); however, the juvenile sea cucumbers in both

treatments lost weight during the 14 d trial. The mean initial weight decreased from 1.91 ± 0.02 g to 1.62 ± 0.03 g equating to an overall mean growth rate of -0.02 ± 0.00 g day^{-1}. The biomass density decreased from the initial stocking density of 1034.00 ± 12.73 g m^{-2} to 874.97 ± 18.31 g m^{-2}.

5.3.3 Gas fluxes

There were no significant differences in the light, dark or net fluxes of DO, DIC or N_2 flux on day minus one. Sediment oxygen consumption was significantly higher during light incubations in the chambers amended with soluble starch (Student's t-test; t = -2.87, p = 0.006), which resulted in a higher net flux of -2,905.84 ± 99.95 µmol m^{-2} h^{-1} of oxygen in the +C treatment compared to -2,511.31 ± 116.81 µmol m^{-2} h^{-1} in the -C treatment (Figure 5.7a). Dissolved oxygen fluxes showed a clear influx into the sediment during light and dark incubations, indicating that respiration dominated over photosynthesis. This trend was confirmed by the lower gross primary production (Figure 5.7d). There were no significant differences in the light, dark or net fluxes of DIC with a mean net efflux of 12,732.34 ± 2,031.69 µmol m^{-2} h^{-1} in the experimental treatments (Figure 5.7b). The mean net flux of N_2 gas, calculated from measurements taken during dark incubation on days seven and 13, was not significantly different between treatments, despite there being an inverse trend between treatments (Figure 5.7c). Carbon supplementation resulted in a net influx of N_2 gas (-245.47 ± 85.94 µmol m^{-2} h^{-1}) into the sediment, indicating that atmospheric nitrogen fixation (N-fixation) dominated over pathways of permanent removal of nitrogen during dark incubations. In contrast, the -C treatment had a small but overall positive net efflux of N_2 (20.92 ± 123.85 µmol m^{-2} h^{-1}), indicating that pathways of N-fixation and removal, such as denitrification or anaerobic ammonium oxidation (anammox), were relatively well balanced, although pathways of removal dominated during dark incubations in the absence of carbon supplementation.

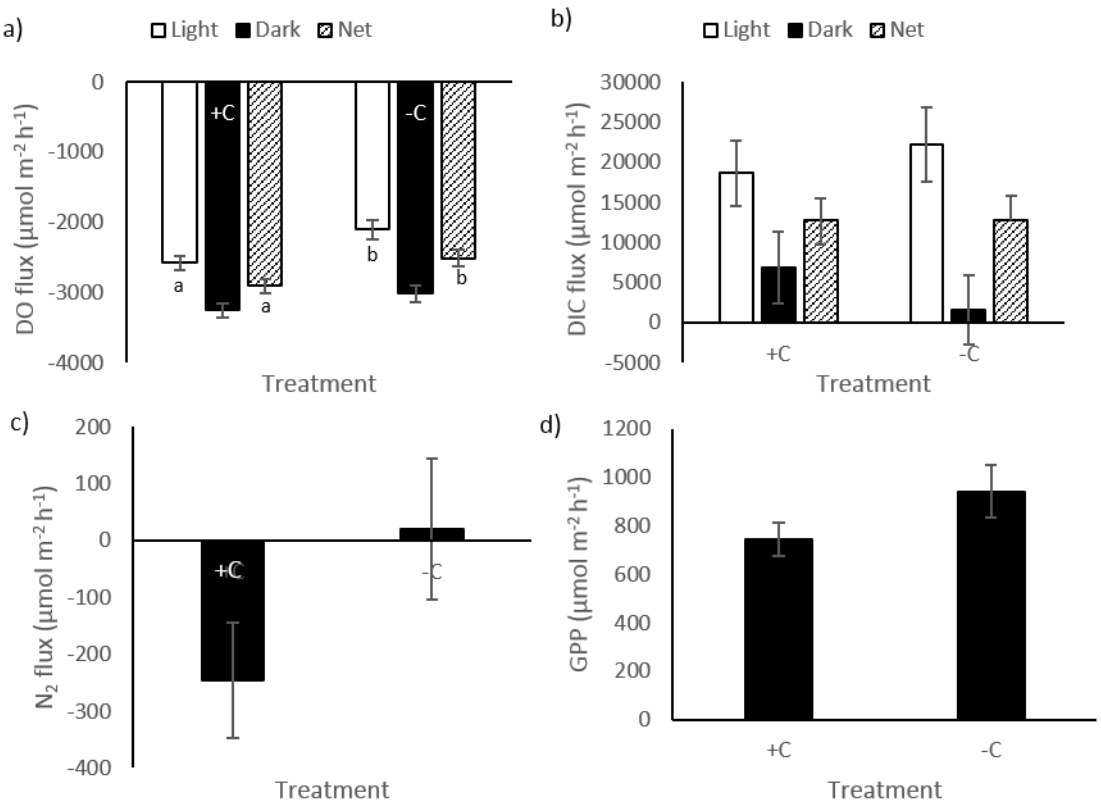

Figure 5.7. Mean (± standard error) net fluxes (in µmol m^{-2} h^{-1}; n = 5) of: a) dissolved oxygen (DO); b) dissolved inorganic carbon (DIC); c) dinitrogen gas (N_2); and, d) gross primary production (GPP) in benthic incubation chambers containing sea cucumbers and subject to either aquaculture waste with (+C) or without (-C) the addition of carbon, incubated under light and dark conditions between day 1 and day 13.

5.3.4 Nutrient fluxes

There was no significant difference in the dark or net fluxes of any of the nutrients measured on day minus one; however, the NH_4^+ fluxes measured during the light incubation were significantly different (one-way ANOVA; $F_{(2, 9)} = 12.73$, $p = 0.002$). The initial cores had a significantly higher efflux of 115.32 ± 11.43 µmol m^{-2} h^{-1} of NH_4^+ compared to the -C treatment where there was a negative flux of -9.77 ± 11.82 µmol m^{-2} h^{-1} across the sediment-water interface. The +C treatment had intermediary values with a mean efflux of 56.03 ± 25.54 µmol m^{-2} h^{-1} of NH_4^+.

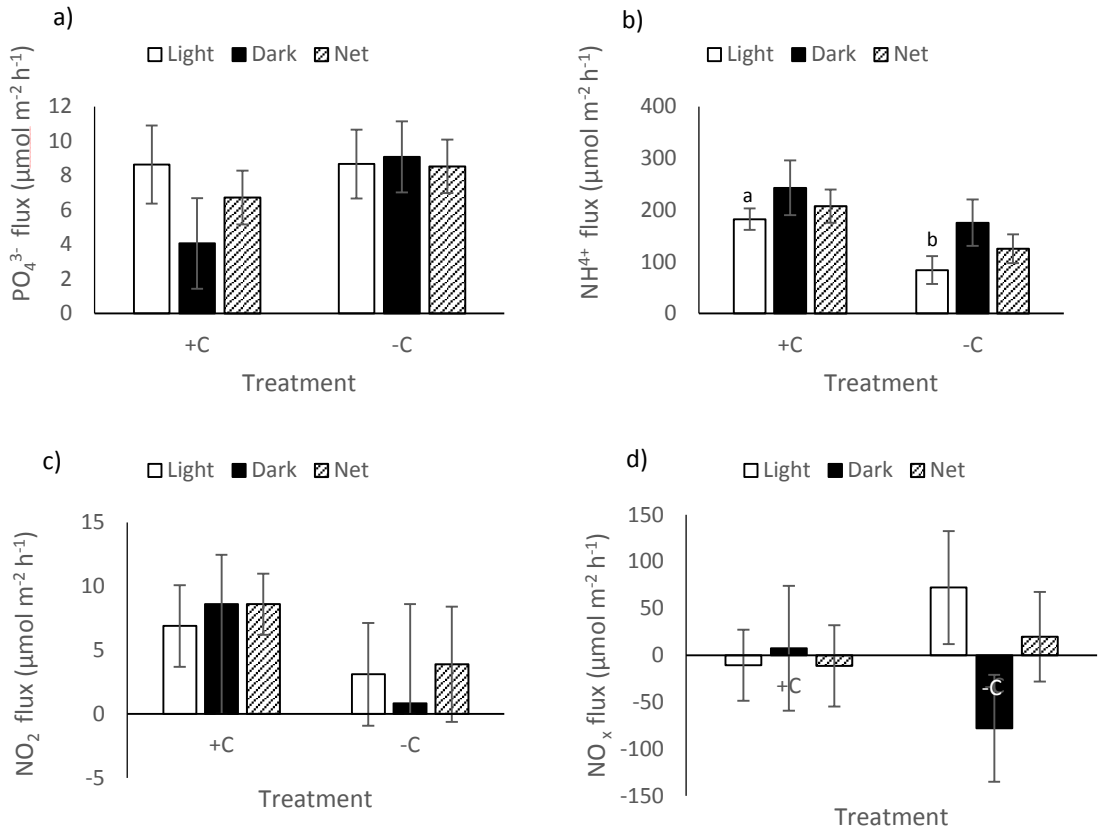

Figure 5.8. Mean (± standard error) benthic light, dark and net fluxes (µmol m^{-2} h^{-1}; n = 5) of: a) phosphate (PO_4^{3-}); b) ammonium (NH_4^+); c) nitrite (NO_2^-); and d) nitrate and nitrite (NO_x) in benthic incubation chambers containing sea cucumbers and subject to either aquaculture waste with (+C) or without (-C) the addition of carbon, incubated under light and dark conditions between day 1 and day 13.

During the experimental period (days 1-13), the nutrient with the highest flux rate was NH_4^+ with rates as high as 855.09 µmol m^{-2} h^{-1} (Figure 5.8b). Mean NH_4^+ efflux was significantly higher in the chambers amended with a carbon source during light incubations compared to the -C treatment (182.25 ± 120.77 versus 83.90 ± 26.70 µmol m^{-2} h^{-1}, t-test; t = 2.93, p = 0.005; Figure 1.7b). Sediment-water exchange of NO_2^-, NO_x and PO_4^{3-} were not affected by the addition of carbon as there were no significant differences in mean light, dark or net fluxes ($p > 0.05$). Mean fluxes of NH_4^+, NO_2^- and PO_4^{3-} were positive, irrespective of diel cycle, indicating net release from the sediment (Figure 5.8 a, b and c); however, NO_x was highly variable with inverse trends in light, dark and net fluxes between experimental treatments (Figure 5.8d).

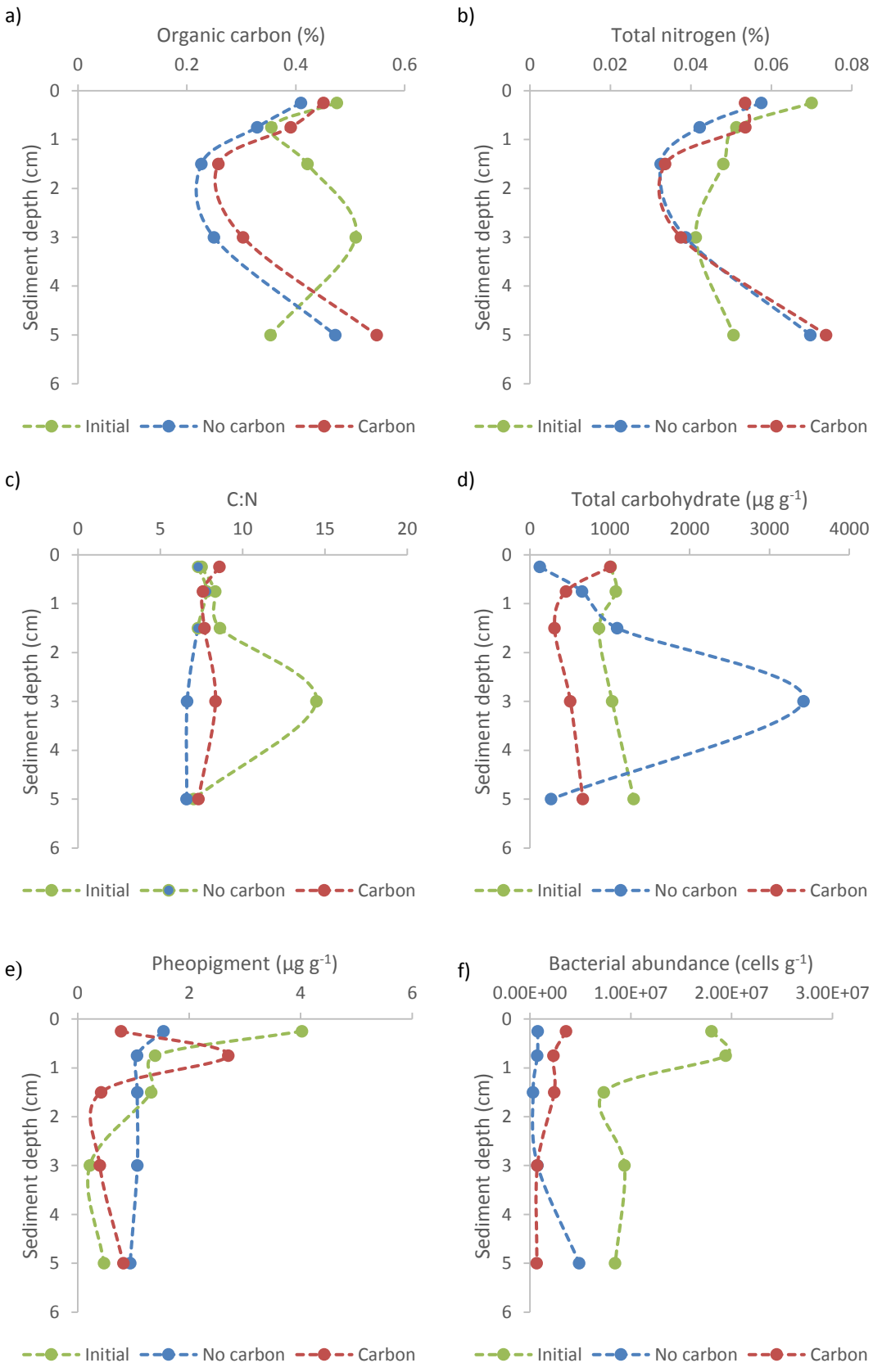

Figure 5.9. Vertical depth profiles of sediment characteristics : a) organic carbon; b) total nitrogen; c) carbon to nitrogen ratio (C:N); d) total carbohydrate; e) phaeopigment and f) bacterial abundance sectioned on day zero, prior to the addition of aquaculture waste (initial) and after the addition of this waste, both with and without carbon supplementation (carbon and no carbon, respectively) on day 14.

5.3.5 Sediment characteristics

The organic carbon content of the sediment decreased over the course of the 14 day experiment, from the levels measured in the initial cores (Figure 5.9a). The largest decrease was observed at the 1.0 – 2.0 cm and 2.0 – 4.0 cm depth intervals spanning the oxic-anoxic interface that is one of the most active zones of organic matter mineralization by heterotrophic microorganisms (Reimers *et al.*, 2013). Vertical profiles of total nitrogen content and the C:N of the sediment sampled on days one and 14 followed a similar trend with the most marked changes occurring at the 1.0 – 2.0 cm and 2.0 – 4.0 cm depth intervals respectively. Carbon addition did not have a significant effect on organic carbon or total nitrogen but sediment depth significantly influenced the organic carbon content (mixed model ANOVA, $F_{(4, 20)} = 3.54$, $p = 0.024$; Figure 1.8a) and total nitrogen (mixed model ANOVA, $F_{(4, 20)} = 3.37$, $p = 0.029$; Figure 5.9b). The organic carbon and total nitrogen content were significantly lower at the 1.0 – 2.0 cm depth interval with mean values of 0.24 ± 0.02 % and 0.03 ± 0.00 % respectively, confirming that the oxic-anoxic interface supported the highest rates of organic matter mineralisation. In contrast, the deepest sectioned depth interval (4.0 – 6.0 cm) had a significantly higher mean organic carbon (0.51 ± 0.08 %) and total nitrogen (0.07 ± 0.01 %). Carbon addition significantly increased the carbon to nitrogen ratio (C:N) of the sediment in the +C treatment (7.90 ± 0.27) compared to the -C treatment (7.12 ± 0.24; mixed model ANOVA, $F_{(1, 20)} = 4.52$, $p = 0.054$).

5.3.1 Remineralisation ratios

Carbon supplementation resulted in mean remineralisation ratios (after exclusion of outliers) of 10.12 ± 2.65 that were approximately twofold higher than chambers receiving particulate aquaculture waste only (5.20 ± 0.08), although the difference was not significant due to the variation in the data (t-test; $t = 1.11$, $p = 0.32$). In the +C treatment, remineralisation ratios were higher than the C:N of the sediment (7.90 ± 0.27), a trend that is consistent with assimilation of nitrogen by heterotrophic bacteria, including N_2 fixation (Eyre *et al.*, 2013). In the -C treatment receiving raw aquaculture waste at a C:N of 5:1, the remineralisation ratios were lower than the C:N in sediment (7.12 ± 0.24), indicating net release of nitrogen.

5.3.2 Sequencing and quality control

A total of 781,701 16S rRNA reads were generated by sequencing triplicate sediment samples from the initial (I) and experimental incubation chambers (+C and -C), sectioned at five depth intervals (n = 45). Due to a low abundance of reads, four samples from replicate c of the 'initial' treatment were removed during sub-sampling and therefore excluded from

further analysis (initial 0.0 – 0.5 cm replicate_c; initial 1.0 – 2.0 cm replicate c; initial 2.0 – 4.0 cm replicate c and initial 4.0 – 6.0 cm replicate c). Subsequent to quality control, primer trimming, size exclusion, and removal of unassigned bacteria, mitochondria and eukaryota, a total of 780,612 sequences in the 41 samples remained.

5.3.3 Microbial community — alpha diversity

Carbon addition, sediment depth or the interaction between the factors (treatment x sediment depth) did not significantly affect the number of OTUs (observed), community richness (Chao and ACE), or diversity measured as Simpson and Inverse Simpson index (mixed model ANOVA; $p < 0.05$; Figure 5.10). Sediment depth significantly influenced Shannon diversity, with the highest diversity of 2.85 recorded in the sediment surface layer (0-0.5 cm) and the lowest diversity with a Shannon index of 1.54 in the 4.0 – 6.0 cm sediment layer (mixed model ANOVA; $F_{(4, 26)} = 3.14$, $p = 0.031$).

The flow cytometry data compared well with data generated from the 16S rRNA amplicon sequencing data. Bacterial abundance (cells g^{-1}), the number of sequences and number of OTUs (observed) was higher in the initial cores than in the experimental cores sampled on day 14, presumably in response to grazing by the sea cucumbers. The number of OTUs (observed) decreased from 286.81 ± 128.13 in the initial cores to 176 ± 65.15 and 181.20 ± 45.90 in the +C and -C treatments respectively. Overall, the diversity of the microbial communities in all treatments was low: Shannon diversity had an overall mean of 2.31 ± 0.13 and Inverse Simpson was 5.79 ± 0.51. There was a marked increase in microbial community richness at the 1.0 – 2.0 cm depth interval, coinciding with the oxic-anoxic interface. In the initial cores, the number of OTUs was 778.00 ± 731.00; the number of OTUs in the experimental cores was similar with 343.33 ± 199.25 and 322.67 ± 307.25 OTUs in the +C and -C treatments respectively. The Chao 1 richness indicator also followed this trend (Figure 5.10).

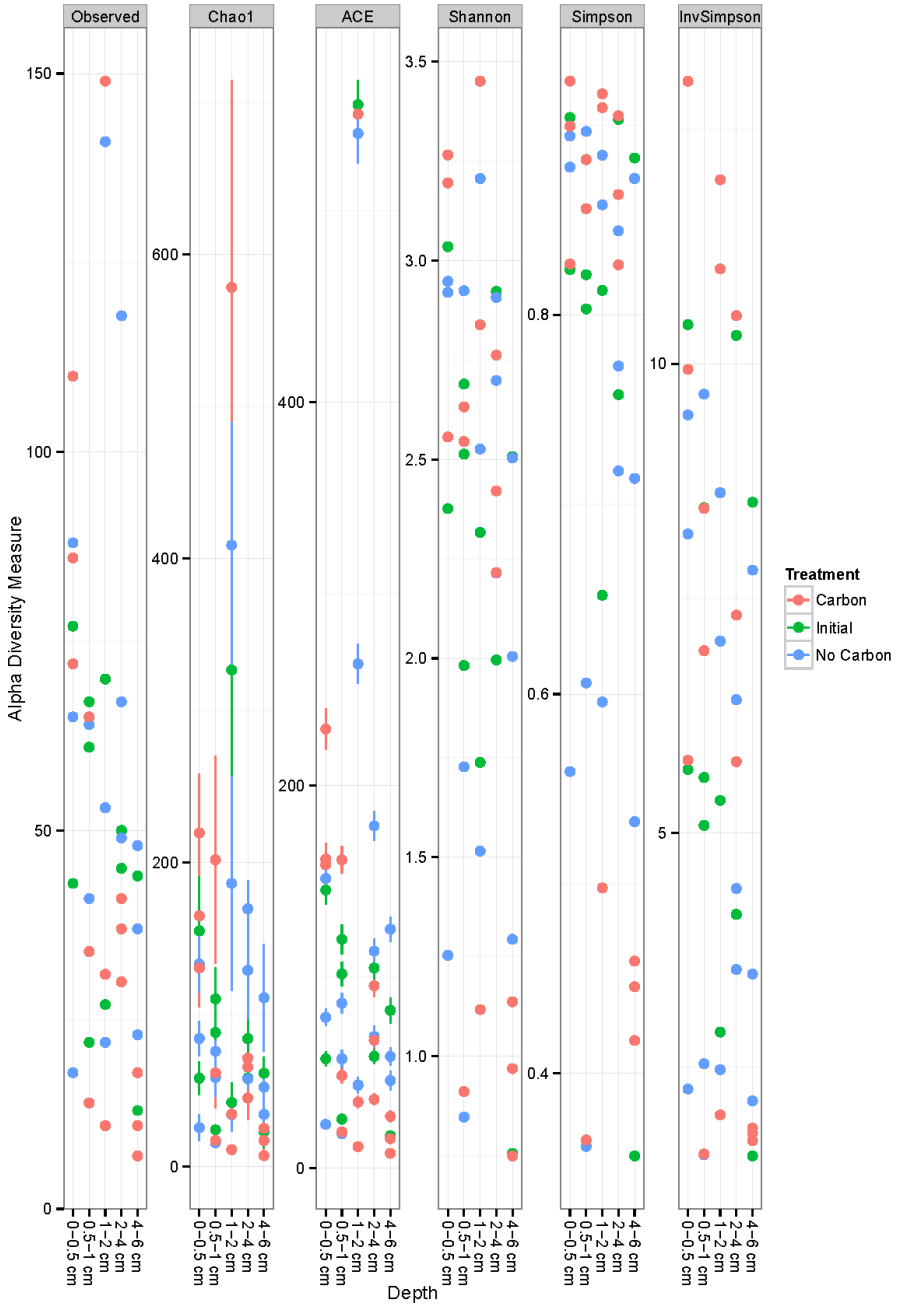

Figure 5.10. Alpha diversity metrics calculated on subsampled data. Observed = the number of operational taxonomic units (OTUs); ACE = abundance-coverage estimator; InvSimpson = Inverse Simpson diversity metric.

5.3.4 Microbial community composition — taxonomy

The majority of sequences (99.8 %) from the 41 samples taken from the initial and experimental treatments were assigned to bacteria, with only 0.12 % of the total reads assigned to archaea. Taxa from four archaeal phyla were present in the samples, including Euryarchaeota, Thaumarchaeota and Woesearchaeota or were unassigned. *Natronorubrum* (Euryarchaeota), a halophilic aerobic chemoorganotroph, was the most abundant genus representing 14 of the 27 archaeal reads (Xu *et al.*, 1999).

The bacterial community contained a total of 18 phyla and five candidate phyla. Proteobacteria and Firmicutes were the two dominant phyla accounting for 47.64 and 34.71 % of the total sequences respectively. Cyanobacteria accounted for 7.42 % of the total sequences. Planctomycetes (2.45 %), Actinobacteria (2.34 %), unclassified Bacteria (2.12 %) and Bacteroidetes (1.33 %) were minor components of the overall community composition. The remainder of the phyla, candidate phyla and the candidate division WPS_2 each represented less than 1 % of the overall community composition. Candidate phyla included Hydrogenedentes (formerly NKB19), Latesbacteria (formerly WS3), Parcubacteria (formerly OD1) and Poribacteria.

5.3.5 Taxa with statistical differences between treatments

In the -C treatment, taxa within the family *Oxalobacteraceae* and the genus *Herbaspirillum* were significantly more abundant (Welch's two-sided t-test; $p < 0.05$; Figure 5.11). In comparison, the genera *Blastopirellula* and *Litorilinea* were significantly enriched in the +C treatment. There were no significant differences in the mean proportion of taxa between experimental treatments at the phylum, class or order level, underscoring the high degree of similarity between the microbial communities in the experimental treatments.

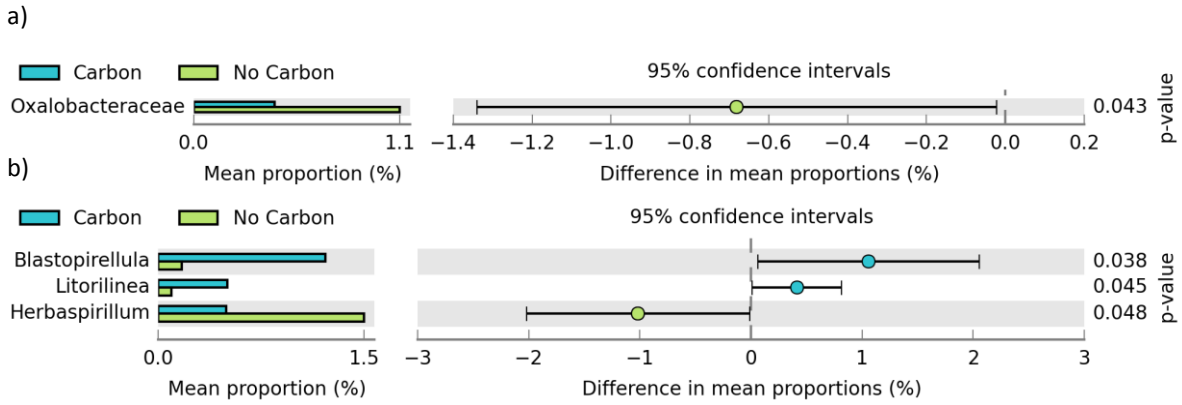

Figure 5.11. The mean proportion (%) and the difference in the mean proportion (± 95 % confidence intervals) of taxa at: a) family and b) genus level between carbon and no carbon treatments . Significant differences in mean proportions were determined using two-sided Welch's t-tests (alpha = 0.05).

5.3.6 Microbial community structure

There was a high degree of similarity between the sediment microbial communities present in the initial cores and the experimental treatments after 14 days, indicated by the lack of separation between samples in the PCoA ordination (Figure 5.12). The first axis of the PCoA that explained 53.4 % of the variation, appeared to be associated with sediment depth, while the second axis, responsible for only 4.7 % of the total variation appeared to be associated with experimental treatment.

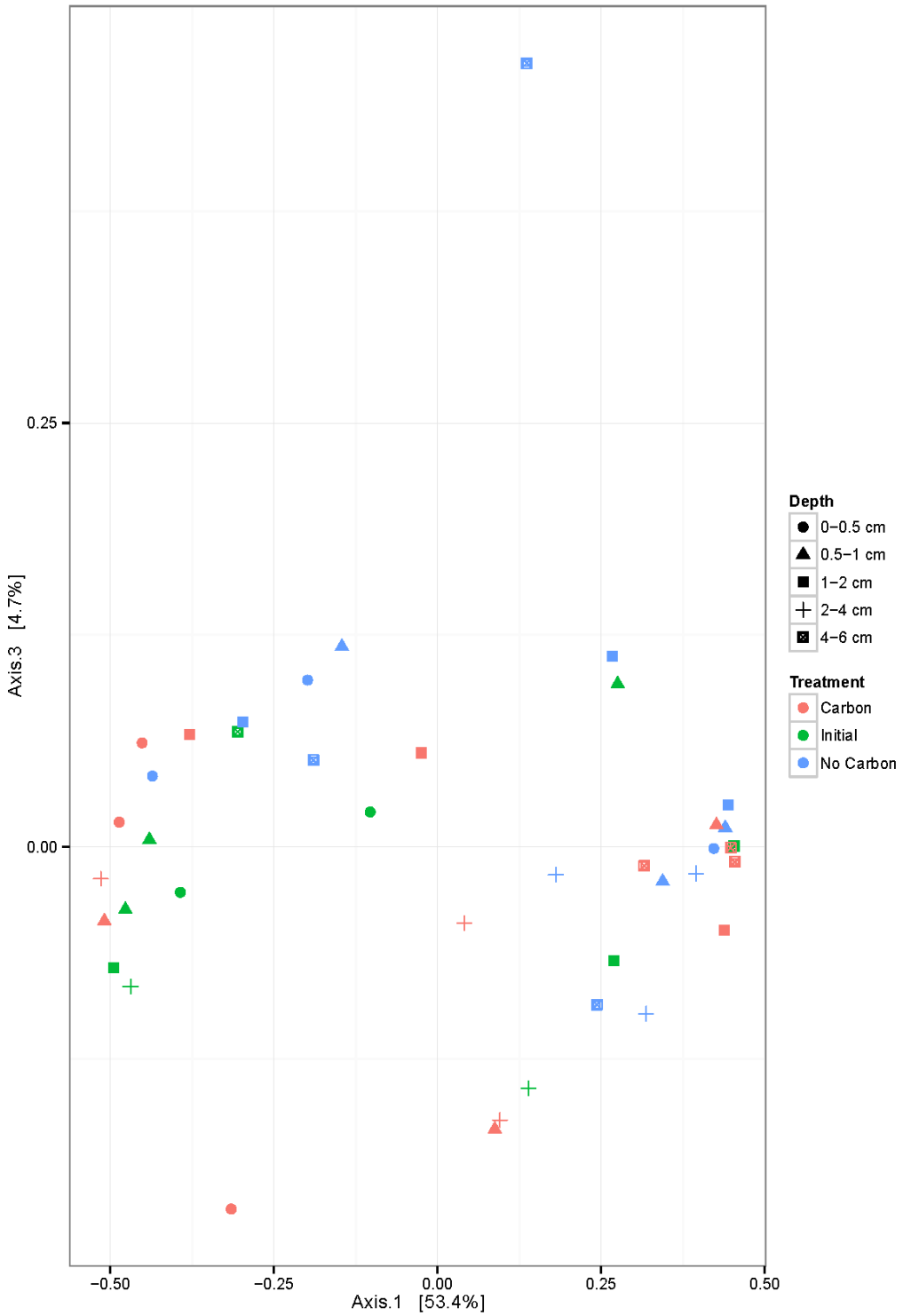

Figure 5.12. Principal component analysis of the microbial community structure between the initial, carbon and no carbon treatments at the five sediment depth intervals performed on a Bray-Curtis of community dissimilarity matrix.

Treatment (i.e. I, +C or -C) did not significantly influence microbial community structure (PERMANOVA; $p < 0.05$; Table 5.3); however, sediment depth had a significant effect on microbial community structure (PERMANOVA; $p = 0.011$; Table 5.3).

Table 5.3. Results of a non-parametric multivariate analysis of variance (PERMANOVA) testing the differences in microbial community structure at the five sediment depths prior to the addition of aquaculture waste (initial) and after the addition of this waste, both with and without carbon supplementation (carbon and no carbon, respectively).

	df	SS	Mean squares	F model	R^2	p
Treatment (T)	2	0.797	0.399	1.195	0.058	0.115
Sediment depth (D)	4	1.705	0.426	1.278	0.123	0.011
T x D	8	2.656	0.332	0.996	0.192	0.494
Residuals	26	8.672	0.334		0.627	
Total	40	13.830			1.000	

5.3.7 Correlation between microbial communities and environmental variables

There was no correlation between the microbial community and environmental data (Mantel test; r = 0.04, p = 0.27). Furthermore, the results of the env.fit procedure (Section 5.2.19) indicated that none of the environmental parameters measured, including light, temperature, sediment characteristics, net gas or nutrient fluxes, were significantly correlated with microbial community structure therefore no vectors were plotted on the PCoA ordination.

5.3.8 Functional gene prediction — nitrogen metabolism

There were no significant differences in the predicted relative abundance of genes mediating the different redox reactions involved in the six different nitrogen transformation pathways (mixed model ANOVA; p > 0.05; Figure 5.13). The relative abundance of predicted genes mediating nitrification peaked at the 0.5 – 1.0 cm depth interval in the -C treatment that coincided with the oxic zone. In the +C treatment, the relative abundance of genes mediating denitrification and dissimilatory reduction of nitrate to ammonia (DNRA) were higher in the deeper sediment layers sectioned at 1.0 – 2.0, 2.0 – 4.0 and 4.0 – 6.0 cm. Overall, DNRA was the dominant pathway (20.52 ± 0.01 %) predicted to occur in all treatments and sediment depths, with the exception of the surface layer (0.0 - 0.5 cm) in the +C treatment, where there was a higher relative abundance of genes mediating denitrification (Figure 5.13). Denitrification was the second most abundant pathway (18.02 ± 0.01 %) predicted to be present, followed by complete nitrification (8.80 ± 0.43 %), indicating that the potential for coupled nitrification-denitrification was present in all treatments. Genes predicted to be involved in N-fixation represented 2.85 ± 0.32 %.

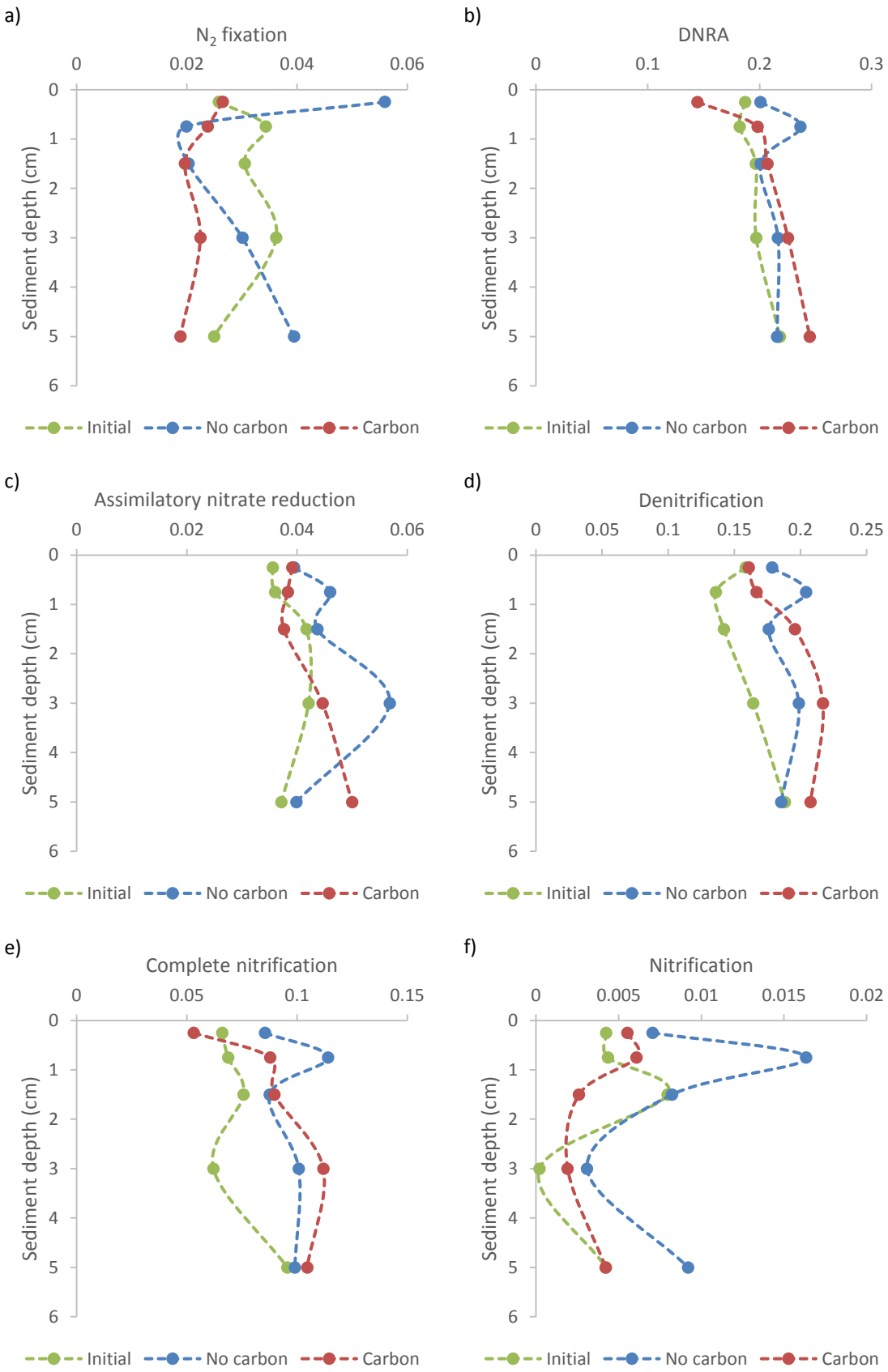

Figure 5.13. Vertical depth profiles of the predicted relative abundance (%) of genes involved in the six nitrogen transformation pathways : a) nitrogen fixation; b) dissimilatory nitrate reduction to ammonia (DNRA); c) assimilatory nitrate reduction; d) denitrification; e) complete nitrification and f) nitrification, under the pathway module of nitrogen metabolism in the KEGG database (Kanehisa *et al.*, 2012).

5.4 Discussion

Effluent originating from intensive land-based aquaculture can impact the marine benthos due to changes in the underlying sediment following the discharge of large amounts of particulate organic matter (Katz *et al.*, 2002). Impacts of organic enrichment on sediment chemistry are reported as changes in total organic matter and/or organic carbon (Nixon, 1995). In this study, comparison of vertical sediment profiles, prior to and at the termination of the experiment, indicated that additions of particulate aquaculture waste did not increase the OC content, total nitrogen and C:N in the presence of deposit feeding sea cucumbers stocked at densities of > 1 kg m^{-2}. Overall, the OC content, total nitrogen and C:N was generally lower in the sediment depth intervals sectioned from the experimental chambers after 14 days of daily additions of aquaculture waste at 400 mmol C m^{-2} day^{-1} compared to the initial cores. This finding is consistent with previous studies that concluded that sea cucumbers are efficient bioturbators that stimulate benthic metabolism and increase rates of organic matter mineralization (Mactavish *et al.*, 2012), providing further support for the integration of sea cucumbers into sediment-based aquaculture bioremediation systems.

Despite the short duration of the trial, there was an overall decrease in mean *H. scabra* biomass. The initial stocking density (1,034.00 ± 12.73 g m^{-2}) was comparable to the final density (1,011.46 ± 75.58 g m^{-2}) achieved in Chapter 4 in treatments amended with aquaculture waste and soluble starch. Differences in environmental conditions rather than feeding regime are likely to be responsible for the differences in growth rates. In this study, the source of particulate aquaculture waste, C:N, and time of feeding were the same as in Chapter 4. Furthermore, the daily waste additions were increased from 200 mmol m^{-2} to 400 mmol m^{-2} in this trial. The differences are thought to be principally linked to differing conditions of irradiation; previous studies by G. Robinson (unpublished data) indicated that light was an important factor regulating *H. scabra* growth, with up to seven-fold decreases in growth rate under lower light conditions. The importance of natural light on *H. scabra* growth has been reported elsewhere (Battaglene *et al.*, 1999). Consequently, all studies presented in this thesis were performed under the same conditions, on the top row of benches under direct light, subject only to daily and seasonal changes in light intensity, photoperiod and temperature (Figure 5.1). However, in this study it was necessary to mitigate risks associated with the high temperatures and strong irradiation that prevail during the austral summer in South Africa, that would have been elevated by the small water volume (0.817 L) and lack of aeration in the incubation chambers. The high temperatures would have increased the rate of sediment oxygen consumption, increasing the risk of hypoxia and impacting the experimental

protocol by necessitating reducing the length of incubations to remain within the recommended maximum linear decrease of 20 % in oxygen concentration (Glud, 2008). To mitigate these risks, the experimental area was shaded from direct sunlight by hanging shade cloth and whitewashing the clear polycarbonate roof panels directly above the area where the study was performed (Figure 5.1c). In this study, the light intensity ranged from 0 to 678.1 lux during the experimental period, which was almost sixty times lower than the mean light intensity of 39,860.30 lux recorded above the tanks situated on the top row of benches under direct sunlight during the trial in Chapter 4 (Figure 5.1c). The lower irradiation and lack of direct light are likely to account for the lack of growth observed in this study, compared to that presented in Chapter 4.

Benthic fluxes of dissolved oxygen and dissolved inorganic carbon (DIC) can provide an indication of the overall benthic metabolism in response to organic enrichment (Veuger *et al.*, 2007). Indicators of organic matter mineralisation, including dissolved oxygen consumption and NH_4^+ production, were significantly higher in the +C treatment, compared to -C chambers, indicating an overall increase in benthic metabolism during daylight hours. The increased rates of mineralisation were reflected in the significantly higher mean C:N ratio of the sediment in the C+ treatment, indicating the preferential mineralisation of N-rich compounds such as proteins and amino acids when the C:N ratio of particulate aquaculture waste was increased from 5:1 to 20:1 (Fenchel *et al.*, 2012). Furthermore, remineralisation ratios in the +C treatment were higher than the C:N of the sediment, indicating that heterotrophic bacteria were assimilating nitrogen (Eyre *et al.*, 2013).

In this study, fluxes of DO and DIC clearly indicated that the sediment was net heterotrophic. During the day, the DIC release from organic matter degradation exceeded DIC consumption from primary production. The net influx of DO into the sediment during light and dark incubations, the net efflux of DIC from the sediment and the lack of diurnal changes in DO fluxes indicated that organic matter degradation (respiration) dominated over photosynthesis. The low rates of photosynthesis by benthic microalgae and/cyanobacteria may have been due to reduced light levels due to shading and grazing by sea cucumbers. In addition, DIC fluxes were four-fold higher than DO fluxes, indicating that the majority of the OC was oxidised by anaerobic pathways (Burford and Longmore, 2001).

It was hypothesised that increasing the C:N ratio through carbon supplementation would mediate a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ leading to an overall decreased in the efflux of NH_4^+. In this study, remineralisation ratios indicated that carbon addition stimulated heterotrophic assimilation of nitrogen; however, net rates of NH_4^+ production were significantly higher in the treatments receiving particulate

organic waste amended with carbon to increase the C:N to 20:1. The production of NH_4^+ can originate from four different nitrogen transformation pathways studied herein, namely; ammonification (degradation of organic nitrogenous waste), nitrogen fixation, assimilatory reduction of nitrate to ammonia (ARNA) and dissimilatory reduction of nitrate to ammonia (DNRA; Figure 5.3). In this study, the increased concentration of NH_4^+ in the treatments supplemented with carbon is unlikely to have originated from ammonification since the waste was added to the chambers on an isonitrogenous basis. The assimilatory reduction of nitrate to ammonia and nitrogen fixation are both assimilatory pathways that occur internally within organisms, and therefore do not contribute to an increase in NH_4^+ concentrations at the sediment-water interface (Gardner *et al.*, 2006). DNRA is therefore the only pathway that had the potential to contribute to increased NH_4^+ production in the +C treatment. The functional prediction of metagenome of sediment microbial communities supported this observation, as DNRA was the dominant nitrogen cycling pathway with the highest relative abundance of genes present in all treatments, closely followed by genes mediating denitrification. While this suggests that DNRA was the most likely pathway contributing to the increased NH_4^+ efflux, the uncertainty associated the prediction of functional genes from 16S rRNA sequences is acknowledged. Since the predictions are based on the taxonomy of OTUs classified according to public databases, even if the predictions are 100 % accurate, this method does not provide unequivocal evidence that these pathways are occurring; it merely demonstrates that the functional potential for these pathways is present.

Nevertheless, an increasing number of recent studies are demonstrating the importance and indeed dominance of DNRA, a previously understudied nitrogen transformation pathway, in nearshore shallow water coastal environments, particularly in tropical ecosystems (Gardner *et al.*, 2006; Fernandes *et al.*, 2012; Song *et al.*, 2014; Decleyre *et al.*, 2015). For example, Fernandes *et al.* (2012) showed that DNRA can account for 99 % of nitrate removal in nitrogen-limited mangrove ecosystems. In marine sediments, DNRA and denitrification are processes that compete for nitrate; however, in comparison to denitrification that results in permanent removal of nitrogen from the system, DNRA retains bioavailable nitrogen in sediments by reducing nitrate to NH_4^+ (Gardner *et al.*, 2006). Since these two nitrogen transformation processes are reductive pathways, mediated by heterotrophic bacteria in the anaerobic zone of redox-stratified sediments, carbon addition can stimulate both denitrification and DNRA (Hardison *et al.*, 2015). In some aquaculture systems, the availability of organic carbon has been known to limit N_2 production, via denitrification; therefore carbon supplementation is employed for the successful operation of denitrifying filters (Roy *et al.*, 2010; Castine, 2013). However, in a study designed to examine the

pathways of nitrogen removal in aquaculture-impacted sediment, Castine (2013) found no significant difference in the rate of N_2 production when sediment sludge slurries were amended with particulate organic matter or methanol as the carbon source. Other studies have found that high rates of organic loading and/or the addition of exogenous carbon sources stimulated DNRA and concluded that high OC loading is a pre-requisite for DNRA to be favoured over denitrification (Capone, 2000; Hardison *et al.*, 2015). In this study, the significantly higher efflux of NH_4^+ in the +C treatment, supported by the metagenome predictions and the influx of N_2 gas, would appear to indicate that OC addition stimulated DNRA over denitrification.

Increasing the availability of OC, a potential electron donor, had the potential to stimulate the four pathways within nitrogen metabolism that involve reduction reactions, including DNRA, ARNA, denitrification and nitrogen fixation (Figure 5.3). All of these pathways, with the exception of denitrification, result in the production of ammonia and therefore contribute to nitrogen retention in the system (Hardison *et al.*, 2015). The factors regulating the balance between denitrification and nitrogen fixation are not well understood; however, the quality and quantity of OC may control the balance between these processes (Fulweiler *et al.*, 2013). Historically, denitrification has been considered to be the main pathway of nitrogen loss, based on deficiencies in mass balance calculations (Seitzinger, 1988). However, in sediment-based systems containing particulate organic waste, such as settlement ponds, the processes for permanent removal of nitrogen accounts for a very small fraction of the total nitrogen that is permanently removed from the system. For example, Castine (2013) found that denitrification and anammox were only responsible for removing 2.5 % of total nitrogen inputs to the settlement ponds in intensive shrimp and barramundi farms.

More recent research has demonstrated that sediment nitrogen fixation, a previously understudied and underestimated process in the marine environment, can equal or exceed N_2 loss in estuarine systems (Newell *et al.*, 2016a). The genetic potential for nitrogen fixation - the assimilatory reduction of N_2 - is widespread within the bacteria and archaea (Zehr and Paerl, 2008; Newell *et al.*, 2016b). Until recently, heterotrophic nitrogen fixation had not been shown to occur in sediments beyond the observation of N_2 uptake (Gardner *et al.*, 2006); however, more recent studies have provided direct evidence by measuring N_2 consumption *in situ* combined with molecular and genomic tools to quantify the presence of the nitrogenase (*nifH) gene* (Baker *et al.*, 2015; Newell *et al.*, 2016b). In this study, indirect evidence for nitrogen fixation is provided by the presence of the nitrogenase gene (*nifH*; K02588) found in all samples and the taxonomic composition of the microbial communities.

Nitrogen fixation can be mediated by photoautotrophic and heterotrophic diazotrophs; indeed, in oceanic and estuarine systems, heterotrophic diazotrophs, including Gammaproteobacteria and Group A cyanobacteria are the dominant organisms (Halm *et al.*, 2012; Bentzon-Tilia *et al.*, 2015). In this study, Cyanobacteria were the third most abundant phylum representing 7.42 % of the total microbial community. In the rhizosphere of seagrass beds, the bulk of nitrogen fixation is mediated by sulphate-reducing bacteria (Welsh *et al.*, 1996). The Deltaproteobacteria class, which contains the majority of sulphate-reducing bacteria, represented a very small proportion (< 0.5 %) of the community; however, Firmicutes were the second most abundant phylum, demonstrating the potential that taxa capable of nitrogen fixation were present.

The addition of exogenous carbon sources has been found to stimulate heterotrophic nitrogen fixation in cyanobacteria and in sulphate reducing bacteria (Welsh *et al.*, 1997; Newell *et al.*, 2016a). In this study, the overall net uptake of N_2 by the sediment in the +C treatment compared to the control receiving only waste that exhibited net N_2 production indicates that carbon supplementation enhanced nitrogen fixation. Similarly to DNRA and denitrification, rates of heterotrophic nitrogen fixation in coastal marine sediments are frequently limited by the availability of suitable OC sources (Nedwell and Azni bin Abdul Aziz, 1980). The enhancement of nitrogen fixation observed here is supported by other manipulation studies demonstrating that the addition of exogenous carbon sources, including glucose, sucrose and lactose, significantly stimulated sediment nitrogen fixation (Welsh *et al.*, 1997; Newell *et al.*, 2016a). A wide range of phototrophic cyanobacteria that fix atmospheric nitrogen are capable of mixotrophic growth; alternating between autotrophic growth with CO_2 as the carbon source, and heterotrophic growth utilising labile organic carbon sources. Similarly, the addition of OC sources, such as lactate, have been demonstrated to support high rates of nitrogen fixation in sulphate-reducing bacteria *Desulfovibro* species in the rhizosphere in seagrass beds due to tight coupling between exudates from photosynthesis fuelling nitrogen fixation (Welsh *et al.*, 1997)

Benthic incubation chambers integrate the exchange of gases and nutrients across the sediment-water interface; thus, while many reactions may be occurring within the sediments, the net outcome of sediment reactions are translated into benthic fluxes. It was anticipated that by combining this traditional approach with the application of high throughput sequencing technologies, this would elucidate the response of sediment microbial communities to carbon addition, by highlighting shifts in taxonomy and functional potential. Benthic flux incubations detected a significant enhancement of NH_4^+ production during light incubations in response to carbon supplementation; however, no significant differences in the microbial community or

predicted nitrogen transformation pathways were observed. In Chapter 3, increasing the availability of rate-limiting electron acceptors, by providing oxygen to the sediment, had a marked effect on the taxonomic composition, structure, metabolic capacity and functional potential of the sediment bacterial community. In contrast, increasing the availability of potential electron donors, by carbon supplementation, did not significantly affect the bacterial community structure. The microbial communities showed a high degree of similarity between treatments, with only significant variations at different sediment depths, likely due to the partitioning of processes within the oxic and anoxic layers respectively. Only a few taxa at family (n=1) and genus level (n=3) were significantly different between treatments in response to carbon supplementation, compared to the 16 phylum level and 86 taxonomic biomarkers that were statistically different between treatments in response to oxygen availability in Chapter 3. Furthermore, none of the environmental parameters, sediment characteristics, gas or nutrient fluxes were significantly correlated with microbial community structure and no significant differences in the relative abundance of predicted genes involved in the major nitrogen transformation pathways were observed. The benthic nitrogen cycle is one of the most complex biogeochemical cycles on earth, characterised by a diverse set of dissimilatory microbial processes (Thamdrup and Dalsgaard, 2008). The lack of significant changes in microbial community structure and functioning may be since processes that contribute NH_4^+ to the sediment were operating concurrently with transformations that removed NH_4^+ from the system, such as assimilation by heterotrophic bacteria; anammox and coupled nitrification-denitrification. Furthermore, organic carbon can fulfil many functions under reducing conditions, i.e. 1) as an electron donor in redox reactions; 2) as a substrate for fermentation or 3) as an organic substrate assimilated by heterotrophic bacteria coupled with NH_4^+ uptake; therefore the effects may have been less discernible.

Recently, there has been a paradigm shift in the understanding of nitrogen cycling in estuarine ecosystems, after research demonstrated that pathways that support retention of nitrogen in sediments dominate over pathways for permanent removal (Newell *et al.*, 2016a). In tropical ecosystems, denitrification and anammox are often outcompeted by pathways for nitrogen retention in marine sediments (Castine, 2013). The imbalance between denitrification and nitrogen fixation is partially responsible for nitrogen limitation in marine ecosystems (Fulweiler *et al.*, 2013; Newell *et al.*, 2016a). Thus, in nitrogen limited systems, such as mangrove and seagrass ecosystems that are the natural habitat of *H. scabra*, DNRA and heterotrophic nitrogen fixation are important processes for retaining nitrogen and sustaining ecosystem productivity (Fernandes *et al.*, 2012; Decleyre *et al.*, 2015; Enrich-Prast *et al.*, 2016). In shallow euphotic sediments, these processes are likely be important mechanisms for

overcoming nitrogen limitation and competition with benthic microalgae and cyanobacteria, by recycling and retaining NH_4^+ in the sediment. The increase in NH_4^+ efflux combined with net influx of N_2 into the sediment, in response to carbon addition indicates that even under nutrient loading rates consistent with eutrophic estuaries (400 mmol C m^{-2} day^{-1} and 240 mg N m^{-2} day^{-1}), pathways that retained N in the system dominated over pathways of permanent removal, underscoring the immense capacity of sediments for assimilating N from land-based intensive aquaculture systems. This finding, coupled with the economic analysis in Chapter 4, demonstrates that significant potential exists to exploit the functional potential of microbial communities in sediment-based aquaculture bioremediation systems to upcycle nitrogenous aquaculture effluents into secondary biomass valued at US$ 14.13 m^{-2}.

5.5 Conclusion

The results of this study highlight the dynamic nature of nitrogen cycling in marine sediments. It was hypothesised that increasing the C:N through carbon supplementation would influence nitrogen regeneration, by mediating a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ by heterotrophic bacteria. The remineralisation ratios indicated that assimilation of nitrogen was occurring in the sediment; however, carbon addition increased the efflux of NH_4^+ rather than decreasing it as hypothesised. Carbon supplementation appeared to stimulate pathways that contributed NH_4^+ to the system, through heterotrophic nitrogen fixation and DNRA. Furthermore, the benthic gas fluxes confirmed that redox-stratified sediments are net heterotrophic systems, dominated by anaerobic respiratory pathways.

This study is consistent with recent research that reports the co-occurrence of nitrogen fixation, DNRA and denitrification in coastal marine sediment systems (Newell *et al.*, 2016a). Together, these data provide new insights into nitrogen cycling processing in the context of nitrogen management. The coupled biogeochemical-molecular approach was useful in providing an overview of the functional potential for different nitrogen cycling pathways; however, given the complexity of nitrogen cycling in marine sediments, future studies should use more targeted molecular approaches, such as metagenomic shotgun sequencing or quantitative polymerase chain reaction (qPCR) in conjunction with stable isotope labelling studies to fully elucidate the pathways of nitrogen cycling in response to C:N manipulation.

Chapter 6. Role of the microbiome in aquaculture waste bioremediation

6.1 Introduction

Bioremediation relies on stimulating the metabolic capacity of endogenous bacterial communities to treat effluent streams. In Chapters 2-5, the addition of rate limiting electron acceptors (oxygen) or donors (carbon), to sediment-based effluent treatment systems integrating the sea cucumber *Holothuria scabra*, was examined. Next generation sequencing (NGS) was used in Chapters 3 and 5 to examine the response of the sediment bacterial communities to oxygenation and carbon addition, respectively. The circulation of oxygenated seawater through the sediment increased the taxonomic and metabolic diversity of bacteria such that the majority of the functional groups necessary for successful bioremediation of aquaculture wastes were present. In Chapter 5, no significant changes in the bacterial community structure or putative function were observed in response to carbon addition; however, the overall trend, supported by nitrogen flux data, indicated that carbon addition stimulated pathways that contribute to the retention of nitrogen in the sediment. In this chapter, NGS was used to examine changes in the structure and predicted function of endogenous bacterial communities within the culture system and gut of *H. scabra* during bioremediation of waste sediment and effluent streams from intensive land-based aquaculture.

Sea cucumbers have complex relationships with sediment microbial communities; however, little is understood about the fundamental aspects of these microbial assemblages in relation to host nutrition (Plante *et al.*, 1990; Plotieau *et al.*, 2013b). Grazing on intertidal or subtidal sediments have been shown to depress bacterial abundance (Plotieau *et al.*, 2013b) and productivity (Moriarty, 1982), while growth rate and metabolism of bacterial communities may be stimulated by bioturbation (Aller, 1994; Mactavish *et al.*, 2012). The relative importance of bacteria as a direct source of nutrition to deposit feeders has been the subject of long-standing debate (Lopez and Levinton, 1987); however, a number of studies highlight the potential for mutualistic nutritional relationships (Plante *et al.*, 1990; Harris, 1993). Nutritional interactions are defined as any interaction in which proximity allows nutrient transfer in one or both directions (Plante *et al.*, 1990; Roberts *et al.*, 2000). Potential sites of sea cucumber-microbial interactions may therefore include food prior to ingestion (ambient sediments), the oral opening and tentacles, the different sections of the digestive tract, cloacal openings, and faeces (Deming and Colwell, 1982; Plante *et al.*, 1990; Roberts *et al.*, 1991; Ward-Rainey *et al.*, 1996; Roberts *et al.*, 2000). Furthermore, bacteria may be transient or indigenous, attached or free-living, and be associated with their animal

counterparts in obligate or facultative relationships (Plante *et al.*, 1990). The open nature of the sea cucumber gut, at the oral and cloacal openings, combined with the physico-chemical similarities with the ambient sedimentary environments (e.g. pH, reduction-oxidation potential, water availability), increases the potential for causal associations with bacteria (Plante *et al.*, 1990).

The nutritional relationships between sea cucumbers and bacteria clearly warrants further research (Mactavish *et al.*, 2012). Despite the increasing accessibility of NGS over recent years, some studies of sea cucumber gut microbiomes (Plotieau *et al.*, 2013b; Bossers, 2015) have applied culture-based approaches which can produces a bias towards the cultivation of opportunistic and pathogenic bacteria. Furthermore, given the low percentage of bacteria that can currently be cultured (~ 2 %), this amounts to a substantial underrepresentation of bacterial richness (Quast *et al.*, 2013). Conversely, studies employing culture-independent techniques have demonstrated the high microbial diversity associated with deposit feeding sea cucumbers, and are beginning to elucidate the key roles of the microbiome in underpinning the growth performance of sea cucumbers (Gao *et al.*, 2014; Sha *et al.*, 2016; Yamazaki *et al.*, 2016).

Sea cucumbers are being increasingly recognised as candidate species for integrated aquaculture production and effluent treatment systems due to their ability to bioremediate sediments and convert aquaculture waste into high value secondary biomass (Watanabe *et al.*, 2012a; Robinson *et al.*, 2015). Despite the increasing number of studies demonstrating the potential benefits of integrating sea cucumbers into aquaculture systems, the mechanisms underlying their bioremediation capacity and the role that microbial communities play remains unclear. This study used Illumina amplicon sequencing to investigate how the gut microbiome of *H. scabra* altered during bioremediation of wastes from land-based aquaculture. The experiment was designed to investigate effects on the gut microbiome and long-term growth performance of *H. scabra* reared on two waste streams originating from land-based intensive aquaculture (shrimp pond sediments and particulate organic waste from a recirculating aquaculture system). An experimental approach was taken to test whether differences existed in diversity, community composition and predicted functional capacity of sediment bacterial communities subject to experimental treatments, contrasting aquaculture-impacted and un-impacted sediment and feed sources, in addition to passage through the gut. It was hypothesized that microbial communities would exhibit high turnover along the gut due to digestion in the midgut, but that communities present in the gut microbiome would play a key role in nutrient provisioning to *H. scabra*, in mutualistic relationships.

6.2 Materials and methods

6.2.1 Study site

This study was conducted at the National Aquaculture Group (NAQUA) located near Al Aleith on the Red Sea coast of the Kingdom of Saudi Arabia 20°13'41.48"N; 40° 8'41.91"E (Figure 6.1) between 11th March and 29th July 2015.

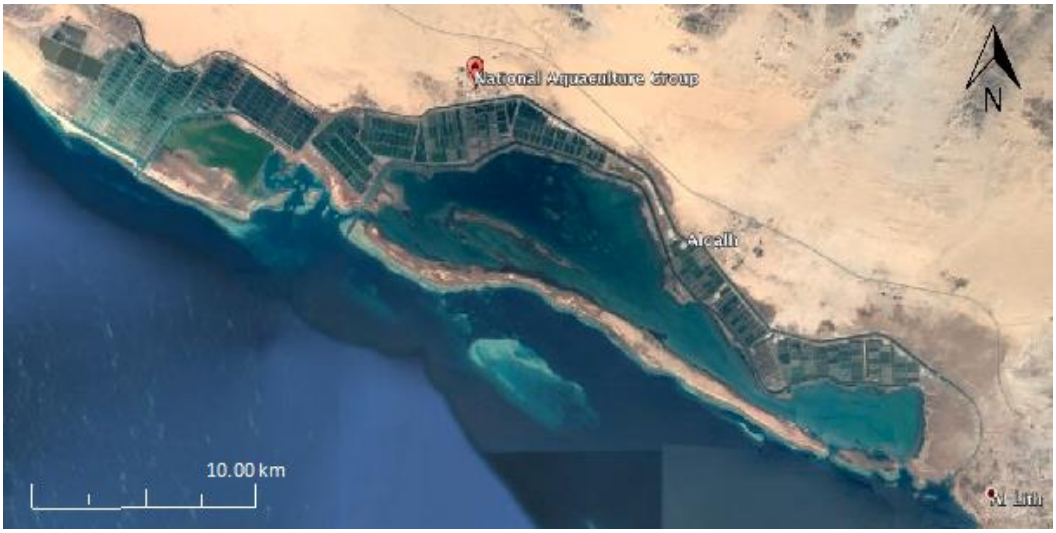

Figure 6.1. A satellite image of the National Aquaculture Group (NAQUA) – the largest vertically integrated desert aquaculture operation in the world, occupying 200 km^2 of land on the Red Sea coast of the Kingdom of Saudi Arabia. Source: Google Earth 2016.

6.2.2 Animal husbandry and acclimation

Hatchery-reared juvenile *Holothuria scabra* were produced by the sea cucumber department at NAQUA from broodstock spawned in November 2014. Prior to the experiment, the animals were held in an indoor greenhouse facility in 1000 L tanks on a flow-through system with ambient unfiltered seawater (temperature 26.0 – 31.1 ^{o}C; salinity 38 – 40 mg L^{-1}). Calcium carbonate sand that was sourced from a nearby sand dune system was added to tanks to a depth of 10 cm. During the acclimation period, the experimental animals were fed a 36 % protein commercial shrimp starter post-larvae diet (SSPL; NAQUA, Kingdom of Saudi Arabia).

6.2.3 Experimental design

A 2 × 2 factorial design was adopted to examine the growth rate, biomass carrying capacity and changes in bacterial community composition in response to:

(1) feed type (formulated feed versus particulate organic aquaculture waste from a finfish recirculating aquaculture system); and

(2) sediment type (un-impacted dune sand versus aquaculture-impacted shrimp pond sediment, Table 6.1).

Table 6.1. Description of the experimental treatments with sediment type crossed with feed type.

Sediment type	Sediment code	Diet	Diet code	Treatment code
Dune sand	DS	Formulated feed	FF	DS_FF
Dune sand	DS	Finfish waste	FW	DS_FW
Shrimp pond sediment	SPS	Formulated feed	FF	SPS_FF
Shrimp pond sediment	SPS	Finfish waste	FW	SPS_FW

Changes in the sea cucumber microbiome with passage through the gut were investigated by sampling from five different locations along the feeding chain: ambient sediment (S); foregut (FG); midgut (MG); hindgut (HG) and faeces (F) (Table 6.2 and Figure 6.2; Feral and Massin (1982); Plotieau (2012)).

Table 6.2. Definition of the five sampling locations for DNA extraction and analysis of organic carbon, total nitrogen and carbon to nitrogen ratios.

Sampling location	Definition	Sample code
Sediment	Sediment directly adjacent to the area where sea cucumbers were actively feeding	S
Foregut	Pharyngeal bulb and the anterior descending intestine	FG
Midgut	Anterior ascending intestine	MG
Hindgut	Posterior descending intestine and the cloaca	HG
Faeces	Faecal mounds in close proximity to actively feeding sea cucumbers	F

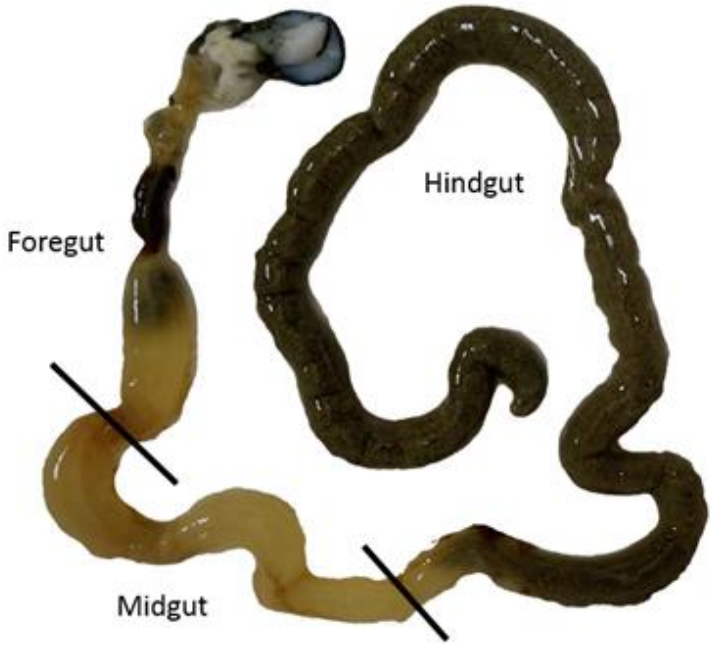

Figure 6.2. The digestive tract of *Holothuria scabra* from the mouth to the anus, divided into the foregut (oesophagus and pharynx), midgut (anterior ascending intestine) and hindgut (Feral and Massin, 1982; Plotieau, 2012). Photo credit Claro Melicano.

6.2.4 Experimental sediments

The two sediment types tested comprised: 1) shrimp pond sediment (SPS) that was impacted by aquaculture; and, 2) dune sand (DS) as a sediment un-impacted by aquaculture as a control. Shrimp pond sediment was collected from a 10-hectare *Litopenaeus vannamei* production pond at the end of a four-month shrimp farming cycle. The sediment was collected after harvesting the shrimp and after the ponds where prepared for the following cycle, which included drying, liming and ploughing (Figure 6.3). The dune sand was collected from a sand dune system adjacent to the farm. No additional preparation steps (i.e. washing, sieving) were undertaken prior to adding the sediments to the experimental tanks. The sediment depth in each tank was 10 cm.

Figure 6.3. a) A 10-hectare shrimp pond after harvesting, b) exhibiting heavily reduced anoxic sediment indicated by the black colour.

6.2.5 Experimental diets and aquaculture waste collection

A commercial formulated shrimp starter feed (FF) for *Litopenaeus vannamei* production (shrimp starter post-larvae SSPL, NAQUA Pty Ltd, Kingdom of Saudi Arabia) was used as a control diet as it is also commonly used to rear *H. scabra* (Pitt *et al.*, 2004; Pitt and Duy, 2005; Watanabe *et al.*, 2012b). The FF diet is a fine grade feed with an average pellet size of 600-800 μm, containing fish meal, soya meal and barley. The fish waste (FW) was particulate organic waste comprising a mixture of uneaten fish feed and/or barramundi (*Lates calcarifer*) faecal waste recovered from the drum filter effluent from a recirculating aquaculture system (RAS) for nursery phase production. The filtration system for the finfish RAS comprised a series of drum filters as a first stage solids removal, a protein skimmer, biological filter and ozonation with a maximum filtration capacity of 200 m^3 h^{-1}, treating water from 20 tanks (65 m^3) with a total flow of 4,650 m^3 h^{-1}. The waste was recovered from the effluent of a series of three drum filters (Hydrotech, Sweden) with a 60 μm mesh (Figure

6.4) via a submersible pump situated in the sump of the waste outlet. The drum filter effluent was pumped into a 1000 L custom-made waste collection tank. The waste was recovered daily by skimming the particulate suspended solids from the surface of the tank, placing it in a 40 μm sieve and refrigerating prior to use (Figure 6.4).

Figure 6.4. The barramundi recirculating aquaculture system including: a) the series of Hydrotech drum filters used to separate the particulate suspended solids (< 60 μm); b) the submersible pump system used to pump particulate waste from drum filter effluent channel to the collection tank; c) the custom made waste collection tank; and d) the concentrated aquaculture waste after collection in a 40 μm screen.

During the experiment the average biomass of fish in the system was 30,842 kg (maximum capacity of 35,000 kg) and the average feeding rate was 1,097 kg d^{-1} (maximum capacity of 1,300 kg d^{-1}). The proximate composition and respective quantities of the different finfish formulated feeds that were fed during the experimental period are detailed in Table 6.3.

The proximate composition of the fish waste and formulated feed were analysed on a wet weight and dry weight basis (Table 6.4). The feed rations were standardised between treatments on an isonitrogenous basis and a conversion factor (wet to dry) of 5.32 was used to calculate the quantity of fish waste to feed daily.

Table 6.3. Proximate composition of the commercial finfish diets fed during the nursery phase production of *Lates calcarifer* during the experimental period (Arascao, Riyah, Kingdom of Saudi Arabia and Skretting, Mugla, Turkey). The 'Gemma Diamond' range are post-weaning diets for juvenile marine fish; 'Protec RC' are specific recirculation hatchery diets that improve fish health. The numbers refer to the diameter (mm) of the feed pellets: 1.5 = 1.5 mm; 1.8 = 1.8 mm; 1P = 2mm and 2P = 4 mm.

	Arasco			Skretting					
	2 mm	3 mm	4 mm	Gemma Diamond 1.5	Gemma Diamond 1.8	Protec RC- 2P	Protec RC- 1P	Protec RC-2PS	Overall mean
Protein (%)	53.55	52.20	49.60	57.80	57.80	50.93	51.30	51.20	51.02
Lipid (%)	10.75	10.75	11.00	13.60	13.60	13.57	12.90	13.50	12.01
Carbohydrate (%)	17.20	16.00	-	8.00	8.00	-	-	-	2.98
Fibre (%)	1.30	1.10	-	0.60	0.60	-	-	-	0.21
Calcium (%)	2.20	2.50	-	2.20	2.20	-	-	-	0.51
Phosphorus (%)	1.90	2.30	-	1.70	1.70	-	-	-	0.45
Ash (%)	12.15	14.25	16.00	13.00	13.00	7.60	7.20	7.30	12.15
Moisture (%)	5.35	5.80	7.60	7.60	7.60	8.07	8.00	8.40	7.55
Total fed (kg)	968.00	23,889.00	60,251.00	118.00	6,661.00	14,210.00	13,908.00	31,884	151,890.00
% of total fed	0.64	15.73	39.67	0.08	4.39	9.36	9.16	20.99	100.00

Table 6.4. Proximate composition of the experimental diets analysed on a wet and dry weight basis and the conversion factor used to adjust the quantity of fish waste fed.

	Fish waste	Formulated feed
Wet weight (g)	8.20	7.86
Dry weight (g)	1.54	7.18
Conversion factor	5.32	N/A
Moisture (%)	81.30	8.60
Crude Protein (%)	43.40	43.20
Total Lipids (%)	9.10	11.20
Organic Matter (%)	67.10	87.60
Organic Carbon (%)	39.00	50.90
Nitrogen (%)	6.95	6.89
Carbon to nitrogen ratio	5.61	7.38

6.2.6 Experimental system and rearing conditions

The four experimental treatments (Table 6.1) were randomly allocated to 12 polyethylene harvest bins (1160 × 960 × 750 mm L x W x H internal dimensions) situated outside in direct sunlight (Figure 6.5). Experimental tanks were subject to a natural photoperiod which increased from 11.55:13:05 L:D (06:36 hours to 18:31 hours, sunrise to sunset) to 13:09:11.51 L:D (05:55 hours to 19:05 hours, sunrise to sunset) as day length increased during the summer season. The water height was set to 0.55 m above the substrate to give a tank water volume of 612.5 L. The tanks were supplied with unfiltered seawater at ambient temperature (24.05 ± 0.56 °C) from a header tank at a flow rate of 1.25 L min^{-1} $tank^{-1}$, equating to a 294 % exchange rate per day. No supplemental aeration was supplied to the tanks. Prior to the start of the experiment, the tanks were left to condition with flow-through unfiltered seawater for 11 days.

Figure 6.5. Experimental tanks situated outside under ambient environmental conditions.

Sea cucumbers were fed their respective treatment diets once per day at 16:00 h. Feed rations and feeding practises were standardised across treatments. Daily rations were calculated at two percent of the total tank biomass for both diets, with the wet-to-dry conversion (5.32) factor applied to calculate the quantity of fish waste. The feed rations were re-calculated and adjusted every 14 days based on the net biomass change per tank during that period.

6.2.7 Growth of Holothuria scabra

At the start of the experiment, animals were grouped into size classes to facilitate assignment of animals to experimental treatments and minimise size variation between treatments. No attempt was made to gut evacuate animals prior to the weight assessments, as the risk of safely maintaining animals in suspended mesh containers in outdoor tanks subject to high temperatures and strong irradiation for 24 h was considered too high. Prior to weighing, animals were drained on a damp cloth for one minute and weighed to the nearest 0.1 g. Animals with a mean (±SE) weight of 50.12 ± 0.07 g were allocated randomly to 12 groups of three individuals per group. The mean initial stocking density across all the experimental tanks was 135.03 ± 0.20 g m^{-2}. Animals were reweighed every 14 d over the 140 d experimental period. Wet weight data were used to calculated growth rate and coefficient of variation (CV) using Equation 2 and Equation 3 respectively (Chapter 2, Section 2.2.5). On day 140, the length and breadth of each animal was measured to the nearest millimetre using a ruler. The wet weight, body length and body breadth were used to calculate body volume (BV) as a spheroid and Fulton's condition factor (K) according to Equations Equation 15 and Equation 16, respectively:

Equation 15 $BV = 4/3 \times \pi \times BL \times (BB)_2$

Equation 16 $K = W_2 / BV \times 10^4$

where BL is the final body length (cm); BB is final body breadth (cm); BV is body volume (cm^2); and W_2 is the final weight wet (g) (Watanabe *et al.*, 2012b).

6.2.8 Water and sediment quality monitoring

Water and sediment quality parameters were monitored every two to three days on alternating replicate tanks from each treatment over the experimental period (total number of observations = 115). Water quality parameters were measured in the mid-water column adjacent to the outflow during mid-morning. The saturation (± 0.01 %) and concentration (± 0.01 mg L^{-1}) of dissolved oxygen was measured electronically in conjunction with temperature (± 0.01 ºC) using a portable dissolved oxygen meter (Handy Polaris 2,

OxyGuard®). pH (± 0.01 pH units) was measured using a portable pH meter (Handy pH, OxyGuard®) and salinity (± 0.01 mg L^{-1}) was measured using a handheld refractometer (Brix Refractometer 2313, Master Series, Atago). Flow rates were calculated using a stopwatch to measure the time (to the nearest second) taken to fill a beaker with 500 mL of water from the tank inflow. Semi-quantitative observations of the sediment surface were made daily by visual estimation to record the main type of and percentage coverage of primary producers colonising the sediment surface. These were classified as benthic diatoms, cyanobacteria, non-filamentous algae and filamentous algae and the percentage coverage of white bacterial mats (e.g. *Beggiatoa*) that had developed on patches of uneaten and decaying feed. If the coverage exceeded five percent of the total sediment surface area the patches were removed by siphoning; however, the feed rations were sufficiently low (< 4 % of total tank biomass per day) to prevent the development of bacterial mats.

6.2.9 Analysis of water and sediment quality parameters

At the end of the trial, water and sediment samples were collected for analysis at the NAQUA Central Analytical Services laboratory. Water samples were collected from the centre of each tank at approximately five centimetres above the sediment-water interface using a 50 mL syringe (10 samples per tank). They were filtered to 1.2 μm using glass microfibre filters (Grade GF/C, Whatman®) into screw cap polycarbonate bottles, refrigerated, and analysed within five days. Standard methods for the examination of water and wastewater were used for the analysis of all water quality parameters (American Public Health *et al.*, 1998). Salinity (0.01 mg L^{-1}) was measured using a benchtop conductivity meter (Orion 145A+, Thermo Scientific; APHA method 2520) and alkalinity (0.01 mg L^{-1}) was measured by end-point titration (APHA method 2320 B). Inorganic nutrients were measured spectrophotometrically according to the following standard methods (0.01 mg L^{-1}): total ammonia-nitrogen (APHA 4500–NH_3), nitrite (APHA 4500–NO_2), nitrate (APHA 4500–NO_3), orthophosphate (APHA 4500–PO_4^{3-}), sulfide (APHA 4500–S), and sulphate (APHA 4500–SO_4^{2-}) (Hach Company, 1989).

At the end of the trial, sediment cores were taken from each tank using a 25 × 2.5 cm (length × diameter) polyvinyl chloride core. The sediment reduction-oxidation (redox) potential (0.01 mV) was measured in millivolts by inserting an oxidation-reduction potential (ORP) probe (Orion 4Star ORP meter) into the base of the core and recording the readings following stabilisation. Sediment samples were collected from the upper two to three millimetres of three replicate tanks and dried to a constant weight at 60 °C for 48 h. Samples were analysed for organic carbon content (0.01 %) using the Walkley-Black method (Boyd

and Tucker, 1992) and total nitrogen (0.01 %) was measured using the Kjeldahl method (AOAC Method 955.04; AOAC (2010).

6.2.10 Sea cucumber dissection, deoxyribonucleic acid extraction, and gut content analysis

Animals (n = 3 per tank) were sampled for measurement of morphometric parameters, dissection and removal of gut contents for DNA extraction and chemical analysis at 15:00 on day 140 at the beginning of their diurnal feeding cycle to ensure that the sea cucumbers were actively feeding. The sea cucumbers from each replicate tank were immediately placed inside plastic bags on ice in insulated containers to slow their metabolism and activity of the digestive enzymes. The top two to three millimetres of ambient sediment (S) where the animals were actively feeding and faeces from recently produced faecal mounds (F) were sampled at the same time as sea cucumbers were removed from the tanks. The sediment and faecal samples were stored on ice in labeled and sealed sterile petri dishes prior to refrigeration and analysis. In the laboratory, after the collection of wet weight and morphometric data, the sea cucumbers were surface sterilized with 70 % ethanol and dissected under sterile conditions. Samples of the foregut, midgut and hindgut (Section 1.2.3; Feral and Massin, 1982; Plotieau, 2012) were removed from the individual sea cucumbers and were placed into sterile, sealed and labeled petri dishes and stored overnight in the refrigerator prior to removal of the gut contents for DNA extraction and analysis of organic carbon and total nitrogen.

In the laboratory, the gut contents were separated from the gut tissue and removed from each compartment. Composite samples were created for each gut compartment by homogenising the gut contents of the three individual sea cucumbers sampled from each replicate tank. Genomic DNA was extracted from approximately 250 mg of the composite samples (Section 6.2.13). The remaining material from each composite samples was analysed for organic carbon and total nitrogen content (Section 6.2.9).

6.2.11 Proximate composition analysis of the sea cucumber body wall

After dissection and removal of the digestive tract, the body wall of the three individual sea cucumber from each replicate tank were pooled to create one composite sample per tank and frozen at -20 °C. The composite samples were ground to a fine powder (~50 µm) with a grinder, and the proximate composition was analysed according to the Association of Official Analytical Chemists' (AOAC) official methods (AOAC, 2010). Moisture (0.01 %) was determined by weight loss after drying at 95 °C for 72 h (AOAC Method 930.15), while ash (0.01 %) was determined by weight loss on combustion after ashing in a furnace for 4 h at 550 °C (AOAC Method 942.05). Crude protein (0.01 %) was analysed using the Kjeldahl

method (AOAC Method 976.05). Crude lipid (0.01 %) was analysed using a Soxhlet extractor (AOAC Method 920.39).

6.2.12 Statistical analyses of water, sediment, growth, and proximate composition data

The mean wet weight (g) of sea cucumbers per replicate tank was tested for normality using Shapiro-Wilk's test and homogeneity of variance using Levene's test. Treatment means were compared using repeated measures analysis of variance (repeated measures ANOVA). Mean values of data that were collected on day 140, including sea cucumber morphometric parameters, biomass density (g m^{-2}), proximate composition, water and sediment quality, were analysed to ensure they met the test assumptions of a parametic test and treatment means were compared using a two-way ANOVA. Tukey's honest significant difference (HSD) post hoc tests were used to compare differences among means of dependent variables. Results were expressed as mean ± standard error and differences were considered significant at $p < 0.05$. Statistical analyses were performed using Statistica version 13.

6.2.13 Deoxyribonucleic acid extraction and importation

Extraction of genomic deoxyribonucleic acid (DNA) from sediment, faecal and gut content samples was carried out on 250 mg of sample using the PowerSoil® DNA Isolation Kit (MoBio, CA, USA) following manufacturer's instructions. Genomic DNA was stored in sealed, labeled Eppendorf tubes at -20 °C prior to being couriered from the Kingdom of Saudi Arabia to the UK. The samples were accompanied by a general import license (IMP/GEN/2008/03; Chapter 5, Section 5.2.11).

6.2.14 Polymerase chain reaction amplification and sequencing of the variable region 4 of the 16S ribosomal ribonucleic acid gene

Library preparation was performed using a modified version of the MiSeq WetLab protocol (Kozich *et al.*, 2013) and amplicons were sequenced on an Illumina MiSeq platform by NU-OMICS (Northumbria University, UK; Chapter 5, Section 5.2.14).

6.2.15 Processing of raw sequence data

The raw fastq files were processed using Mothur (version 1.37.0) based on the Schloss MiSeq SOP (Chapter 5, Section 5.2.15) with the following variations. The positive and negative controls were examined for the presence of contaminating operational taxonomic units (OTUs). The genus *Escherichia* assigned to OTU 004 was removed using the command remove.otulabels. In addition, taxons classified as 'chloroplast', 'mitochondria', 'unknown', 'archea' and 'eukaryota' were removed using the remove.lineage command. The count.groups command was used to determine the minimum number of reads per sample for normalisation.

In order to standardize sequencing effort, all samples were subsampled to 1,606 (the minimum number of sequences per sample) using the sub.sample command, which ensured that all replicate samples were retained. The subsampled OTU table (shared file) and assigned taxonomy (cons.taxonomy.file) were merged using the make.biom command and used for all downstream statistical analyses.

6.2.16 Calculation of guanine and cytosine content of bacterial communities

To determine whether the experimental factors of sediment and feed type had an impact on the percentage of nitrogenous bases in the sediment samples, the guanine-cytosine (GC) content of each of the fastq files was analysed using FASTQC software (FastQC, Babraham Bioinformatics, UK). The mean GC content of the sediment samples in the experimental tanks were analysed using a two-way ANOVA and Tukey HSD post-hoc tests to identify significant differences.

6.2.17 Alpha diversity and rarefaction curves

To observe the sampling efficiency of each sample, rarefaction curves were produced from the raw sequence data generated using the rarefaction.single command in Mothur. Alpha (within-sample) diversity metrics for coverage, species richness, diversity and evenness were generated by the summary.single command by subsampling to the lowest number of reads per sample (n = 1,606). The number of sequences that were sampled for an OTU definition and Good's coverage were computed (Kozich *et al.*, 2013). Richness estimators included the total number of OTUs observed in a sample (S_{obs}) and Chao1 (Chao, 1984). Two different diversity indices were calculated, including Shannon diversity and Inverse Simpson. Alpha diversity metrics were compared across treatments using permutational analysis of variance (PERMANOVA) with sediment, diet and sampling location as fixed factors and tank as a random factor nested within sediment and diet. Statistical analyses were performed using PRIMER-E version 7 (Clarke and Gorley, 2015).

6.2.18 Beta diversity

Beta diversity at the OTU level was performed using correspondence analysis (CA) in the vegan package (Oksanen *et al.*, 2016) in RStudio to determine whether bacterial community structure was influenced by the experimental factors (sediment and feed type) and sampling location (sediment, foregut, midgut, hindgut and faeces). Correspondence analysis is a multivariate technique that is particularly suited to situations where species or OTUs display unimodal relationships in response to an environmental gradient (Ramette, 2007), such as the hypothesised high turnover of OTUs along the gut. Prior to computing all beta diversity

analyses, OTUs with five or less observations were removed from the dataset, which reduced the total number of OTUs in all 60 samples from 7,519 to 1,184. A Hellinger transformation was applied to the OTU table to ensure the community data was suitable for analysis by linear methods (Ramette, 2007). The transformation calculates the square root of the relative abundance and thus gives weight to variables with low counts and many zeros, which is common in microbial ecological data (Legendre and Legendre, 1998).

6.2.19 Taxonomic composition of bacterial communities

Changes in the relative abundance of bacterial taxa in response the experimental factors of sediment and diet type were examined at the phylum level. As Proteobacteria was the dominant phyla, the subclasses within the Proteobacteria were also analysed.

6.2.20 Characterisation of the **Holothuria scabra** *microbiome*

To provide a comprehensive overview of the microbiota associated with the ambient sediments, gut compartments and faecal mounds, the top 100 OTUs present in each of the five sampling locations were identified, resulting in a total of 365 OTUs. The number of reads for each OTU was converted to relative abundance and after assigning the taxonomy, to genus level where possible, the list was filtered and subtotalled to combine OTUs with the same taxonomic assignment. The final table, representing 138 taxa, was annotated with information pertaining to their gram stain, motility, nutritional classification, main energy and carbon source, ecology, and habitat, based on a thorough review of available literature (Appendix B).

6.2.21 Turnover of operational taxonomic units along the gut

To examine OTU turnover with passage through the gut, a custom built script was developed by S. Rushton and W. Reid in the School of Biology at Newcastle University. The ‘dredge’ function in the MuMIn package in RStudio was used to select the best model based on the lowest Akaike information criterion (AIC; Burnham and Anderson, 2002). This modelling approach enabled detection of the nature of the relationships with passage through the gut (i.e. quadratic or linear, positive or negative) and whether there was a significant difference between the experimental factors of feed and sediment type (Appendix B).

6.2.22 Identification of a core microbiome in the midgut

Characterisation of the top 100 OTUs in all sampling locations (Section 6.2.20 and Appendix B) revealed that the midgut compartment harboured bacterial taxa that were not present at any other sampling location. To further explore the existence of a core microbiome within the midgut, the ‘metastats’ function in Mothur was used to conduct pairwise comparisons between the ‘midgut’ group and the other sampling locations (sediment, foregut,

hindgut and faeces). The OTUs that were significantly different between groups were converted to presence/absence data and used to compile a list of OTUs that were present in the midgut communities only. To facilitate presentation and interpretation, the OTU identifiers were removed and OTUs were merged in the case of multiple taxonomic assignments

6.2.23 Correlations with environmental variables

Correlation between the bacterial community structure and the environmental metadata were explored by canonical correspondence analysis (CCA) using the vegan package in RStudio (Oksanen *et al.*, 2016). Canonical correspondence analysis (CCA) is an extension of correspondence analysis that constrains OTU abundance data in the ordination based on the environmental variables measured (Zuur *et al.*, 2007). The environmental factors influencing the microbial communities of ambient sediments and faecal mounds (tank level) and the gut compartments were explored separately since water and sediment quality parameters recorded at the tank level of each replicate could not be said to be linked to bacterial communities in the gut compartments and vice versa. The scaling was set to zero to plot the OTUs and samples on separate plots to facilitate visualisation and interpretation. The 'envfit' function in the vegan package in RStudio was used to determine which environmental parameters were significantly correlated with community structure (Chapter 5, Section 5.2.19) and vectors corresponding to the significant environmental parameters were plotted onto the CCA ordinations.

6.2.24 Prediction of the metabolic capacities of bacterial communities

The Tax4Fun package in RStudio was used to predict the metabolic capacities of the bacterial communities from the 16S rRNA data (Chapter 5, Section 5.3.8) with the following modifications. The fctProfiling option was set to FALSE in order to predict the metabolic capacities of the metagenomes based on pre-computed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway reference profiles (Aßhauer *et al.*, 2015). To facilitate analysis, the KEGG Orthologs (KO) were classified according the BRITE hierarchy with three levels: module, category and pathway respectively in descending order of hierarchy. The KOs within the modules of 'human diseases' and 'non-organismal systems' were removed as they are not relevant in microbial ecology. Shade plots in PRIMER were used to explore patterns in the different modules and finally, only KEGG Pathways within the module of 'metabolism' were retained for analysis. All pathways within the categories of 'amino acid metabolism' 'carbohydrate metabolism', 'energy metabolism', 'nucleotide metabolism' and 'metabolism of other amino acids' were plotted in a shade plot. The relative abundances of gene

frequencies were square root-transformed to down-weight the influence of excessively abundant reads and used to calculate a Bray–Curtis dissimilarity matrix on the sample data (Bray and Curtis, 1957). The shade plot was seriated based on Bray Curtis resemblance matrices for samples and variables respectively and samples were re-ordered based on the hierarchical cluster produced from the sample resemblance matrix (Clarke and Gorley, 2015).

6.3 Results

6.3.1 Water and sediment quality

There were no significant interactions nor significant differences in mean flow rate, exchange rate, and water salinity, temperature, dissolved oxygen concentration, alkalinity, nitrite and sulphate between treatments on day 140 (two-way ANOVA; $p < 0.05$; Table 6.5). Sediment type and diet type interacted to influence the pH of the water at the sediment-water interface, with a more alkaline pH of 8.63 ± 0.12 recorded in the shrimp pond sediment and formulated feed treatment (two-way ANOVA; $F_{(1,\ 8)} = 16.5$, $p = 0.004$). There was no interaction between factors for orthophosphate (two-way ANOVA, $p < 0.05$; Table 6.5); however, feed-type on its own had a significant influence on orthophosphate. The concentration of orthophosphate was very low in the treatments where sea cucumbers were fed the formulated feed (0.01-0.02 mg L^{-1}) but was significantly higher in the treatments receiving fish waste (two-way ANOVA; $F_{(1,\ 8)} = 16.52$, $p = 0.036$) with the highest concentration recorded in the shrimp pond sediment and fish waste treatment (Table 6.5). Feed type and sediment type interacted significantly for nitrate (two-way ANOVA, $p = 0.011$). The concentration of nitrate was significantly lower in the dune sand and fish waste treatment (two-way ANOVA; $F_{(1,\ 8)} = 10.70$, $p = 0.011$). Total ammonia-nitrogen and sulphide were below the limit of quantification (LOQ) in all treatments (0.1 mg L^{-1}). Nitrite was below the LOQ in the fish waste diet treatments only and there were no significant differences in alkalinity, nitrite or sulphate between treatments.

The shrimp pond sediment was significantly more reduced than the dune sand treatments with a mean redox potential of -84.23 ± 13.03 mV compared to -33.38 ± 3.62 mV (two-way ANOVA; $F_{(1,\ 8)} = 22.85$, $p = 0.001$). Diet and sediment type interacted to significantly affect the content of organic matter, organic carbon and total nitrogen in the surface sediment samples (two-way ANOVA; $p < 0.05$; Table 6.5). The dune sand treatments receiving fish waste had the lowest total nitrogen content of 0.03 ± 0.00 % (two-way ANOVA; $F_{(1,\ 8)} = 13.64$, $p = 0.006$).

Table 6.5. Mean (± standard error) water and sediment quality parameters measured on day 140 in tanks with various combinations of dune sand (DS), shrimp pond sediment (SPS), formulated feed (FF) and fish waste (FS). Different superscripts with each row indicate significant differences (two-way ANOVA, $p < 0.05$); ns = not significant.

	DS_FF	DS_FW	SPS_FF	SPS_FW	p-value		
					Sediment (S)	Feed (F)	S x F
Water quality							
Flow rate (L min^{-1})	1.21 ± 0.06	1.22 ± 0.21	1.45 ± 0.17	1.31 ± 0.07	ns	ns	ns
Exchange rate (%)	382.25 ± 17.71	388.49 ± 66.83	460.62 ± 54.69	415.82 ± 20.95	ns	ns	ns
Salinity (ppt)	39.33 ± 0.13	39.29 ± 0.28	39.18 ± 0.26	39.66 ± 0.10	ns	ns	ns
Temperature (°C)	27.47 ± 0.09	27.27 ± 0.03	27.33 ± 0.19	27.37 ± 0.20	ns	ns	ns
Dissolved oxygen (mg L^{-1})	1.77 ± 0.37	2.87 ± 0.27	3.03 ± 0.62	2.93 ± 0.43	ns	ns	ns
pH	8.06 ± 0.07^{a}	8.12 ± 0.01^{a}	8.63 ± 0.12^{b}	8.13 ± 0.01^{a}	0.003	0.012	0.004
Alkalinity (mg L^{-1})	134.00 ± 3.06	137.33 ± 3.53	134.67 ± 0.67	134.67 ± 2.67	ns	ns	ns
Ammonia (mg L^{-1})	0.10	0.10	0.10	0.10	*Below level of quantification*		
Nitrite (mg L^{-1})	0.01 ±	0.01 ±	0.02 ±	0.01	ns	ns	ns
Nitrate (mg L^{-1})	1.40 ± 0.07^{ab}	1.11 ± 0.13^{a}	1.34 ± 0.07^{ab}	1.69 ± 0.11^{b}	0.029	ns	0.011
Orthophosphate (mg L^{-1})	0.01 ^{a}	0.06 ± 0.03^{ab}	0.02 ± 0.01^{a}	0.14 ± 0.03^{b}	ns	0.004	ns
Sulfide (mg L^{-1})	0.10	0.10	0.10	0.10	*Below level of quantification*		
Sulphate (mg L^{-1})	3,133.33 ± 260.34	3,366.67 ± 272.85	2,833.33 ± 176.38	3,066.67 ± 66.67	ns	ns	ns
Sediment quality							
Redox (mV)	-31.17 ± 6.09^{a}	-35.60 ± 4.85^{a}	-62.87 ± 17.15^{ab}	-105.60 ± 9.90^{b}	0.001	ns	ns
Bulk density (g cm^{3})	1.86 ± 0.01	1.86 ± 0.01	1.86 ± 0.02	1.87 ± 0.00	ns	ns	ns
Benthic algae (%)	30.00 ± 7.64	25.00 ± 7.64	33.33 ± 4.41	40.00 ± 2.89	ns	ns	ns
Organic matter (%)	0.39 ± 0.09^{a}	0.41 ± 0.08^{a}	1.08 ± 0.08^{b}	1.54 ± 0.09^{c}	0.000	0.022	0.030
Organic carbon (%)	0.23 ± 0.05^{a}	0.24 ± 0.05^{a}	0.63 ± 0.05^{b}	0.90 ± 0.05^{c}	0.000	0.022	0.034
Nitrogen (%)	0.05 ± 0.01^{a}	0.03 ± 0.00^{b}	0.06 ± 0.00^{a}	0.07 ± 0.01^{a}	0.002	ns	0.006
C:N	4.36 ± 0.58^{b}	9.42 ± 1.62^{ab}	11.39 ± 1.41^{a}	12.98 ± 1.96^{a}	0.007	ns	ns

Sediment and feed type interacted to influence the organic carbon content of the sediment (two-way ANOVA; $F_{(1, 8)} = 6.56$, $p = 0.04$). The shrimp pond sediment had a significantly higher organic carbon content than the dune sand treatments where the organic carbon content was low (mean 0.233 ± 0.031 %). The treatment that combined both types of aquaculture waste (SPS_FW) had the highest organic carbon content with a mean of 0.90 ± 0.05 %. Incidentally, this treatment also had the lowest redox potential, the highest C:N and total nitrogen content, although these differences were not significant. There was an inverse relationship between the sediment redox potential and C:N between the two sediment types (Table 6.5). The shrimp pond sediment was significantly more reduced than dune sand and had a significantly higher C:N (12.19 ± 1.14 versus 6.89 ± 1.37; Table 6.5). Finally, there was no significant difference in bulk density or the percentage coverage of benthic algae between treatments (two-way ANOVA; $p<0.05$; Table 6.5).

6.3.2 Sea cucumber growth, biomass density, and proximate composition

The growth rate of *H. scabra* was significantly different between treatments (repeated measures ANOVA; $F_{(9, 72)} = 2.37$, $p = 0.021$) with a significant interaction between diet, sediment and time. Diet, sediment type and time also had a significant effect on the mean biomass density (g m^{-2}) of *H. scabra* produced during the trial (repeated measures ANOVA; $F_{(10, 80)} = 5.65$, $p < 0.0001$). Diet had the strongest influence on the overall growth rate and final biomass density, with the formulated feed out-performing fish waste. Throughout the trial, *H. scabra* reared on dune sand, fed with the formulated feed out-performed the other treatments, achieving a significantly higher mean density of 529.56 ± 9.53 g m^{-2} (Figure 6.6).

Sea cucumbers fed with particulate organic waste achieved similar densities at the end of the trial (336.63 ± 35.06 g m^{-2} on dune sand versus 345.53 ± 26.17 g m^{-2} on shrimp pond sediment). These densities were not significantly different to the density of sea cucumbers reared on shrimp pond sediment fed with formulated feed (407.12 ± 36.00 g m^{-2}). Despite differences in growth rate and biomass density between treatments, there was no significant difference in condition factor or proximate composition of the body wall ($p < 0.05$; Table 6.6).

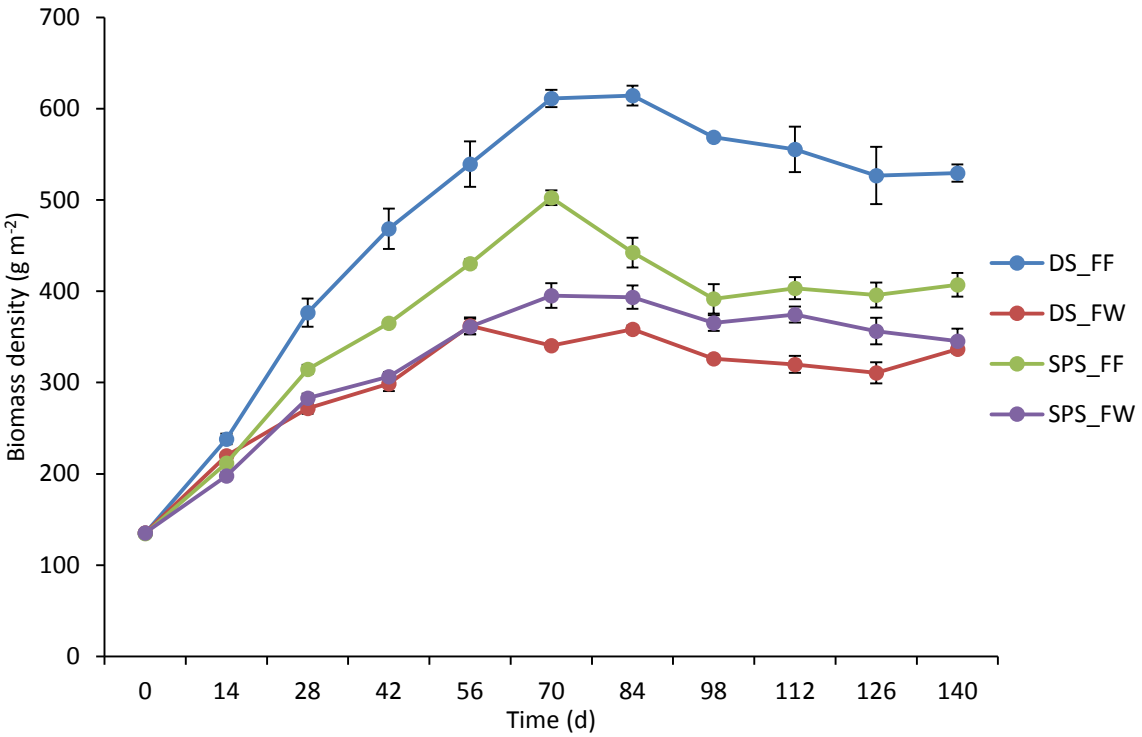

Figure 6.6. Mean (± SE) cumulative biomass density of *Holothuria scabra* (n = 3) reared for 140 days on different sediment and feed types, contrasting aquaculture-impacted sediments recovered from ashrimp pond (SPS) with a dune sand (DS) and fish waste (FW) recovered from a finfish recirculating system with formulated feed (FF).

6.3.3 Environmental variables associated with ambient sediment, gut content and faecal samples

The fixed factors of sediment type, feed type and sampling location interacted to significantly influence the content of organic carbon and total nitrogen along the sea cucumber feeding chain (multifactor ANOVA; $p<0.001$; Figure 6.7). There was a significantly higher peak in organic carbon (4.46 ± 0.56 %) and total nitrogen (0.46 ± 0.01 %) content in the midgut samples taken from sea cucumbers reared on both types of aquaculture waste (shrimp pond sediment and fish waste). Carbon and nitrogen followed similar trends, with the lowest concentrations in the sediment and faecal samples, significant increases in concentration in the fore- and midguts with intermediate concentration in the hindgut. The C:N was significantly influenced by sediment and sampling location (multifactor ANOVA; $F_{(4,\ 40)} = 5.15$, $p = 0.0019$). Overall, the C:N was higher in the shrimp pond sediment and lowest in the dune sand (Table 6.5). The ambient sediment in the dune sand and formulated feed treatment that supported the highest growth rate had the lowest C:N of 9.17 ± 1.13 while the sediment in the treatment with both sources of aquaculture waste had the highest C:N of 22.40 ± 5.32.

Table 6.6. Mean (± standard error) growth performance and proximate composition of sea cucumbers measured on day 140 in tanks with various combinations of dune sand (DS), shrimp pond sediment (SPS), formulated feed (FF) and fish waste (FS). Different superscripts with each row indicate significant differences (two-way ANOVA, $p < 0.05$); ns = not significant.

	DS _FF	DS _FW	SPS _FF	SPS _FW	Sediment (S)	Feed (F)	S x F
Sea cucumber growth							
Average weight (g)	174.25 ± 9.90^{b}	110.67 ± 4.95^{a}	134.95 ± 7.08^{ab}	118.60 ± 5.69^{a}	ns	0.002	ns
Coefficient of variation (%)	8.78 ± 0.66^{a}	11.23 ± 0.93^{ab}	11.24 ± 0.96^{ab}	12.22 ± 0.79^{b}	0.014	0.013	ns
Length (mm)	169.22 ± 8.97^{b}	147.89 ± 2.76^{ab}	148.22 ± 1.16^{ab}	131.89 ± 4.96^{a}	0.009	0.008	ns
Width (mm)	54.11 ± 48.33^{b}	1.18 ± 1.50^{ab}	50.00 ± 1.71^{ab}	46.33 ± 0.67^{a}	0.050	0.007	
Condition factor	0.96 ± 0.08	0.86 ± 0.04	0.99 ± 0.08	1.08 ± 0.03	ns	ns	ns
Biomass density (g m^{-2})	469.42 ± 26.68^{b}	298.14 ± 13.33^{a}	363.54 ± 19.07^{ab}	319.50 ± 15.33^{a}	ns	0.002	ns
Growth rate (g d^{-1})	2.14 ± 0.14^{b}	1.15 ± 0.11^{a}	1.51 ± 0.12^{ab}	1.20 ± 0.09^{a}	ns	0.002	ns
Proximate composition							
Protein (%)	51.19 ± 4.03	42.12 ± 9.88	65.65 ± 13.77	42.89 ± 4.07	ns	ns	ns
Lipid (%)	1.77 ± 0.31	2.80 ± 1.15	2.12 ± 0.52	1.62 ± 0.31	ns	ns	ns
Ash (%)	54.29 ± 8.31	52.25 ± 7.41	55.50 ± 3.72	53.31 ± 1.63	ns	ns	ns
Moisture (%)	84.50 ± 1.31	80.80 ± 3.80	85.63 ± 1.56	79.70 ± 2.48	ns	ns	ns

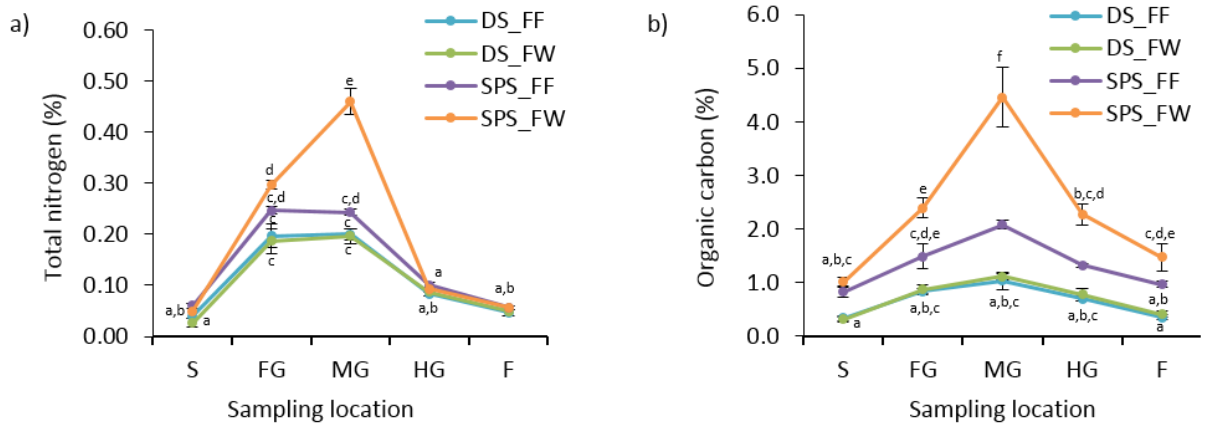

Figure 6.7. Changes in mean (± SE) (a) total nitrogen and (b) organic carbon of sediment samples with passage through the holothurian gut. S = sediment; FG = foregut; MG = midgut; HG = hindgut; F = faeces. DS = dune sand; SPS = shrimp ponds sediment; FF = formulated feed; FW = fish waste.

6.3.4 Sequencing quality control

A total number of 4,952,808 PCR amplicons spanning the V4 hyper-variable region of the 16S rRNA gene were generated by sequencing triplicate samples from five sampling locations in each of the four experimental treatments (n = 60). Subsequent to quality control and removal of contaminating OTUs, a total of 3,144,522 optimised reads remained.

6.3.5 Guanine-cytosine content of bacterial communities – ambient sediments

The overall distribution of the GC content in the shrimp pond sediments was skewed to the right and had a different shape to the dune sand treatments, which exhibited a bimodal distribution (Figure 6.8).

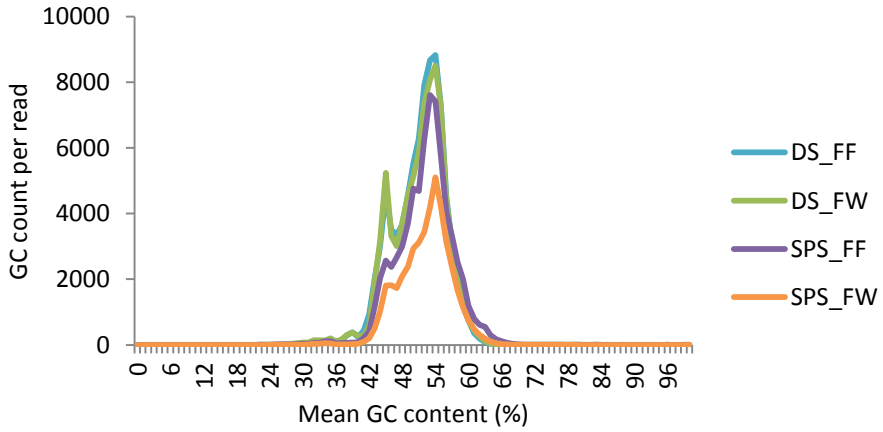

Figure 6.8. Guanine-cytosine (GC) distribution of all sequences from the ambient sediment samples of the four experimental treatments.

The mean percentage of nitrogenous bases, measured as GC content, was significantly higher in the shrimp pond sediment than the dune sand sediment (two-way ANOVA; $F_{(1, 20)}$ = 27.44, $p < 0.0001$; Figure 6.9). The dune sand treatments had an identical mean GC content of

51.33 ± 0.21 % irrespective of the diet type. The highest GC content of sediment bacterial communities was observed in the treatment combining both types of aquaculture waste (shrimp pond sediment and fish waste) with a mean of 52.83 ± 0.31. The shrimp pond sediment and formulated feed treatment had a mean GC content of 52.33 ± 0.21 %. Sediment redox potential was a significant predictor of the guanine-cytosine (GC) content of the ambient sediment bacterial communities (multiple regression, $F_{(5, 6)} = 21.79$, $r^2 = 0.95$; $p < 0.001$).

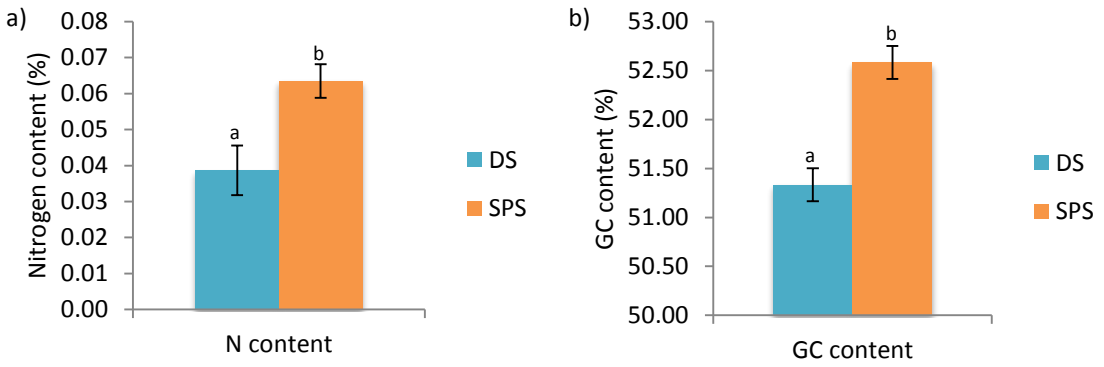

Figure 6.9. The mean (± standard error) (a) nitrogen content and (b) the guanine-cytosine (GC) content of bacterial communities in the ambient sediments of the dune sand (DS) and shrimp pond sediment (SPS).

6.3.6 Rarefaction analysis

Rarefaction analysis demonstrated that sediment and faecal samples had rarefaction curves that continued to climb indicating that bacterial communities in sampling locations external to the gut were not sampled to saturation and contained unexplored richness (Figure 6.10). In general, the number of OTUs decreased with passage through the gut, with the lowest richness in midgut and hindgut communities. The rarefaction curves for the mid- and hindgut communities of sea cucumbers reared on dune sand sediments were the only curves that reached the plateau phase indicating an adequate depth of sampling. The number of OTUs between treatments did not follow predictable trends with passage through the gut, for example, some of the replicates from the shrimp pond sediments, which had the lowest richness in the sediment communities, exhibited an increase in richness in the foregut and midgut communities with the steepest curves and highest richness. In the midgut samples, there was a clear separation of curves between the two sediment types, with the lowest richness in the shrimp pond sediment treatments which reached the curvilinear phase, while the dune sand midgut samples continued to climb, indicating that these communities were not sampled to saturation (Figure 6.10).

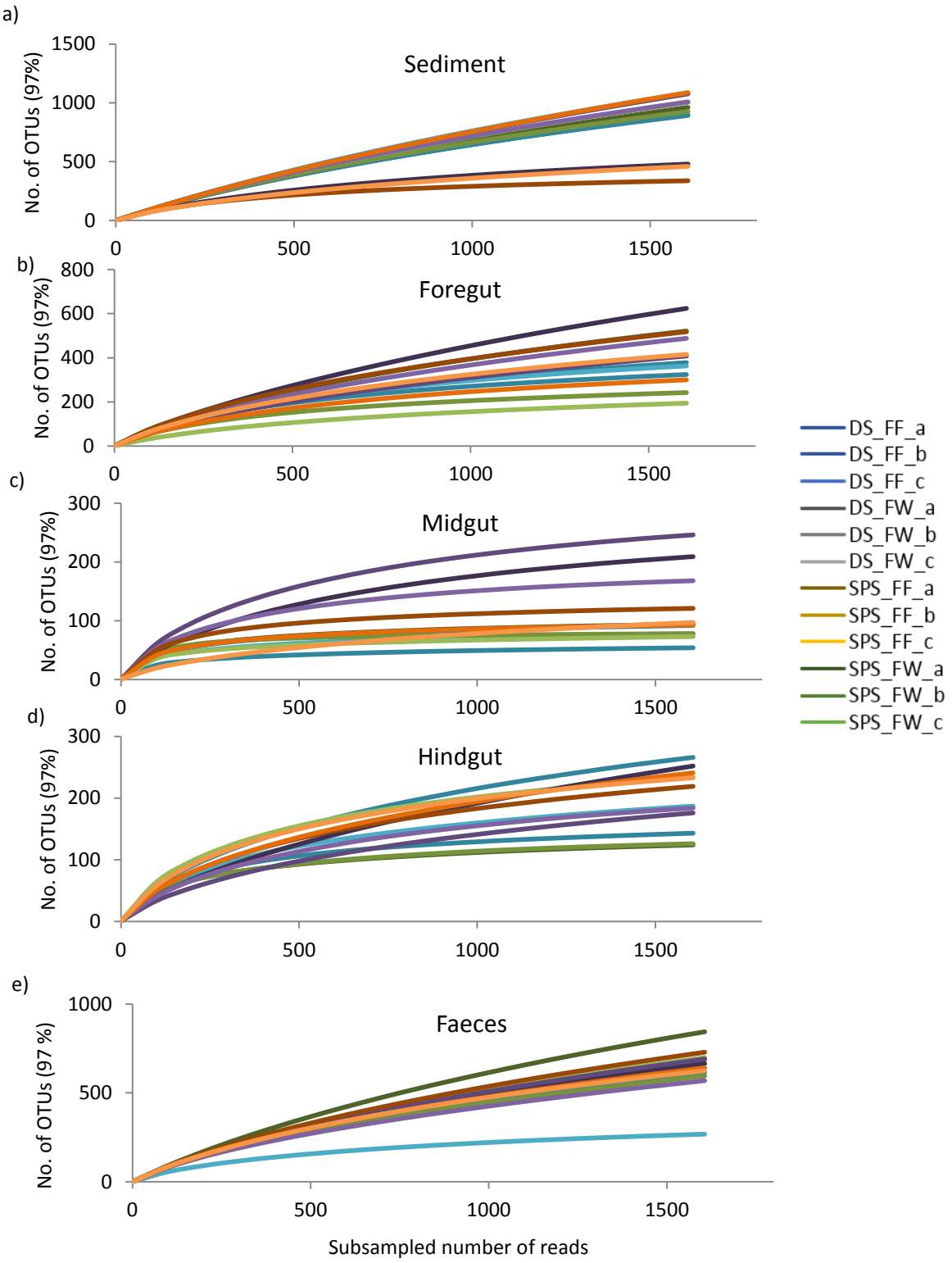

Figure 6.10. Rarefaction curves for the replicate samples from each of the four experimental treatments for each sampling locations along the feeding chain: a) sediment; b) foregut; c) midgut; d) hindgut; and e) faeces. The number of reads is normalised by subsampling to 1,606 reads. SPS = shrimp pond sediment; DS = dune sand; FW = fish waste; FF = formulated feed.

6.3.7 Alpha diversity

Sampling location had a significant effect on all alpha diversity metrics, including the number of OTUs, Chao richness estimator, Inverse Simpsons and Shannon diversity (PERMANOVA; $p < 0.001$). The number of OTUs and community richness (Chao) was highest in the ambient sediments and subsequently decreased with passage through the gut, to reach the lowest richness in the midgut (Figure 6.11). There was an increase in the number of OTUs in the hindgut and a proliferation in the number of OTUs and community richness in the faecal mounds.

As with the rarefaction curves, marked differences were observed between the different sediment types. The dune sand treatments supported the richest bacterial communities in the ambient sediments; however, the number of OTUs and Chao richness were higher in the shrimp pond sediment treatments at all sampling locations along the gut. The most noticeable difference between treatments was observed in the sea cucumber midgut communities. Midgut communities from the dune sand treatments had a low and comparable richness with 86.33 ± 15.67 OTUs and 96.33 ± 3.76 OTUs in the formulated feed and fish waste treatments respectively. Sea cucumbers reared on the shrimp pond sediment and fed fish waste had intermediate richness with 153.67 ± 31.7 OTUs; however, sea cucumbers reared on the shrimp pond sediment and fed with the formulated feed had the richest midgut communities with 303.33 ± 31.7 OTUs.

Similarly, the dune sand supported the highest bacterial diversity in the ambient sediments with comparable Shannon diversity indices between feed types (6.48 ± 0.02 in formulated feed treatments versus 6.43 ± 0.03 for fish waste treatments) and in the faecal mounds. Overall, the diversity of bacterial communities ingested from shrimp pond sediment in all gut compartments was higher than the dune sand gut communities. The foregut and midgut communities in sea cucumbers reared on shrimp pond sediment with formulated feed had the highest diversity; however, sea cucumbers reared on both type of aquaculture waste had the most diverse hindgut communities (Figure 6.11).

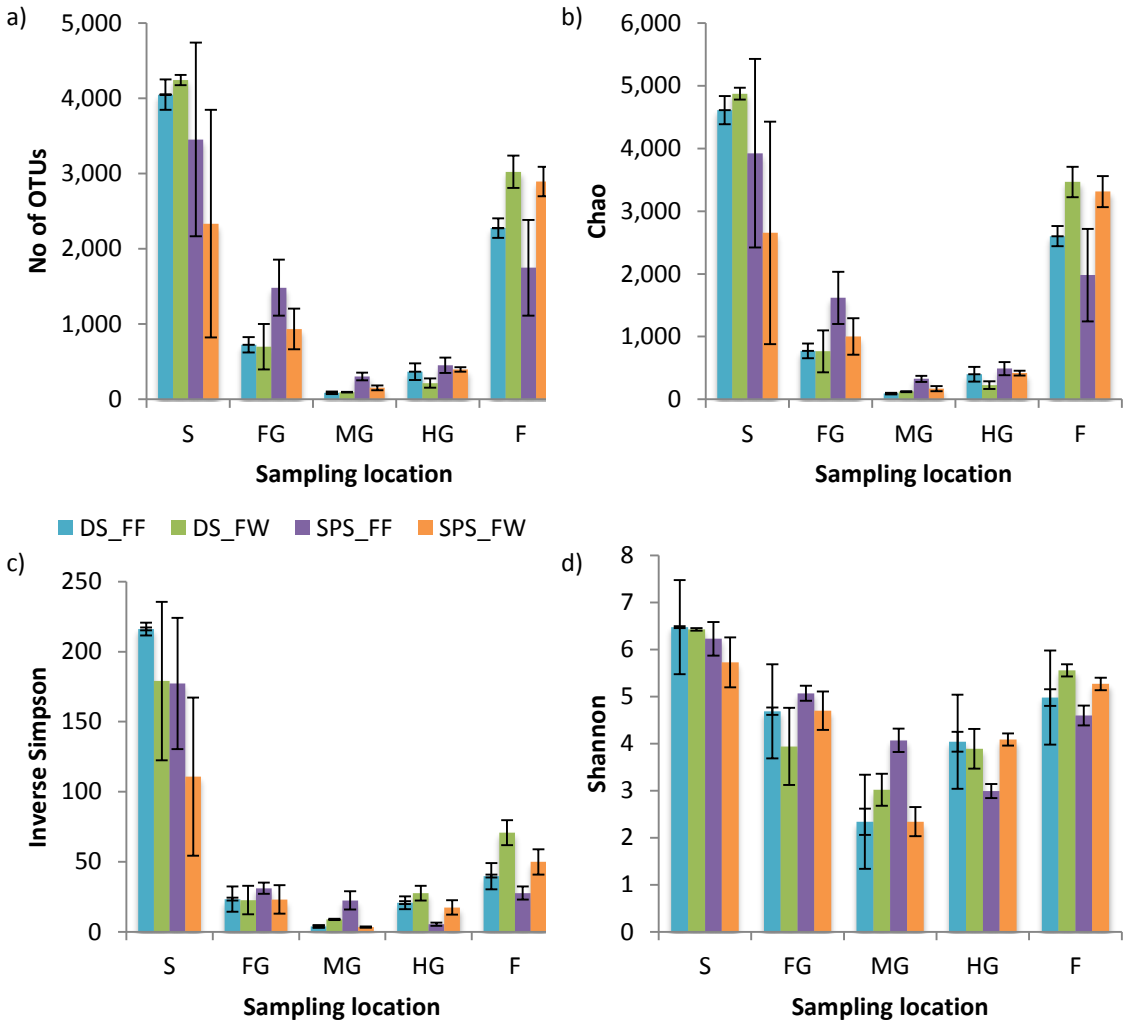

Figure 6.11 Mean (± standard error) alpha diversity metrics for (a) number of operational taxonomic units (OTUs), (b) Chao richness, (c) Inverse Simpson and (d) Shannon diversity. S = sediment; FG = foregut; MG = midgut; HG = hindgut; and F = faeces.

6.3.8 Phylum level taxonomy

Taxonomic analysis detected 20 phyla and 11 candidate phyla. Candidate phyla that have recently been given provisional names, although they are not yet recognised officially as they have no cultured representatives (Rinke *et al.*, 2013) represented less than 0.3 % of the total sequences. They included Candidatus, Saccharibacteria (formerly TM7), Hydrogenedentes (formerly NKB19), Latesbacteria (formerly WS3), Microgenomates (formerly OP11), Parcubacteria (formerly OD1), and Poribacteria, a recently discovered new candidate phylum found living in symbiosis with sponges (Fieseler *et al.*, 2004). Sequences belonging to the candidate divisions BRC1 (from bulk roots and soil), SR1, WPS-2, and ZB3 represented only 0.13 % of the total sequences.

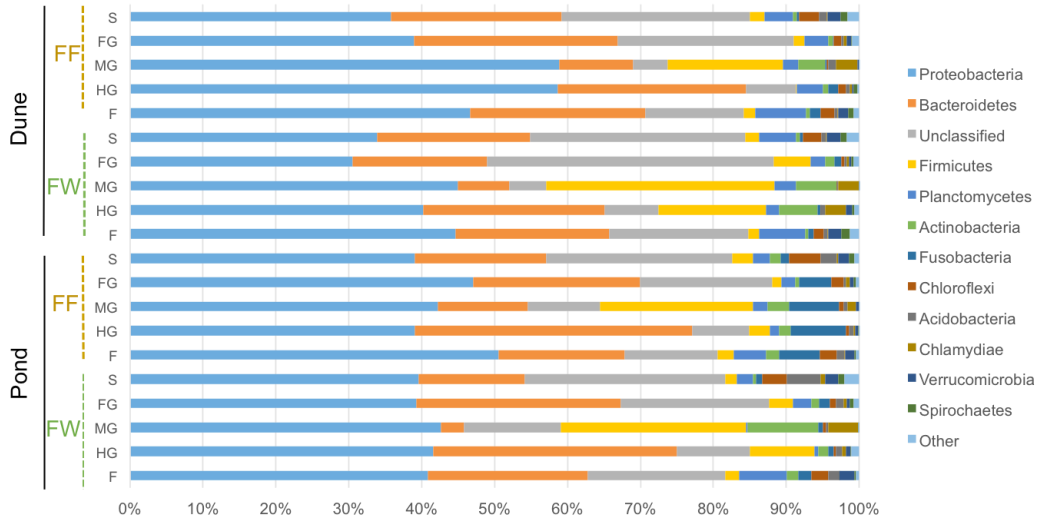

Figure 6.12. Relative abundance of the major phyla between treatments. Phyla that represented less than 10 % of the overall total abundance were classified as 'other'. Pond = shrimp pond sediment; Dune = dune sand; FF = formulated feed and FW = fish waste. S = sediment; FG = foregut; MG = midgut; HG = hindgut; and F = faeces.

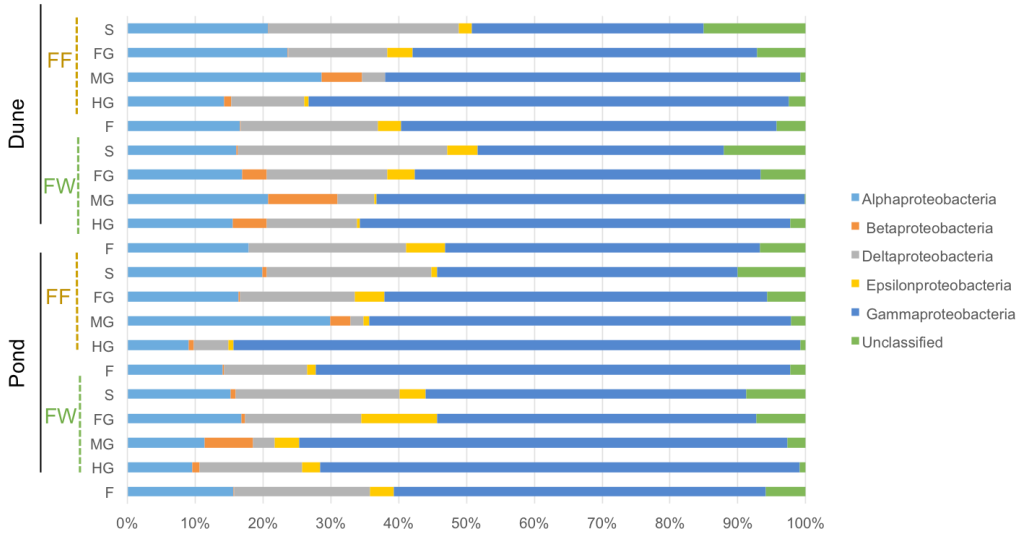

Figure 6.13. Relative abundance of the Proteobacteria classes between treatments. Pond = shrimp pond sediment; Dune = dune sand; FF = formulated feed and FW = fish waste. S = sediment; FG = foregut; MG = midgut; HG = hindgut; and F = faeces.

Proteobacteria was the dominant phylum (42.72 ± 1.59 %) followed by Bacteroidetes (20.68 ± 1.90 %), Firmicutes (7.34 ± 2.06 %) and Planctomycetes (3.10 ± 0.43 %), that together with the unclassified sequences represented over 90 % of the bacterial community composition in all samples (Figure 6.12). Gammaproteobacteria (20.92 ± 2.52 %) were the dominant class within the Proteobacteria, followed by Deltaproteobacteria (13.74 ± 2.16 %) and Epsilonproteobacteria (4.53 ± 1.42 %; Figure 6.13).

6.3.9 Presence of core microbiome

Of the taxa that were significantly different between the five sampling locations, 165 OTUs were present in the midgut samples only; i.e. these taxa were not found in any of the other sample locations. These OTUs, which constitute the core midgut microbiome, represented seven phyla including Actinobacteria (n = 4), Bacteroidetes (n = 18), Chlamydiae (n = 13), Firmicutes (n = 42), Planctomycetes (n = 4), Proteobacteria (n = 51), Verrucomicrobia (n = 1) and a total of 32 OTUs that remained unassigned (Table 6.7).

Table 6.7. Assigned taxonomy of the 165 statistically significant operational taxonomic units that were present in the holothurian midgut only.

Phylum	Order	Genus
Actinobacteria	Actinomycetales	*Brevibacterium*
		unclassified
	Bifidobacteriales	*Bifidobacterium*
Bacteroidetes	Bacteroidales	*Bacteroides*
		Barnesiella
		Parabacteroides
		Porphyromonas
		Paraprevotella
		Prevotella
		Alistipes
	Cytophagales	unclassified
	Flavobacteriales	*Chryseobacterium*
	Flavobacteriales	*Muricauda*
	unclassified	unclassified
Chlamydiae	Chlamydiales	*Parachlamydia*
		unclassified
Firmicutes	Bacillales	*Gemella*
		Staphylococcus
		Planifilum
	Lactobacillales	unclassified
		Enterococcus
		Lactobacillus
		Streptococcus
	Clostridiales	*Anaerococcus*
		Anaerostipes
		Blautia
		Clostridium_XlVa
		Coprococcus
		Dorea
		Roseburia
		Ruminococcus2
		unclassified
		Clostridium_XI

Table 6.7. *Continued.*

Phylum	Order	Genus
	Clostridiales	unclassified
		Clostridium_XI
		Clostridium_IV
		Faecalibacterium
		Oscillibacter
		unclassified
	Erysipelotrichales	unclassified
	Selenomonadales	*Veillonella*
	unclassified	unclassified
Planctomycetes	Planctomycetales	*Pirellula*
Planctomycetes	Planctomycetales	unclassified
Alpharoteobacteria	Rhizobiales	unclassified
	Rhodobacterales	*Rubellimicrobium*
	unclassified	unclassified
Betaproteobacteria	Burkholderiales	unclassified
	Burkholderiales	*Sutterella*
	Neisseriales	*Neisseria*
Deltaproteobacteria	Bdellovibrionales	*Vampirovibrio*
	Desulfobacterales	unclassified
Epsilonproteobacteria	Campylobacterales	*Campylobacter*
	Campylobacterales	*Sulfurovum*
Gammaproteobacteria	Enterobacteriales	unclassified
	Legionellales	*Coxiella*
	Legionellales	*Legionella*
	Pseudomonadales	*Pseudomonas*
	unclassified	unclassified
	Xanthomonadales	*Luteimonas*
Proteobacteria	unclassified	unclassified
unclassified	unclassified	unclassified
Verrucomicrobia	Verrucomicrobiales	*Akkermansia*

The midgut core microbiome was dominated by the Proteobacteria and Firmicutes. All five classes of the Proteobacteria were present; Alphaproteobacteria contained unclassified OTUs, within the order Rhizobiales that are symbiotic nitrogen-fixing bacteria. An additional two OTUs were assigned to Rhodobacterales (*Rubellimicrobium*). Betaproteobacteria, previously identified as being numerically abundant in midgut samples (Figure 6.13), contained four OTUs belonging to the families *Alcaligenaceae*, *Oxalobacteraceae* and *Sutterellaceae* (order Burkholderiales) and the genus *Neisseria*. Only two OTUs classified within the core midgut microbiome belonged to the Delta and Epsilon classes, including the predatory bacteria *Vampirovibrio*, an unclassified *Desulfobulbaceae*, *Campylobacter* and *Sulfurovum*. The majority of the OTUs within the Protebacteria (n = 34) belonged to the

Gammaproteobacteria. The OTUs classified to genus level included *Coxiella, Legionella, Pseudomonas* and *Luteimonas*. Two OTUs were unclassified within the families *Enterobacteriaceae* and *Pasteurellaceae* and the remaining 17 were unclassified Gammaproteobacteria and five unclassified Proteobacteria.

Within the Firmicutes, the second most abundant phylum, OTUs were affiliated to the orders Bacillales, Lactobacillales, Clostridiales, Erysipelotrichiales and Selenomonadales. Clostridiales were the most abundant order containing 29 OTUs belonging to *Lachnospiraceae* (n = 19) and *Ruminococcaceae* (n = 7). All genera with the Lachnospiraceae family including *Anaerostipes, Blautia, Clostridium_XlVa, Coprococcus, Dorea, Roseburia* and *Ruminococcus2* and *Ruminococcaceae* including *Clostridium_IV, Faecalibacterium,* and *Oscillibacter* are common host-associated bacteria present in the intestines of humans and animals (Appendix 2). Lactic acid bacteria (Lactobacillales) were also key members of the core midgut microbiome including five OTUs assigned to the genera *Enterococcus, Lactobacillus* and *Streptococcus*, which perform homolactic fermentation of glucose to pyruvate (Appendix 2; Pessione, 2012).

In addition to the Firmicutes, the phyla Actinobacteria and Chlamydia had a higher relative abundance in the midgut (Figure 6.12 and Appendix B). All OTUs within the phyla Chlamydiae belonged to the order Chlamydiales, and were unclassified or assigned to *Parachlamydia*, which are non-motile obligate intracellular bacteria that naturally infect free-living amoebae (Horn, 2010). Within the Actinomycetales, four OTUs were assigned to *Brevibacterium, Bifidobacterium* and one was unassigned.

Bacteroidetes were relatively diverse in the midgut with 13 OTUs assigned to the genera *Bacteroides, Barnesiella, Parabacteroides, Porphyromonas, Paraprevotella, Prevotella* and *Alistipes* within the order Bacteroidales. Additional OTUs present only in the midgut were assigned to *Chryseobacterium, Muricauda* and the family *Flammeovirgaceae*. All four OTUs assigned to Planctomycetes were classified as *Planctomycetaceae,* including the genus *Pirellula* and the only OTU in the phyla Verrucomicrobia was assigned to *Akkermansia*. In total, 32 OTUs (19 % of the total) that were present only in the holothurian midgut remained unassigned, indicating the extent of uncharacterized diversity in deposit feeder guts.

6.3.10 Microbial community structure

Correspondence analysis indicated that the microbial communities were different between sampling locations along the feeding chain and between the different sediment types (Figure 6.14). The major trend in the data was along the first axis (CA1), which explained

11.51 % of the total variation. This axis represents the changes in micr obial community structure across the different sampling locations due to the turnover of OTUs along the holothurian gut (Figure 6.14).

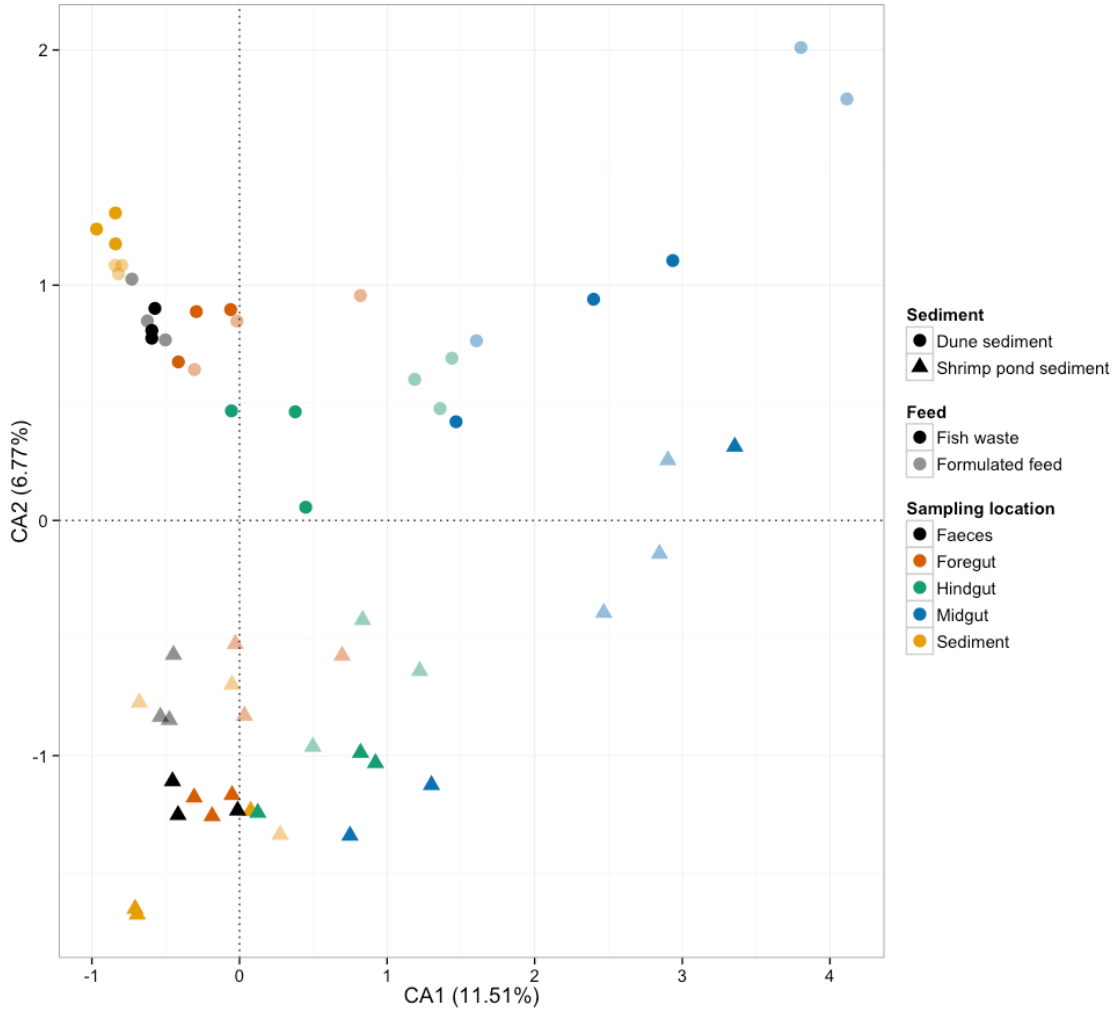

Figure 6.14. Correspondence analysis of bacterial community structure performed at the operational taxonomic unit (OTU) level (n = 1,187). Axis one (CA 1) and axis two (CA 2) explained 11.51 % and 6.77 % of the total inertia respectively. The experimental factors are illustrated in the legend: sediment (symbol shape; circle = dune sediment; triangle = shrimp pond sediment) and diet type (symbol shade; light = formulated feed; dark = fish waste) for the five sampling locations (symbol colour; yellow = sediment, orange = foregut, blue = midgut, green = hindgut, black = faeces).

The ambient sediment and faecal samples clustered together and were in close proximity to one another, indicating that the microbial communities in the faecal mounds are similar but distinct to the sediment communities. The greatest difference in microbial community structure was observed between the foregut and hindgut compared to the midgut. There was high beta diversity in the midgut communities indicated by the wide spread of points, as a result of the decrease in alpha diversity observed in the midgut communities.

The origin of the sediment had a stronger influence on bacterial community composition than feed type as the bacterial communities were separated by sediment type. The sediment type (either dune or shrimp pond sediment) appeared to be influencing CA2, explaining 6.77 % of the total variation (Figure 6.14). Although the clustering of sediment, foregut and faecal samples from the shrimp pond sediment treatments was not as distinct as the dune sand communities, the changes in bacterial community structure followed an identical pattern with passage through the gut.

6.3.11 Correlation of community structure with environmental variables (tank level)

For the tank level variables, canonical correspondence analysis (CCA), showed that sediment reduction-oxidation (redox) potential, orthophospahte, C:N and pH were the factors that most strongly correlated with microbial communities in the ambient sediments and faecal mounds (Figure 6.15). The CCA ordination showed clear partitioning among the four treatments indicating that the different sediments had different microbial communities associated with them. The communities were also associated with the particular environmental variables. The first axis (CCA1) explained 8.22 % of the total variance and separated the samples by sediment type, while CCA2 separated the samples by diet type and explained 7.36 % of the variance. Sediment redox potential correlated with communities in the sediment and faecal samples in the dune sand treatments fed with fish waste. Microbial communities from the shrimp pond sediment and fish waste treatment were mainly associated with pH. The organic carbon content of the sediment and the orthophosphate concentration at the sediment-water interface had the strongest association with communities in samples from the shrimp pond sediment and formulated feed treatment.

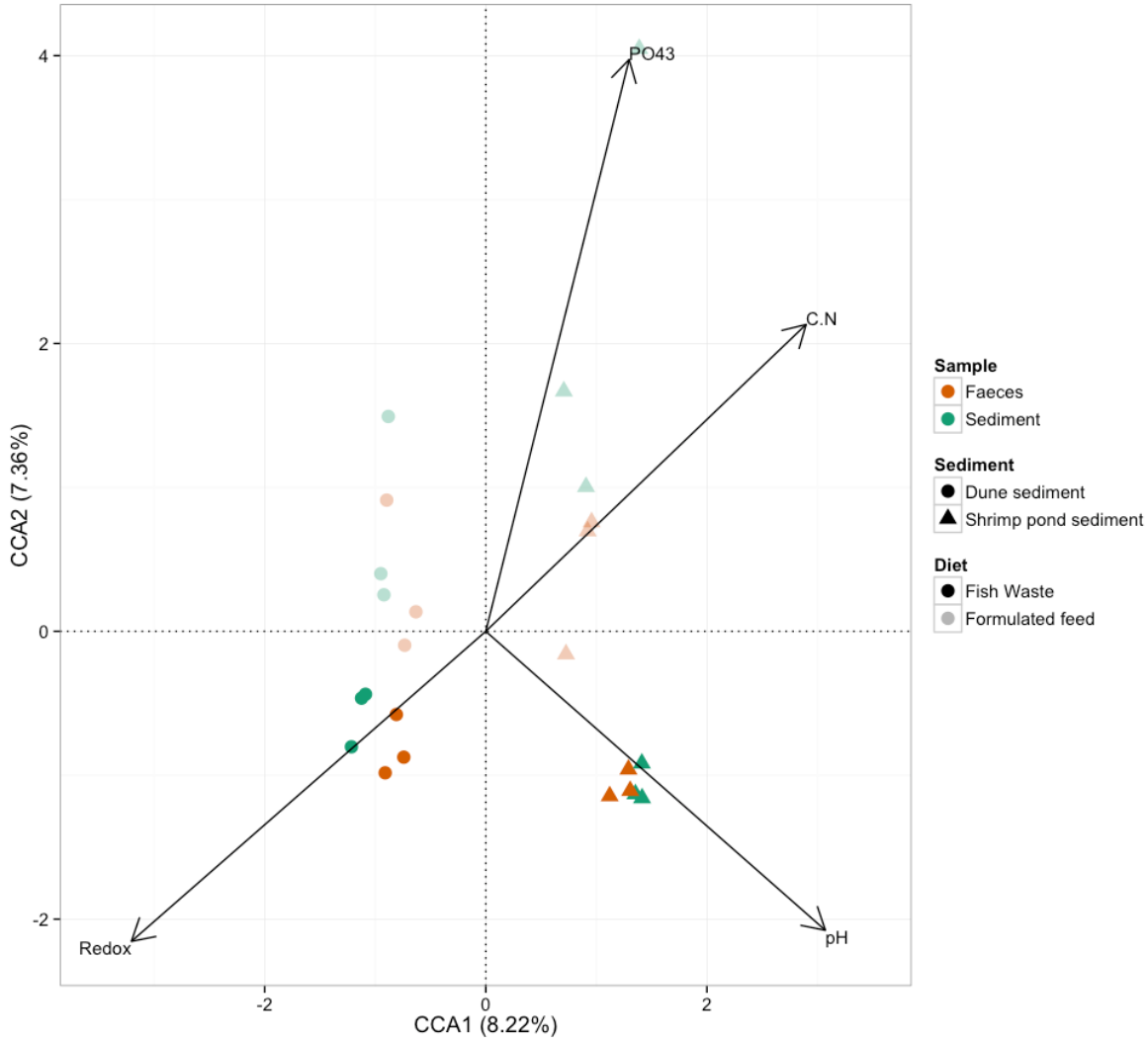

Figure 6.15. Canonical correspondence analysis performed on the tank level environmental variables associated with the bacterial communities in the ambient sediment and sea cucumber faecal mounds. Axis one (CCA 1) and axis two (CCA 2) explained 8.22 % and 7.36 % of the total inertia respectively. The experimental factors are illustrated in the legend: sediment (symbol shape; circle = dune sediment; triangle = shrimp pond sediment) and diet type (symbol shade; light = formulated feed; dark = fish waste) for the five sampling locations (symbol colour; green = sediment, orange = faeces). C:N = carbon to nitrogen ratio. PO43 = orthophosphate.

6.3.12 Correlation with environmental variables (gut compartments)

Organic carbon and total nitrogen were the only environmental variables measured on samples from the gut compartments. Organic carbon exerted an influence along axis 1 and total nitrogen exerted an influence on axis 2 (Figure 6.16). The first and second canonical axes explained only seven and four percent of the total variation in the OTU data respectively (Figure 6.16). Total nitrogen content appeared to be mainly associated with the midgut bacterial communities of sea cucumbers reared on shrimp pond sediment and fed with formulated feed and fish waste.

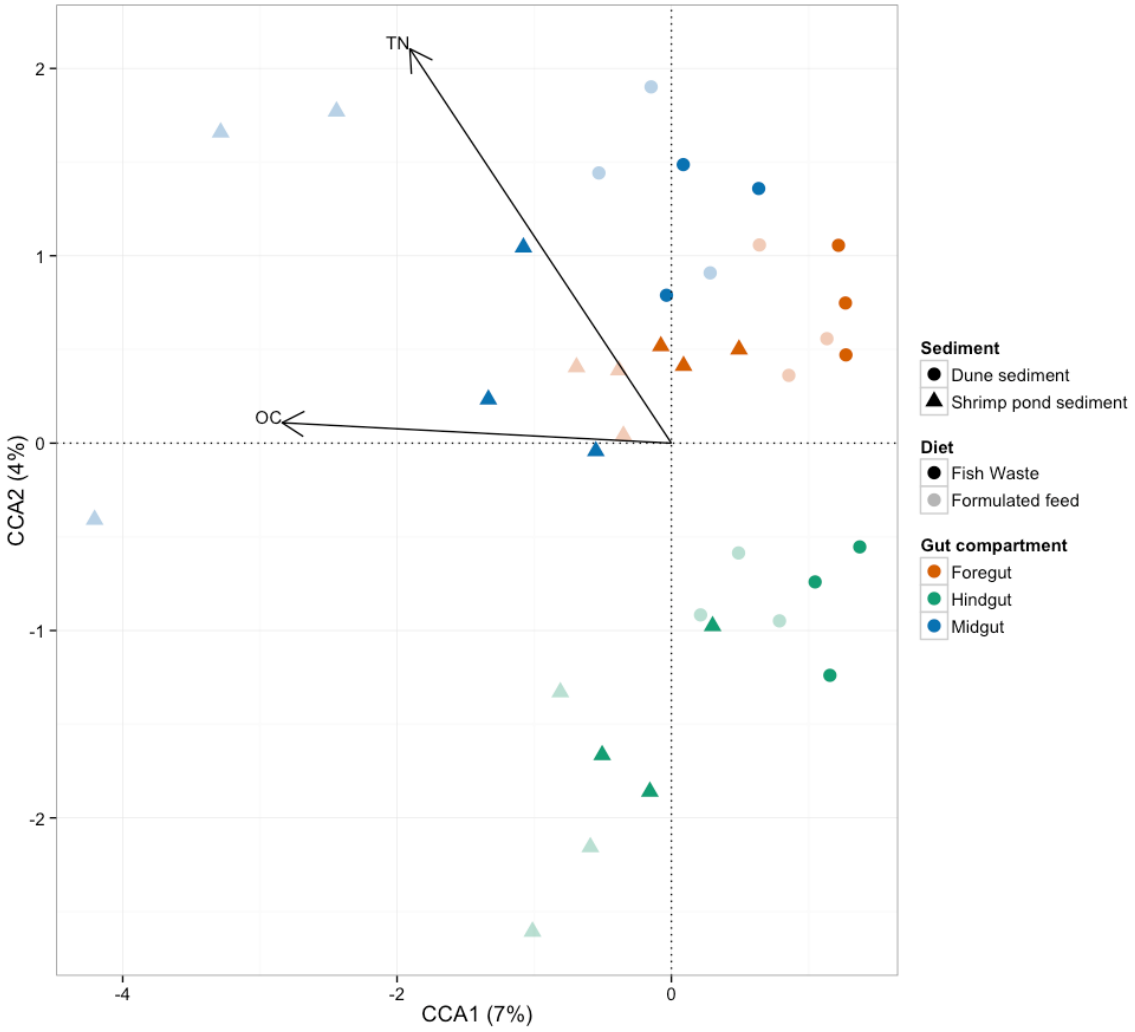

Figure 6.16. Canonical correspondence analysis performed on the gut compartment samples and their respective environmental variables. Axis one (CCA 1) and axis two (CCA 2) explained 7 % and 4 % of the total inertia respectively. The experimental factors are illustrated in the legend: sediment (symbol shape; circle = dune sediment; triangle = shrimp pond sediment) and diet type (symbol shade; light = formulated feed; dark = fish waste) for the five sampling locations (symbol colour; orange = foregut, blue = midgut, green = hindgut). TN = total nitrogen; OC = organic carbon.

6.3.13 Predicted metabolic capacity of the bacterial communities

The relative abundance of KEGG pathways belonging to the categories of 'nucleotide metabolism', 'amino acid metabolism', 'carbohydrate metabolism' and 'metabolism of other amino acids' is presented in Figure 6.17. 'Purine' and 'pyrimidine metabolism', the only two pathways within 'nucleotide metabolism', had the highest relative abundance and the strongest enrichment of all of the pathways examined. The predicted enhancement of 'purine metabolism' was much stronger compared to 'pyrimidine metabolism', which was enhanced mainly in midgut and hindgut samples (Figure 6.18). 'Nitrogen metabolism', was the third most important enhanced KEGG pathway after 'purine' and 'pyrimidine metabolism' (Figure 6.17 and Figure 6.18).

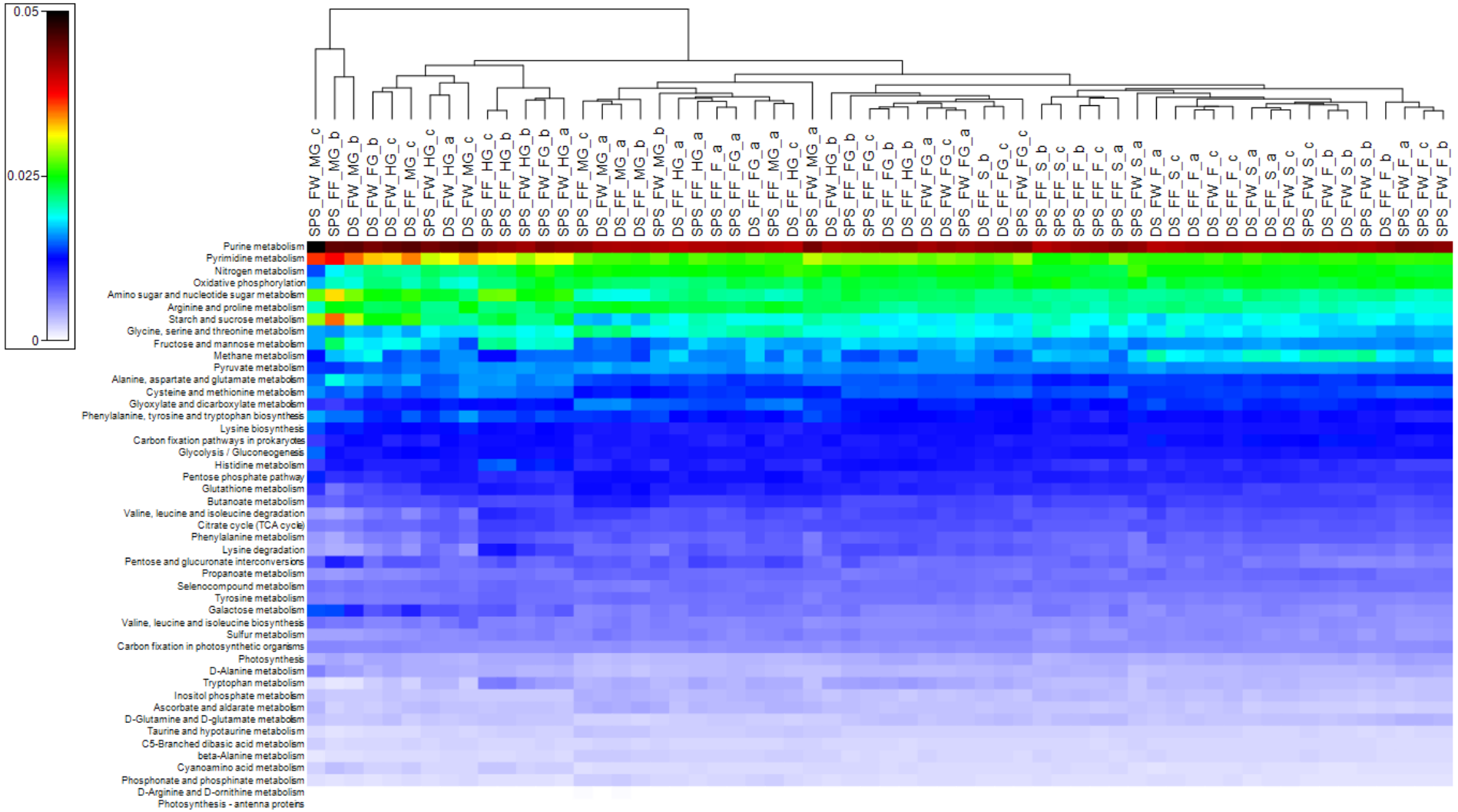

Figure 6.17. Heatmap of KEGG pathway modules within 'metabolism' (Kanehisa *et al.*, 2012) that were predicted to be enriched across samples. DS = dune sand; SPS = shrimp ponds sediment; FF = formulated feed; FW = fish waste. S = sediment, FG = foregut, MG = midgut; HG = hindgut and F = faeces. The lower case letters a, b, c are replicates.

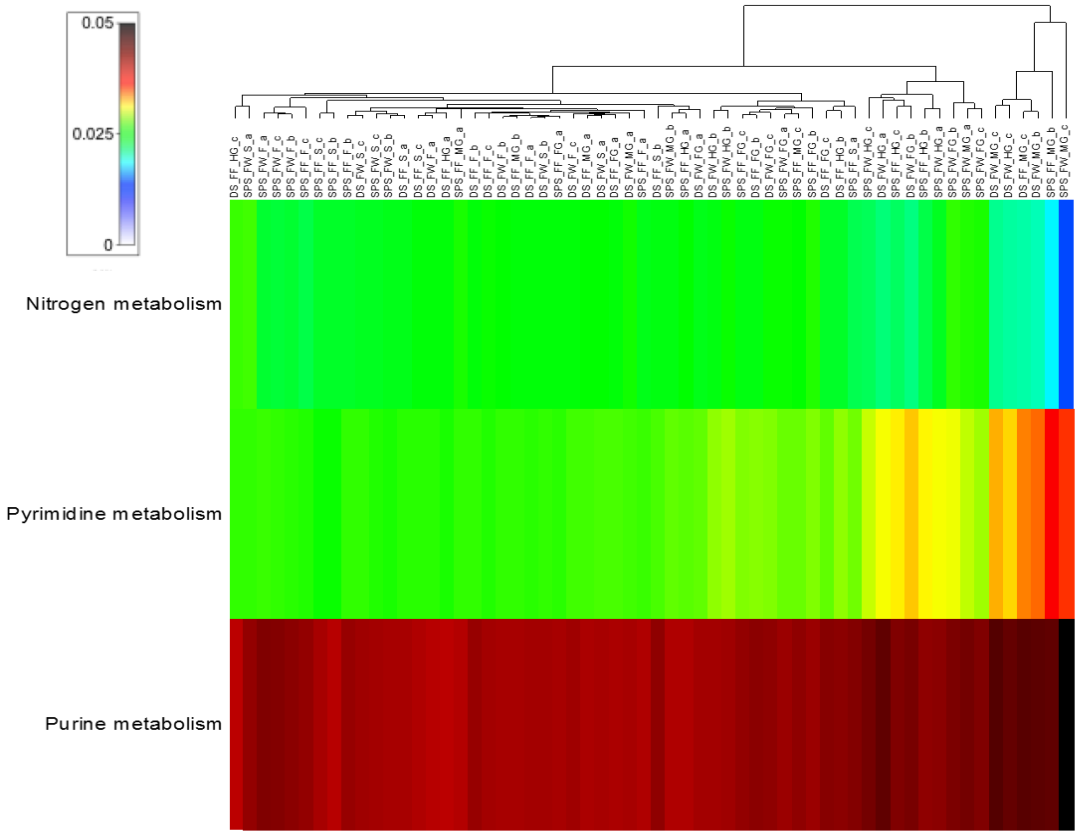

Figure 6.18. Heatmap of KEGG pathway modules of 'nitrogen', 'purine' and 'pyrimidine metabolism' predicted to be enriched among all samples.

'Nucleotide metabolism' was significantly influenced by passage through the gut (multifactor ANOVA, $p < 0.05$), increasing from the ambient sediment to reach the highest levels in the midgut (0.060 ± 0.002 %), before decreasing in the faecal mounds. Similarly, both pathways of 'purine' and 'pyrimidine metabolism' within 'nucleotide metabolism' were significantly affected by gut passage, reaching peak levels of 0.036 ± 0.001 % and 0.024 ± 0.001 % respectively in the midgut (multifactor ANOVA, $p < 0.05$). The predicted capacity for bacteria to metabolise purine bases was highest in the midgut samples of sea cucumbers reared on shrimp pond sediment and fed with finfish waste (Figure 6.18).

The relative abundance of predicted genes within the categories of 'amino acid metabolism' and the 'metabolism of cofactors and vitamins' were significantly affected by sediment type and sampling location along the feeding chain (multifactor ANOVA, $p < 0.05$; Figure 6.19). Overall, 'amino acid metabolism' was higher in the gut contents than in the ambient sediments and faecal mounds for both sediment types. There was no change in 'amino acid metabolism' with passage through the gut of sea cucumbers reared on dune sand; however, there was a decrease in 'amino acid metabolism' in the midgut of animals reared on the shrimp pond sediment, which then increased significantly to the highest relative abundance in the hindgut (0.126 ± 0.002 %; Figure 6.19).

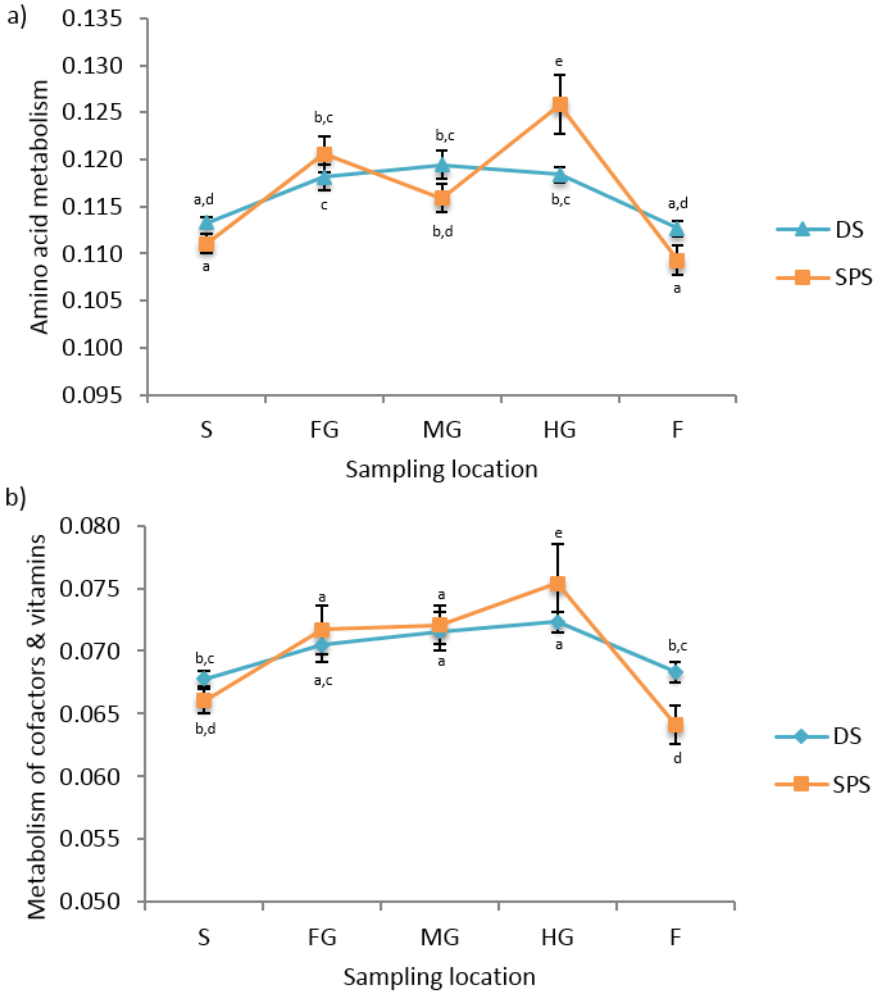

Figure 6.19. Changes in the level two categories for 'metabolism' that were significantly influenced by the interaction of sampling location and sediment type. DS = dune sand; SPS = shrimp ponds sediment. S = sediment, FG = foregut, MG = midgut; HG = hindgut; and F = faeces.

6.4 Discussion

The economic profitability and environmental sustainability of sea cucumber aquaculture can be increased by utilising particulate organic waste to replace formulated feeds (Chapter 4). However, the commercial viability of integrating sea cucumbers as agents of bioremediation into land-based intensive aquaculture systems is dependent upon the ability to farm them at high densities *in situ*. In this chapter, *Holothuria scabra* was produced at commercially viable densities (> 300 g m^{-2}) fed entirely on waste streams from an intensive land-based aquaculture company in Saudi Arabia. Although the best growth performance was achieved on the sediment with a low organic load (dune sand) in combination with the formulated feed, there was no significant difference in growth rate or biomass density between the other experimental treatments, indicating that there is potential to rear *H. scabra* on waste streams to densities of > 300 g m^{-2}. The overall growth rate of 1.20 g d^{-1} and final

biomass density of 320 g m^{-2} that was achieved, and more importantly maintained, over the final two months, was comparable to previous growth rates attained using commercial diets and dune sand sediments (Chapter 2 and Robinson *et al.*, 2015). Since economic viability is affected by growth rate and biomass carrying capacity, this growth trial lends support for the integration and cultivation of *H. scabra* into existing land-based aquaculture systems. Furthermore, the ability to reuse and recycle nitrogen-rich aquaculture waste streams as the sole inputs for high-density production has the potential to substantially reduce or even eliminate the discharge of aquaculture effluent to the marine environment.

The experimental factors of sediment and feed type interacted to significantly affect sea cucumber growth. The highest growth rate and biomass density was achieved on the dune sand sediment (formulated feed treatment) that had the highest redox potential (-31.17 ± 6.09 mV) and the lowest C:N of 4.36 ± 0.58. Overall, the formulated feed supported a higher growth rate and biomass density than the finfish waste, despite being fed on an isonitrogenous basis. The differences in growth may have manifested due to differences in the particle sizes which may have affected the availability of the diets to the sea cucumbers. The particulate organic waste was recovered from a drum filter with a 60 µm screen and collected using a 40 µm sieve. The very fine particle size of <40 µm may potentially have increased the nutrient leaching and mineralization rate compared with the formulated feed which ranged from 600-800 µm and had a high water stability due to the inclusion of binders in its formulation.

Diet is known to influence microbiome structure and function in animals and humans, with concomitant effects on health and growth (Conlon and Bird, 2015; Schmidt *et al.*, 2016). For deposit feeding sea cucumbers that ingest sediment and extract their nutrition from allochthonous and autochthonous sources of organic matter contained therein, ‘diet’ encompasses both experimental factors tested in this experiment, namely feed and sediment type (Lopez and Levinton, 1987). Sediment and feed type interacted to affect the growth and biomass density as well as the structure of the bacterial communities. Although diet and sediment type were both important in structuring bacterial communities, the origin of the sediment appeared to have a stronger influence on community structure than feed type. In the canonical correspondence analysis the bacterial communities in the ambient sediments were clearly separated by sediment and feed type. The sediment redox potential, sediment C:N, orthophosphate and pH at the sediment-water interface were associated with differences in bacterial communities between sediment types. Overall, the shrimp pond sediment was more reduced (-84.23 ± 13.03 mV) than the dune sand and had a higher C:N of 12.18 ± 1.14. The lower redox potential is likely to have decreased the rate of organic matter mineralization

leading to a higher C:N due to burial of more refractory organic matter (Brune *et al.*, 2000; Kristensen, 2000).

The most important factor influencing the alpha and beta diversity of bacterial communities was passage through the sea cucumber gut. Sampling location along the sea cucumber feeding chain had a significant effect on all alpha diversity metrics, including the number of operational taxonomic units (OTUs), community richness, and diversity. Diversity and the number of OTUs was highest in the ambient sediments prior to ingestion, and decreased with passage through the gut to reach the lowest diversity in the midgut before progressively increasing in the hindgut and faecal mounds. This pattern of abundance and diversity has previously been described for deposit feeders, including sea cucumbers (Ward-Rainey *et al.*, 1996; Plante and Shriver, 1998b; Plante and Shriver, 1998a; Gao *et al.*, 2014).

A number of studies have concluded that sea cucumbers, including *H. scabra*, feed selectively on bacteria since the foregut contents contain a higher number of bacteria than ambient sediments (Moriarty, 1982; Zamora and Jeffs, 2011). It has also been suggested that sea cucumbers select and/or culture bacteria in the foregut prior to digestion Plotieau *et al.* (2013b). However, methodologies used in these studies were often flawed due to inability to accurately sample the upper layer of sediment that sea cucumbers ingest, which can lead to underestimations of bacterial abundance in the sediment and over estimations of increased concentration in the foregut (Roberts *et al.*, 2000). In this study, the low alpha diversity and high beta diversity in the midgut lend support to the hypothesis that the midgut is the principal site of digestion and rebuts the hypothesis posed by Plotieau *et al.* (2013b) that *H. scabra* feeds selectively, since no increases in alpha diversity metrics were observed in the foregut. This aligns with previous observations of Uthicke and Karez (1999) that members of the genus *Holothuria* had weak or no selectively.

A number of techniques including measurement of muramic acid concentrations, use of labelled radio and stable isotopes, culture dependent and culture independent techniques have been used to demonstrate digestion and assimilation of bacteria in the midgut of sea cucumbers (Yingst, 1976; Moriarty, 1982; Gao *et al.*, 2014; Plotieau *et al.*, 2014). Labile food sources such as bacteria have relatively high intrinsic food values because they can be digested and absorbed quickly with assimilation efficiencies of ~ 40 % (Moriarty, 1982); therefore, maximum absorption efficiency is reached even during a short gut-residence time (Moriarty, 1982; Lopez and Levinton, 1987). Plotieau *et al.* (2013b) found that the number of bacteria in the digestive tract of *H. scabra* decreased significantly in the midgut and estimated that the overall assimilation efficiency was 59 % for bacterial digestion. The significant

overall reduction in bacterial diversity and the number of OTUs present in the midgut compared to the other sampling locations can be therefore be attributed primarily to digestion.

Following digestion in the midgut, Plotieau *et al.* (2013b) observed that the number of bacteria remained stable throughout the rest of digestive tract, whereas in the current study, a slight increase in the number of OTUs was observed in the hindgut followed by a rapid increase in the faecal mounds. A number of authors have reported rapid proliferations of bacteria in the hindgut of holothurians (Deming and Colwell, 1982; Ward-Rainey *et al.*, 1996; Gao *et al.*, 2014); however, the origin and status of bacteria remains unclear. Sibuet *et al.* (1982) found that a substantial fraction of bacteria consumed by deep sea holothurians survived the initial digestive processes and increase in abundance in the hindgut and faeces. Alternately, it has been suggested that bacteria may colonise the hindgut from the sediment, since the physico-chemical characteristics are not dissimilar between the two environments (Plante *et al.*, 1990) or that hindgut communities are in fact symbiotic resident bacteria attached to the epithelial lining (Deming and Colwell, 1982).

The faecal pellets of deposit feeders are undoubtedly sites of high microbial activity (Hargrave, 1976); however, it has been demonstrated that recolonization of faecal casts appears to be dominated by migration of bacteria from underlying sediments as opposed to repopulation by survivors of ingestion (Plante and Wilde, 2001). Bacterial colonisation of faecal pellets was previously thought to benefit deposit feeders through coprophagy; however, in aspidochirotid sea cucumbers it may represent an important component of a nutritionally bipolar feeder strategy (Jaeckle and Strathmann, 2013). For *H. scabra*, this may provide a mechanism to permit differential digestion and continuous feeding over a 24 hour cycle, whereby oral deposit feeding at night is alternated with anal suspension feeding during periods of inactivity during daylight hours. During the dark hours, *H. scabra* exhibits high levels of feeding activity, combined with rapid throughput of food through the gut (Mercier *et al.*, 1999), thereby maximising the rapid digestion and absorption of labile food resources. When sea cucumbers are buried, faeces is produced but gut passage is reduced, increasing the residence time of food in the hindgut (Mercier *et al.*, 1999; Lavitra, 2008), potentially favouring the digestion of more refractory food resources (Plante *et al.*, 1990). Furthermore, the bidirectional anal excretory and respiratory flows may enable the faecal mounds to function as an 'external rumen' in *H. scabra*, providing a further example of collective-cooperation in nutritional provision between microbial communities and a deposit feeder (Plante *et al.*, 1990; Harris, 1993). Excreted ammonium from the cloacal opening would supply a bioavailable source of nitrogen to bacterial communities colonising the faecal pellets, balancing the stoichiometry to permit the breakdown of more refractory organic matter

egested in the faeces. In the opposite direction, the dual respiratory/feeding current would permit the simultaneous uptake and assimilation of the organic matter breakdown products in the respiratory trees. This aspect of symbiotic microbial-deposit feeder interactions warrants further research.

In this study, the presence of a unique bacterial community in the midgut of *H. scabra* was detected and characterised (Table 6.7 and Appendix B). This is the first study to utilise next generation sequencing to describe the presence of a core microbiome in the midgut of a holothurian. Gao *et al.* (2014) utilised 454 pyrosequencing to analyse the composition and structure of the fore- and hindgut contents of *Apostichopus japonicus*; however, unfortunately they did not examine the midgut bacterial community. Plotieau *et al.* (2013b) conducted a detailed analysis of bacterial communities transiting the *H. scabra* gut in which bacterial abundance was enumerated by DAPI staining and phylotypes were characterised using fluorescent in situ hybridizsation. They found that the midgut community was dominated by *Vibrio* species, which represented 87 % of the clones; however, the use of culture-based techniques prior to cloning will have biased the bacterial community structure by increasing the number of opportunistic cultivable bacteria. Similarly, Bossers (2015) employed a culture-based step prior to sequencing the bacterial communities in the gut contents of *H. arguinensis* and found that the microbiome was dominated by *Vibrio* and *Pseudoalteromonas* species. Based on DGGE gels, Bossers (2015) did not find any significant difference in bacterial community composition between gut compartments, sediment and faeces or between the experimental diets tested and concluded that there was no evidence for adaption of the gut microbiome to diet. However, Amaro *et al.* (2012) did find significant differences in bacterial community composition between deep sea holothurian gut contents and the surrounding sediments and estimated that approximately 14 % of the total OTU richness was associated exclusively with the holothurian. This is the only study to date that supports the current findings detailed herein of a core gut microbiome in holothurians.

Microbial symbionts play key roles in complementing the nutrition of their host. The core microbiome in the holothurian midgut was dominated by chemoorganotrophic taxa that are commonly found in association with human and animal intestines. The midgut core microbiome contained 29 OTUs belonging to the order Clostridiales (Firmicutes) that contains polysaccharolytic taxa that play an important role in the fermentation of complex polysaccharides such as starch and fibre. These taxa and may be important in breaking down refractory sources of organic matter, such as seagrass detritus, in the natural habitat of *H. scabra.* The families *Lachnospiraceae* and *Ruminococcaceae* that were dominant in the core microbiome are positively correlated with the production of short chain fatty acids, including

formate, acetate, propionate, butyrate, and valerate (Daniel *et al.*, 2014). These fatty acids can play a key role in host nutrition by supplying energy to enterocytes lining the intestine and are known to be important in deposit feeder nutrition (Alongi and Hanson, 1985; Lin *et al.*, 2016).

Functional gaps exist in the sea cucumber digestome i.e. all the genes involved in digestion, including a lack of cellulase and chitinase enzymes (Feral and Massin, 1982), suggesting a role for collaboration with microbes in cellulose degradation. Bacteria within the genus *Ruminococcus*, that are important in ruminants where they digest a wide range of plant cell wall polysaccharides, including cellulose, were also present in the core midgut microbiome. *Ruminococcus* and *Coprococcus* are strictly anaerobic chemoorganotrophs that require fermentable carbohydrates to grow, and produce acetate, formate, succinate, lactate, and ethanol, which have been isolated from the rumen, large bowel, or cecum of many animals, including humans (Ezaki, 2009). *Prevotella*, an anaerobic and moderately saccharolytic bacterium that is among the most numerous microbes cultivable from the rumen and hindgut of cattle and sheep, where they help the breakdown of proteins and carbohydrates, was also present (Shah *et al.*, 2010). Since *H. scabra* lacks the enzyme cellulase, these saccharolytic bacteria may fulfill a functional role in the digestion of complex refractory sources of organic matter from seagrasses and terrestrial sources of complex carbohydrates in their natural habitat. Research on deep sea holothurians has demonstrated that bacteria present in the foregut and midgut are able to utilise a greater array of organic compounds, particularly carbohydrates, than bacteria present in ambient sediments (Roberts *et al.*, 2000).

In addition to bacteria being capable of fermenting complex carbohydrates, the midgut core microbiome was also characterised by bacteria with strong proteolytic activity and affinity for nitrogen-rich compounds. The most abundant amino acid fermenters belong to the Proteobacteria, *Clostridium* clusters and the *Bacillus-Lactobacillus-Streptococcus* groups that metabolize peptone and amino acids to organic acids. In the midgut core microbiome, *Clostridia, Peptostreptococci, Fusobacterium, Bacteroides, Propionibacterium, Actinomyces*, and also including Gram-positive cocci (e.g. *Peptococcus, Streptococcus, Ruminococcus, Megasphaera*) were important genera involved in amino acid metabolism using amino acids as energy and carbon sources (Davila *et al.*, 2013). Lactobacillales that have limited biosynthetic capabilities but strong proteolytic activity were also dominant members of the midgut microbiome. Bacteria with flexible metabolisms included *Corynebacterium*, commonly found as commensals in terrestrial and marine animals that are chemoorganotrophic, aerobic or facultative anaerobes capable of switching between

fermentative or oxidative metabolisms (Bernard and Funke, 2012). Only a few chemolithoautotrophic bacteria were identified, including *Sulfurovum* which grow by oxidising sulphur compounds coupled to the reduction of nitrate or molecular oxygen (Inagaki *et al.*, 2004). Two OTUs that could not be classified to family or genus levels were affiliated with nitrogen-fixing bacteria within the order Rhizobiales (Zehr and Bombar, 2015).

Host-microbial interactions in terrestrial organisms are well studied; however, marine examples have received less attention (Harris, 1993). Detritivore-microbial associations in the marine environment are likely, due to the similarity in physico-chemical conditions that prevail between the gut and ambient sediments (Plante *et al.*, 1990). The presence of a core microbiome has been discovered in association with terrestrial invertebrates including earthworms (Aira *et al.*, 2015), termites (Benjamino and Graf, 2016), and cockroaches (Schauer *et al.*, 2014), where the intestinal microbiota is metabolically active and plays a significant role in host physiology and metabolism. Further investigation of the role of holothurian symbionts in nitrogen fixation, re-use and recycling is warranted given the importance of the role in terrestrial counterparts (Schauer *et al.*, 2014; Benjamino and Graf, 2016).

In this study, nitrogen appeared to be an important environmental parameter associated with the structure, metabolism and genomic composition of bacterial communities. Rapidly degradable nitrogenous wastes and ammonia are the predominant forms of waste generated by intensive land-based aquaculture; the removal of ammonia-nitrogen is therefore one of the most important processes in aquaculture bioremediation (Antony and Philip, 2006; Chávez-Crooker and Obreque-Contreras, 2010). In the ambient sediments the total nitrogen and guanine-cytosine (GC) content of bacterial communities in the shrimp pond sediment was significantly higher than communities sampled from the dune sand treatments. Multiple regression analysis indicated that GC content was positively correlated with redox potential of the ambient sediment. The heavily reduced shrimp pond sediment had a significantly lower redox potential that would have increased the efflux of total ammonia-nitrogen (Thamdrup and Dalsgaard, 2008), with additional sources contributed from leaching from uneaten feed and sea cucumber excretion (Uthicke, 2001; Fenchel *et al.*, 2012). In all of the treatments, the concentration of total ammonia-nitrogen at the water-sediment interface was below the limit of quantification (<1 mg L^{-1}), despite a multitude of potential sources of ammonia. The experimental treatment receiving both types of aquaculture waste treatment (SPS_FW) had the highest total nitrogen content in the sediment, the highest concentration of nitrate at the sediment-water interface, and interestingly, supported bacterial communities with the highest GC content. This finding suggests an important link between sediment redox potential and

GC content, with increased supply of NH_4^+ under more reduced sediment provisioning nitrogen for nucleic acid synthesis.

Ammonia, nitrites and nitrates constitute the principal forms of reactive nitrogen available for incorporation into nucleic acids; however, ammonium is the preferential source of inorganic nitrogen assimilated by marine bacteria (Church, 2008). There is evidence to suggest that the GC content of prokaryotes — a measure of the percentage of nitrogenous bases in their genomes — is linked to the availability of nutrients in the environment (McEwan *et al.*, 1998). The nucleic acids contain different numbers of nitrogen atoms: thymine (T) contains two nitrogen atoms, cytosine (C) three, while adenine (A) and guanine (G) contain five (McEwan *et al.*, 1998). The GC bonds therefore contain eight nitrogen atoms while AT bonds contain only seven (McEwan *et al.*, 1998). Bragg and Hyder (2004) found that the GC content of prokaryotes is related adaptively to constraints on nitrogen availability in the environment and is positively correlated with average nitrogen use. For example, elevated GC content in plant symbionts has been related to their ability to fix atmospheric nitrogen and provision their host with nitrogen (McEwan *et al.*, 1998). The significantly higher GC content of bacterial communities in the shrimp pond sediments and the positive correlation with the significantly higher nitrogen content in the ambient sediment, may be an example of the adaptive capacity of microbial communities to respond to nitrogen - a key limiting element in marine coastal ecosystems (Fernandes *et al.*, 2012).

The composition of nucleic acids and proteins is also known to influence nutrient use (Bragg and Hyder, 2004). The highest nitrogen and GC content of microbial communities was observed in the ambient sediments of the treatment with both types of aquaculture waste (shrimp pond sediment fed with fish waste). In the midgut of sea cucumbers reared in this treatment, there was a significantly higher concentration of total nitrogen in the midgut and a strong enrichment in the relative abundance of genes predicted to be involved in nucleic acid metabolism. Marine benthic deposit feeders such as sea cucumbers are commonly food limited and nitrogen is thought to be the key nutrient limiting deposit feeder growth (Tenore and Chesney, 1985; Lopez and Levinton, 1987). Symbiotic bacteria in the sea cucumber core midgut microbiome, with a metabolic capacity to respond to increased nitrogen content in ambient sediments, may be an example of a nutritional interaction that enables deposit feeders to overcome nitrogen limitation in the marine environment (Tenore and Chesney, 1985). Under conditions of nitrogen limitation, purine bases may be preferentially digested, since purine bases contain twice the nitrogen content (N = 4) compared to pyrimidine bases (N = 2) (Berg and Jørgensen, 2006). For example, estuarine bacteria that are generally nitrogen-limited, have a high potential for cycling purines and their intermediates due to their lability

and may preferentially metabolize purine bases over pyrimidine bases (Berg and Jørgensen, 2006). Since microbial nucleic acids are highly digestible (~85 %), the degradation of purine bases to urea is one of the main sources of nitrogenous wastes (Berg and Jørgensen, 2006). These results pose the hypothesis that the peak in nitrogen content in the midgut was released from the degradation of purine bases, during digestion of bacterial communities with high GC content ingested from ambient sediments.

The degradation of purines to uric acid is common to all organisms and can occur under aerobic or anaerobic conditions (Vogels and Van der Drift, 1976). Bacteria capable of both pathways of purine degradation were present in the midgut core microbiome. Some species of *Brevibacterium, Paracoccus* and *Staphylococcus*, that were found in the midgut only are involved in aerobic degradation of purines and are able to use uric acid as their sole nitrogen and carbon source (Vogels and Van der Drift, 1976). Additionally, *Corynebacterium* is able to grow aerobically using the pyrimidine bases thymine or uracil as the only carbon, nitrogen, and energy source (Vogels and Van der Drift, 1976). The OTUs belonging to three of the *Clostridium* genera clusters that are able to degrade purines anaerobically were also a major part of the core holothurian midgut microbiome. Various members of the genus *Pseudomonas* that were numerically abundant in the midgut core microbiome are able to grow on purines either as a sole nitrogen source or as a nitrogen and carbon source (Vogels and Van der Drift, 1976). The presence of these taxa in the core midgut microbiome, together with the enhancement of predicted functional genes for purine metabolism, give support to the hypothesis that symbiotic bacteria play a key role in provision of nitrogen to their host.

In the majority of natural ecosystems, including marine and terrestrial environments, sources of inorganic nitrogen are severely limited (Capone, 2000). Degradation of purines to uric acid is common to all species but the degradation of uric acid varies between species and environments (Usuda *et al.*, 1994). In the marine environment, where water conservation is not an important factor, pathways for the excretion of nitrogenous wastes are primarily ammonotelic (Jangoux and Lawerence, 1982). In bacteria and marine invertebrates, including holothurians, uric acid is degraded to urea, which is then converted to ammonia and carbon dioxide by a complex enzymatic pathway (Bennet-Clark, 1973; Usuda *et al.*, 1994). The ammonia generated by the deamination reactions can be utilized as a nitrogen source in biosynthesis or excreted. In terrestrial systems, invertebrates such as termites, employ ureotelism (urea production) and uricotelism (uric acid production), as the two main pathways for excretion of nitrogenous wastes from proteins and nucleic acid metabolism (Potrikus and Breznak, 1980).

Many terrestrial invertebrates inhabiting nitrogen-limited environments, such as

termites and cockroaches, have evolved a symbiotic hindgut consortia that play a key role in nitrogen provisioning to the host via nitrogen re-use and recycling from uric acid (Brune, 2006; Sabree *et al.*, 2009). In these organisms, uric acid, originating from the degradation of purine bases, has been found to function as a metabolic reserve of nitrogen (Potrikus and Breznak, 1980; Sabree *et al.*, 2009). In wood-feeding termites (*Reticulitermes flavipes*), uric acid is stored in the fat bodies from where it can be mobilised to the hindgut where symbiotic bacteria ferment the uric acid to ammonia, CO_2 and acetate (Potrikus and Breznak, 1980). The holothurian digestive tract is known to excrete uric acid and crystals of uric acid have been observed in the respiratory trees and coelomic fluid of some holothurian species, including *H. tubulosa* (Jangoux and Lawerence, 1982). Crystalline deposits of uric acid have been found in zooxanthellae (marine symbiotic microalgae) in association with a number of hosts including anthozoans, scyphozoans, hydrozoans, bivalve molluscs, foraminifera and ciliates (Clode *et al.*, 2009). These deposits are thought to be associated with nitrogen metabolism of the symbionts which may represent a store of nitrogen that can be rapidly mobilized, thus allowing symbionts to flourish in nitrogen-limited environments (Clode *et al.*, 2009).

Elevated concentrations of nitrogen are associated with nitrogen re-use and recycling in the hindgut of terrestrial invertebrates such as termites and cockroaches (Brune, 2006). They habour endosymbionts that are able to respond to surplus quantities of nitrogen by storing it as uric acid which may then be mobilized in future for the synthesis of essential biochemical compounds (Sabree *et al.*, 2009). For example, the cockroach *Shelfordella lateralis* endosymbiont *Blattabacterium* can produce all of the essential amino acids, various vitamins, and other required compounds from a limited palette of metabolic substrates including urea, glutamate and ammonia (Sabree *et al.*, 2009). In this study, the predicted relative abundance of genes involved in amino acid metabolism and metabolism of co-factors and vitamins were enriched in the hindgut of sea cucumbers reared on the shrimp pond sediment. The synthesis of essential amino acids has been attributed to symbiotic bacteria in other organisms, including ruminants, herbivores and terrestrial insects, which have metabolic pathways that involve anabolism of amino acids. In the midgut, there was a peak in abundance of a number of bacterial genera, including *Prevotella* and *Streptococcus* (Table 2.1), which are key rumen bacteria capable of performing *de novo* amino acid biosynthesis in the cow rumen (Atasoglu *et al.*, 1998). It is possible that bacteria in the holothurian mid and hindgut may be playing a key role in *de novo* amino acid biosynthesis by recycling the nitrogen from purine degradation into the synthesis of amino acids and essential vitamins and co-factors.

Despite the significantly increased nitrogen content and GC content of bacterial communities in the ambient sediments prior to ingestion, enhanced purine metabolism in the midgut and the elevated metabolism of amino acids, vitamins and cofactors in the hindgut of sea cucumbers reared on shrimp pond sediments, this did not translate into differences in growth rate, proximate composition or condition factor. Despite sea cucumbers being frequently cited as nitrogen-limited, nitrogen does not feature in the main storage products that are commonly described. The main forms of nutrient storage in *H. scabra* are lipids and carbohydrates stored within the body wall and intestine (Krishnan and Krishnaswamy, 1970). It is possible that uric acid storage in the respiratory trees of aspidochirotid holothurians may represent a hitherto undocumented nitrogen store that may be mobilized by symbiotic microorganisms, thereby enabling deposit feeders to overcome nitrogen limitation.

6.5 Conclusion

Understanding the relationships between community structure and function is one of the key themes in microbial ecology (Santo Domingo *et al.*, 1998). The study confirmed the hypothesis that microbial communities would have high turnover along the gut with the lowest relative abundance and diversity in midgut due to digestion. The study highlighted the important role that endogenous bacteria play in nitrogen metabolism and the remediation of aquaculture wastes. The core midgut community was taxonomically and metabolically diverse and exhibited an enhanced capacity to respond to increasing nitrogen availability, a key nutrient commonly considered to limit deposit feeder growth by increasing purine and nitrogen metabolism. The significant increase in amino acid metabolism and metabolism of vitamins and co-factors in the hindgut of sea cucumbers reared on shrimp pond sediments supports the hypothesis that the gut microbiome plays a key role in nutrient provisioning to *H. scabra*.

The presence of a core midgut microbiome with an adaptive metabolic capacity to respond to the availability of limiting nutrients in the marine environment has important ecological and evolutionary implications. Symbiotic associations with bacteria that play a fundamental role in nutrient provisioning and recycling may be one of the reasons that sea cucumbers have become one of the dominant guilds in marine habitats ranging from the polar regions to the tropics and the shallow waters to the deep sea. A more in-depth study using labelled stable isotope combined with metagenomic shotgun sequencing is required to fully characterise the structure and function of the sea cucumber microbiome, likely to comprise a consortium of bacteria, archaea, fungi and eukaryotic protists and encompass all the organs that play a role in nutrient acquisition, including the body wall and respiratory trees.

Chapter 7. Synthesis

7.1 Synthesis of key findings

Bischoff (2012) defined a series of pre-requisites for the successful integration of detritivores into land-based intensive recirculating aquaculture systems (RAS); including, the need for high survival rates, the ability to grow on aquaculture-derived particulate organic matter (POM) as the sole feed source, and a net financial gain. The research presented in this thesis demonstrates that *Holothuria scabra* can be reared successfully with high survival rates (97 – 100 %) at commercially viable densities on waste from a marine RAS facility. The final biomass density of *H. scabra* fed aquaculture waste in combination with starch of 1,011.46 ± 75.58 g m^{-2}, was approximately four-fold higher than the average carrying capacity in the wild (250 g m^{-2}; Purcell and Simutoga, 2008) and was directly comparable to the final density of 1,028.50 ± 117.46 g m^{-2} achieved using a commercially formulated feed (Chapter 3). Furthermore, the cost-benefit analysis indicated that supplementation of waste with soluble starch as a carbon source yielded a net financial return of 122.29 % compared to the formulated feed. Two key outcomes of this research are: 1) demonstrating the potential to farm sea cucumbers at high density on aquaculture waste; and, 2) the ability to completely replace formulated feeds in sea cucumber aquaculture.

This thesis has demonstrated the potential to harness the concerted actions of microbial communities and deposit feeders in sediment-based systems to upcycle nitrogenous-rich effluent into high value biomass. The application of bioremediation technologies, centred on biostimulation, demonstrated that microbial communities exhibited differential responses to the addition of rate-limiting electron acceptors (oxygen) and the supply of organic carbon sources, with concomitant effects on biogeochemical cycling and deposit feeder growth. Increasing the availability of electron acceptors (oxygen) positively affected bacterial communities by increasing overall diversity and metabolism (Chapter 3); however, it resulted in stunted growth rates, significantly limiting the biomass yield of *H. scabra* (Chapter 2). In contrast, increasing the availability of electron donors (organic carbon substrates), significantly increased the growth rate and biomass density *H. scabra* (Chapter 4), but did not have a significant effect on microbial community structure (Chapter 5). The hypothesised mechanism underpinning the differences in sea cucumber growth involves changes in the pathways of nitrogen cycling mediated by sediment bacteria under contrasting redox regimes.

The sediment reduction-oxidation (redox) potential was a key driver of the differences in bacterial community structure (Chapter 3) and a significant predictor of sea cucumber growth and biomass density (Chapter 2). The redox-stratified sediment supported faster *H. scabra* growth rates and yielded a significantly higher biomass relative to fully oxygenated sediment (Chapters 2 and 3). It was hypothesised that the fully oxic sediment conditions altered the pathways of both carbon and nitrogen cycling, which affected the availability of food resources for *H. scabra* (Chapter 2); however, re-evaluation in light of the findings in Chapter 6 suggests that changes in nitrogen cycling under the differing redox conditions may have been more important than carbon oxidation.

The percolation of oxygenated water in the manipulated sediment systems was effective in maintaining the sediment under fully oxic conditions. The fully oxic conditions appeared to alter the pathways of nitrogen cycling, by favouring the oxidation reactions of nitrification. Analysis of the bacterial community indicated that nitrifying bacteria within the phylum Nitrospira had a high relative abundance in the fully oxic treatment but were not present in redox-stratified sediments. The significantly higher nitrite concentration, the positive sediment redox potential and the increased microphytobenthic production provided further indications that nitrification was the main nitrogen transformation pathway occurring. It is hypothesised that the step-wise oxidation of NH_4^+ to nitrate resulted in the removal of a large amount of bioavailable nitrogen (NH_4^+) and was the proximal cause for limitation of growth and biomass density of *H. scabra.*

As oxygen is the most thermodynamically efficient electron acceptor in redox reactions, oxygen availability it is one of the most important factors influencing sediment microbial communities (Reimers *et al.,* 2013). Oxygenation of the sediment resulted in a high taxonomic and functional diversity of bacteria with a diverse range of dissimilatory metabolisms required to successfully bioremediate aquaculture wastes (Antony and Philip, 2006). There was a clear differentiation in the trophic status of the sediment bacterial communities subjected to contrasting redox regimes; the majority of the biomarkers identified in the oxic sediment were autotrophs, whereas the redox-stratified sediments predominately supported heterotrophic communities (Appendix A; Table A1.1 and A1.2). The changes in community structure and function under contrasting redox regimes influenced deposit feeder growth, indicating that reducing conditions are more favourable for deposit feeder growth.

Increasing the carbon to nitrogen ratio from 5:1 to 20:1 through carbon supplementation significantly improved the growth rate and biomass carrying capacity of sea cucumbers reared on particulate organic aquaculture waste (Chapter 4); however, the proximal cause for increased growth rates and biomass production were not clearly

elucidated. Increasing the availability of labile organic carbon substrates did not significantly affect the bacterial community structure or the predicted relative abundance of functional genes involved in different nitrogen transformation pathways (Chapter 5). It was hypothesised that increasing the C:N would increase the metabolic demand for nitrogen by heterotrophic bacteria, thus promoting the assimilation of NH_4^+ into bacterial biomass. However, the benthic flux incubation study indicated that carbon addition significantly increased the efflux of NH_4^+ from the sediment. Thus, while carbon supplementation did not mediate a shift in nitrogen cycling, from net mineralisation to net assimilation as hypothesised, it did appear to stimulate other heterotrophic pathways, including the assimilatory fixation of N_2 gas (nitrogen fixation) and dissimilatory reduction of nitrate to ammonium (DNRA).

Microbial communities differed in the magnitude of their response to biostimulation through oxygen addition compared to the effect of organic carbon supply. The differential response of microbial community structure and function, in response to the availability of oxygen and carbon, may be explained by the types of microbial metabolism that were stimulated under the differing redox conditions. Organic carbon has a number of functional roles for microbial communities; as a potential electron donor in reducing reactions; as a substrate for fermentation; or, as a substrate that is directly assimilated for biosynthesis. Thus in redox-stratified sediments where anaerobic respiratory and fermentative metabolisms predominate and NH_4^+ is abundant, increasing the availability of labile organic carbon sources may stimulate a multitude of dissimilatory or assimilatory pathways, making it more difficult to unravel subtle changes in community structure and functioning. Bioenergetics is of greater importance in dissimilatory metabolism, since energy-releasing (dissimilatory) metabolic processes are generally coupled redox reactions (Fenchel *et al.*, 2012). Increasing the availability of oxygen as the most thermodynamically efficient terminal electron acceptor, therefore had a greater response on community metabolism. In contrast, assimilatory metabolism that contributes to biosynthesis, does not involve redox reactions, since it requires nitrogen to be in its lowest oxidation state. The redox-stratified sediments then appeared to provide favourable conditions to promote assimilatory pathways, further enhanced by the availability of carbon that resulted in increased growth performance of sea cucumbers.

Sediment redox potential was identified as a significant driver of holothurian biomass density in Chapters 2 and 4, and changes in microbial community structure in Chapters 3 and 6. Reducing conditions in marine sediments are known to favour nutrient transformation pathways that are reduction reactions, mediated by microbial communities in anaerobic respiratory pathways (Fernandes *et al.*, 2012). In marine sediments, nitrogen transformation pathways that are reduction reactions include DNRA, assimilatory reduction of nitrate to

ammonia, denitrification, and nitrogen fixation, while the predominant pathway for anaerobic organic carbon oxidation is sulphate reduction. The 16S rRNA gene sequencing in Chapters 3 and 5 confirmed that sulphate reduction and DNRA were the main carbon and nitrogen reduction pathways present respectively in redox-stratified sediments.

Increasing the availability of potential electron donors, through carbon supplementation, to microbial communities in redox-stratified sediments, significantly increased the efflux of NH_4^+ during the daytime and appeared to enhance nitrogen transformation pathways that contribute to nitrogen retention within the system, including nitrogen fixation and DNRA (Chapter 5). Increased organic carbon loading has been observed to stimulate DNRA over denitrification and heterotrophic nitrogen fixation in previous studies (Welsh *et al.*, 1997; Nizzoli *et al.*, 2010; Stuart *et al.*, 2016). The commonality between these pathways is the production of NH_4^+, which is the most bioavailable form of reactive nitrogen. Furthermore, since the assimilation of NH_4^+ occurs via the glutamine synthetase/glutamate synthase pathway, which requires four carbon atoms for every atom of nitrogen assimilated; the uptake of NH_4^+ is dependent on the availability of organic carbon (Church, 2008). In the absence of oxygen, ammonium accumulates in anoxic sediment layers since the dissimilatory reaction to transform NH_4^+ requires oxygen; thus, under anoxic conditions, nitrogen predominates in its lowest oxidation state (Hargreaves, 1998). From a thermodynamic perspective, marine bacteria preferentially assimilate nitrogen in its most reduced form, for incorporation into nucleic acids, amino acids, and proteins (Church, 2008). The benthic flux incubation study (Chapter 5) was sufficiently sensitive to detect the significantly higher rates of NH_4^+ from sediments amended with an exogenous carbon source; however, in the other experiments the NH_4^+ concentrations measured at the water-sediment interface were generally low or below the level of quantification. As nitrogen is a key nutrient limiting productivity, marine bacteria have developed very efficient mechanisms for NH_4^+ uptake (Bragg and Hyder, 2004; Church, 2008); in marine costal sediments, assimilation and incorporation into bacterial biomass consumes approximately 30 % of net NH_4^+ production (Blackburn and Henriksen, 1983).

In addition to the physiological and ecological adaptations that bacteria have developed to overcome nitrogen limitation, there is increasing evidence that bacteria also exhibit adaptions at the genome level in response to resource availability (Bragg and Hyder, 2004). In Chapter 3, the bacterial communities in the redox-stratified sediments had a significantly higher relative abundance of genes involved in purine and pyrimidine (nucleic acid metabolism) and nitrogen metabolism. The ecological and genomic flexibility of bacterial communities to respond to increases in nitrogen availability was further evidenced in

Chapter 6. Microbial communities in the aquaculture impacted sediment (shrimp pond sediment and fish waste), which were characterised by having the lowest redox potential and the highest nitrogen content, had a significantly higher guanine and cytosine (GC) content, reflecting a higher proportion of nitrogenous bases (Bragg and Hyder, 2004). This adaptive capacity was further evidenced by the metabolic response of the core sea cucumber midgut microbiome following ingestion and digestion of nitrogen-rich aquaculture waste streams by the increased and preferential metabolism of purine bases. The potential for mutually beneficial microbial-deposit feeder nutritional interactions and the role of symbiotic bacteria in nitrogen re-use and re-cycling was further highlighted by the increase in predicted pathways for amino acid metabolism and metabolism of vitamins and cofactors in the hindgut of sea cucumbers reared on nitrogen-rich shrimp pond sediment. Furthermore, the presence of unidentified taxa within the *Rhodobacteraceae* and Rhizobiales as part of the midgut microbiome, suggests that endosymbionts in marine invertebrates may play additional roles in overcoming nitrogen limitation through nitrogen fixation such as occurs in their terrestrial counterparts (Zehr and Bombar, 2015).

'Nutrition is a pervasive aspect of the biology of echinoderms' (Sloan and Campbell, 1982). In spite of two decades of breeding and research, the biological and ecological requirements of tropical sea cucumbers, including feeding mechanics and food assimilation of *H. scabra* in captivity remain understudied (Purcell *et al.*, 2012a). Deposit-feeders are commonly food limited; yet despite decades of research, the principal food sources and modes of deposit feeder nutrition are still uncertain (Levinton, 2013). A number of early studies indicated that microbes provide most or all of the organic nitrogen required by deposit feeding polychaetes (Rice, 1979; Cammen, 1980); however, it is generally believed that bacteria can supply only a small fraction of the energy requirements of macrofaunal deposit feeders (Kemp, 1980). This study demonstrates that in attempting to define the 'food' value of the sediment for deposit feeders as a biochemical parameter of bulk sediment such as the carbon to nitrogen ratio; protein; calories; dissolved and particulate organic matter; bacteria and detritus (Moriarty, 1982; Lopez and Levinton, 1987), the role of microbial communities in deposit feeder nutrition may have been overlooked.

A broad range of behavioural and physiological adaptations have been described for deposit feeders to enable them to exploit their food resources and maximise growth (Cammen, 1979; Phillips, 1984). Collaboration with symbiotic microbes for nutritional provisioning from inorganic nitrogen sources, principally NH_4^+, may be more important than other forms of nutrition previously thought to be important for deposit feeder growth. Indeed (Plotieau *et al.*, 2014) demonstrated that *H. scabra* was capable of assimilating inorganic nitrogen as

NH_4^+. Re-examination of the results of the Chapters 2 and 3 lends support to the hypothesis that NH_4^+ may be the primary food source for endosymbiotic bacteria. This hypothesis would explain the limited biomass densities and stunted growth of *H. scabra* reared on fully oxic substrates where NH_4^+ was converted to nitrate during nitrification – thus removing the food source for the symbiotic bacteria.

Redox-stratified sediments are net sources of ammonium (Hargreaves, 1998); however, deposit feeding macrofauna, including sea cucumbers, can increase NH_4^+ efflux by up to 50 % through bioturbation (Henriksen *et al.*, 1980) and further enhance the availability at the sediment-water interface through excretion (Uthicke and Klumpp, 1997; Uthicke and Klumpp, 1998). This study provides evidence that the role of sea cucumbers in enhancing the availability of their food resources may extend beyond the concept of 'microbial gardening' in ambient sediments, to microbial communities living in intimate nutritional relationships within their core microbiome. Close associations between deposit feeders and microbes have been known for decades; however, in this era of high throughput sequencing, molecular methods are revealing that we are on the cusp of demonstrating the true nature of host-symbiont interactions.

Modelling of nutrient flows in RAS indicated that bacteria and detritivores independently have very poor nitrogen retention compared to other trophic groups, with retention rates for nitrogen of 7 % and 0.06 % respectively (Schneider *et al.*, 2005; Verdegem, 2013). However, Schneider *et al.* (2005) acknowledged the nutrient assimilation capacity of detritivores may have been underestimated due to the limited availability of data. In this study, no attempt was made to quantify nitrogen retention in sea cucumber biomass; however, the high density production of *H. scabra* demonstrated here indicates the potential to harness the concerted actions of microbial communities and deposit feeders for the bioremediation of nitrogen-rich wastes originating from land-based aquaculture when sediments are taken into account. Additionally, targeting of specific tissues may have led to an underestimation of nitrogen retention in deposit feeders, especially if hitherto undiscovered stores of nitrogen, e.g. uric acid, are present such as occurs in terrestrial insects and other marine invertebrates (Potrikus and Breznak, 1980; Clode *et al.*, 2009; Sabree *et al.*, 2009).

Marine sediments occupy 70 % of the Earth's surface and contain one of the largest pools of nitrogen, bound as ammonium in sediments (Thamdrup and Dalsgaard, 2008; Fenchel *et al.*, 2012). Globally, coastal estuaries and marine sediments are significant sinks for terrestrial nitrogenous inputs; however, in recent decades land-based intensive aquaculture has increased the inputs of biologically available nitrogen to marine ecosystems (Nixon, 1995; Gardner *et al.*, 2006; Hardison *et al.*, 2015). The data presented in Chapter 5, indicate

that marine calcium carbonate sediments have a high assimilative capacity for organic matter, even under nutrient loading rates consistent with eutrophic estuaries (400 mmol C m^{-2} day^{-1}). The nitrogen loading rate used in Chapter 4 was standardised at 120 mg N m^{-2} day^{-1}, which was a mid-high (100 – 150 mg N m^{-2} day^{-1}) ration for deposit feeders. In Chapter 5 this was doubled to 240 mg N m^{-2} day^{-1}, which comes close to approximating the ammonia excretion rate of 300 mg N m^{-2} d^{-1} from feeding a 32 % protein diet (Hargreaves, 1998). There is a growing body of research that is demonstrating that, in tropical sediment systems, processes that recycle and retain NH_4^+ dominate as mechanisms of overcoming nitrogen limitation (Fernandes *et al.*, 2012; Song *et al.*, 2014; Newell *et al.*, 2016a). The significant increase in NH_4^+ combined with the net influx of N_2 into the sediment (Chapter 5), indicates that pathways of nitrogen retention dominated over pathways of permanent removal, thereby underscoring the high capacity for sediments to assimilate nitrogen from land-based intensive systems.

The suitability of heterotrophic systems for microbial-driven bioremediation systems integrating deposit-feeding sea cucumbers was identified at the start of this thesis (Chapter 1). The benthic flux incubation experiment demonstrated that sediments, with and without carbon addition, were net heterotrophic (Chapter 5), while the data from long-term growth studies (Chapters 2 and 4) provides evidence that predominately reducing conditions in redox-stratified sediments are the most suitable environment for deposit feeder growth. Under anaerobic conditions, the growth efficiencies of bacteria are lower, hence a large proportion of energy is lost during catabolism and rather than being incorporated into biomass it ends up as metabolites (Fenchel *et al.*, 2012). This leads to intense recycling of nitrogen within the microbial communities. Redox-stratified sediments are thus primarily net heterotrophic systems in which nutrients are recycled *in situ*

In nitrogen-limited systems, such as mangrove and seagrass ecosystems (*H. scabra'*s natural habitat), recent studies have indicated that DNRA and heterotrophic nitrogen fixation are important processes for retaining nitrogen and sustaining ecosystem productivity (Fernandes *et al.*, 2012; Decleyre *et al.*, 2015; Enrich-Prast *et al.*, 2016). Fernandes *et al.* (2012) hypothesised that mangroves are closed systems, where nitrogen is recycled and re-used, with the net effect of nitrogen conservation and retention in the system. The same type of nitrogen re-use and recycling has been demonstrated within the hindguts of termites, which can also be considered as closed systems, where the loop in the nitrogen cycle is closed when microbial biomass is digested and/or the products of nitrogen storage are assimilated into cellular components (Brune, 2006). It appears that symbiotic bacteria in the holothurian

microbiome play a similar role in nitrogen recycling, re-use and provisioning to the host as a mechanism to overcome nitrogen limitation.

In conclusion, the potential to remediate nitrogenous wastes was demonstrated at multiple levels within the microbial−deposit feeder aquaculture bioremediation system. Firstly, within the capacity of sediment microbial communities to recycle, re-use and retain nitrogen under high loading rates; secondly, as the genome-level response of indigenous bacterial communities within ambient sediments in response to high nitrogen content; thirdly, within the enhanced metabolic capacity of the sea cucumber microbiome to respond to increased nitrogen availability and overall, the enhanced growth rates and biomass carrying capacities of *H. scabra* reared on predominately reducing sediments. Taken together, these mechanisms to overcome nitrogen-limitation, displayed from genome level to ecosystem level effects, highlight the potential that exists to exploit the metabolic capacity and functional potential of microbial communities in sediment-based aquaculture bioremediation systems to upcycle waste sources of nitrogen into high value biomass.

7.2 Overview of the proposed technology and applications of the research

This thesis proposes a novel aquaculture bioremediation system to treat effluent from intensive land-based aquaculture operations that is a hybridisation of a number of approaches currently championed to improve aquaculture sustainability; namely, integrated multi-trophic aquaculture (IMTA), microbial-based systems, and bioremediation techniques. The manipulated sediment-based system integrating a deposit feeding sea cucumber is designed to receive particulate organic wastes that are concentrated and removed during the first treatment step (solids removal) in marine RAS. However the concepts developed herein are broadly applicable and transferrable to flow-through systems, where particulate wastes can be separated by mechanical filtration prior to exiting the facility; suspended solid wastes recovered from zero-exchange biofloc systems and heterotrophic bacterial biomass produced from technological developments in next generation RAS, such as denitrifying filters and anaerobic digesters. The system described herein therefore has a wide range of applications for the treatment of solid and particulate wastes (sediments, biofloc sediments, concentrated suspended solids, sludge) from land-based marine aquaculture, including flow-through, semi-closed, re-circulating and zero exchange aquaculture operations. It is envisaged that the technology described here will be operated as an independent downstream effluent treatment module to treat particulate organic wastes and used for high-density production of deposit feeding sea cucumbers.

Beyond the treatment of particulate organic wastes, this system may have the potential to offer holistic wastewater treatment in RAS. The use of next generation sequencing in this thesis demonstrated that a broad range of functional groups of autotrophic and heterotrophic bacteria that are commonly employed in water treatment technologies in RAS to process particulate and dissolved organic and inorganic wastes (Rurangwa and Verdegem, 2015) were present in the sediments under contrasting redox-regimes. Classification of taxonomic biomarkers (according to their dissimilatory metabolisms and oxygen-related ecophysiology), revealed that nitrification and sulphide oxidation were significantly enriched in the oxic sediments (Chapter 3), while bacteria performing sulphate reduction, denitrification and DNRA dominated in the redox-stratified sediments. Taxa that perform anaerobic ammonium oxidation (anammox) and methanogenesis, which are also used in RAS, were not detected here, although unidentified OTUs within the phylum Planctomycetes were present.

Society's desire to exert greater control over the environmental conditions of the primary culture species produced in RAS is centred on the use of different functional groups of microorganisms for complete removal of organic and inorganic nutrients to ensure the provision of clear, highly oxygenated water. Consequently, RAS performance relies on the separation of these different electron donors (carbon) to replicate the environmental conditions for optimum growth (Rurangwa and Verdegem, 2015). Compared to a RAS, the microbial — deposit feeder aquaculture bioremediation system, that replicates naturally occurring pathways of carbon and nitrogen cycling present in marine sediments, fulfils many of the functions that are typically housed in individual compartments, e.g. solids removal and coupled nitrification-denitrification (Table 7.1).

As molecular methods have developed and replaced culture-based microbial ecology, our understanding of the interdependency of microorganisms that exist as communities has increased. There is intense re-use and recycling of nutrients within the microbial communities present in redox-stratified sediments, such that the metabolite of one acts as a substrate for another. By separating the component processes into different units within a RAS, the energy and pumping costs not only increase in direct proportion of the number of filtration units, but the transfer efficiencies and overall functional potential to the concerted actions of the microbial communities is likely reduced.

Table 7.1. Overview of the different functional compartments in a recirculating aquaculture system (RAS) compared to a redox-stratified sediment-based system. DOM = dissolved organic matter; DNRA = dissimilatory reduction of nitrate to ammonia.

Function	**RAS**	**Sediment**	**Sea cucumber**
Solids removal	Sand/drum filter	Mechanical filtration/bacterial hydrolysis	Direct: ingestion & assimilation Indirect: bioturbation
DOM removal	Foam fractionator	Mineralisation (heterotrophic bacteria)	Direct absorption across body wall; anal suspension feeding
Nitrification ($NH_4^+ \rightarrow NO_2^-$; $NO_2^- \rightarrow NO_3^-$)	Biofilter	Oxic layer (chemolithotrophic bacteria) Assimilation of NH_4^+ by microphytobenthos and bacteria	Excretion of NH_4^+ enhances microalgae & bacteria growth which assimilate NH_4^+
Denitrification; DNRA; Anammox ($NO_3^- \rightarrow N_2$; $NO_3^- \rightarrow NH_4^+$; $NH_4^+ + NO_2^- \rightarrow N_2$)	Denitrifying filters Anammox filters;	Anoxic layer (heterotrophic bacteria; denitrifiers & DNRA)	Enhanced capacity for nitrogen metabolism in core midgut microbiome
Regulation of pH	Water exchange, chemical buffers	Buffering by $CaCO_3$ sediment	Dissolution of $CaCO_3$ in gut, increases pH

The current generation of recirculating aquaculture systems are suited to high value marine finfish that require finely controlled water quality (Badiola *et al.*, 2012); therefore, it is unlikely that existing water treatment technologies will be supplanted by low-tech solutions such as this sediment-based system. Nevertheless, in the current era of innovative RAS technology development, there is potential to conceive the integration of the system described herein as a downstream hybrid production and effluent treatment unit dovetailed onto partial re-use and conventional RAS practices. The potential to pass the discharge water containing POM and effluent water through a downstream sediment-based treatment system may have the capacity to reduce particulate and dissolved nutrients prior to discharge or re-use.

Furthermore, given the functional overlap between RAS filtration and processes naturally present in calcium carbonate sediments, deposit feeder aquaculture bioremediation systems may offer the potential to overcome two key problems associated with RAS; 1) decreasing pH due to the increase in H^+ concentration following reduction of NH_4^+ by ammonia-oxidising bacteria in the first step of nitrification in biofilters, and 2) high nitrate levels produced by nitrite-oxidising bacteria, in the final step of nitrification. Use of chemolithotrophic bacteria in fixed-film biological filters results in the consumption of alkalinity and the production of CO_2; processes that together contribute to a decrease in pH of

recirculating seawater (Ebeling *et al.*, 2006). RAS that employ biological filers for nitrification often have to dose the system with sodium bicarbonate to maintain alkalinity levels and employ CO_2-stripping towers. The buffering capacity of carbonate-based substrates would be an important attribute in the land-based bioremediation system, particularly in RAS that have to be dosed to counter pH decreases due to the nitrification activity in the biological filter.

Conventional RAS rely on water exchange (<10 %) to remove NO_3^- produced during the step-wise oxidation of ammonia to nitrite and nitrate in the biological filter (Ebeling *et al.*, 2006; Badiola *et al.*, 2012). Next generation RAS (<1 % water exchange) are developing systems for the permanent removal of nitrogen from the system by adding additional components such as filters to house anammox and denitrification (Martins *et al.*, 2010), while ecologically-driven systems such as IMTA are focusing on the integration of macroalgae for nitrate removal (Neori *et al.*, 2004; Metaxa *et al.*, 2006). In addition, some intensive biofloc systems rely on the nitrification activity of the floc to control water quality, which creates the same problem and thus requires the addition of denitrifying filters (Hargreaves, 2013). The former technological developments not only increase costs, but permanently remove nitrogen – the most valuable nutrient, and also have the potential to contribute to climate change by increasing emissions of nitrous oxides (Tsukuda *et al.*, 2015). Alternatively, the culture of micro- or macroalgae requires large surface areas, which are not compatible with RAS in many countries where land is scarce. It is possible that the high-density sediment-based system described herein is capable of maintaining nitrate at levels below the threshold for discharge since redox-stratified sediments are a net sink for nitrite and nitrate (Hargreaves, 1998). Additionally, DNRA has been shown to account for up to 99 % of nitrate removal in mangrove ecosystems (Fernandes *et al.*, 2012) which have been proposed as ecologically-driven aquaculture bioremediation systems (Turcios and Papenbrock, 2014).

The sediment-based aquaculture bioremediation systems described herein are relatively low-tech systems with limited engineering that utilise cheap, readily available materials such as calcium carbonate 'builders' sand to provide a high surface area for microbial colonisation. The risk of exceeding the assimilative capacity of the system can be mitigated through the inclusion of an undergravel filtration system that can be activated to increase mineralisation rates and 'flush' the system if required. The limited engineering and low materials cost means that microbial − deposit feeder aquaculture bioremediations systems can offer a simple, low cost option to diversify production, increase revenue, and reduce environmental impacts (particularly in lower income countries in the tropics). The economic and technical advantages of the system include, but are not limited to:

- No cost to transport and dispose of sludge
- Reduction in the energy costs and surface area required for filtration systems treatment;
- Reduced costs for purchase of buffering chemicals
- Production of high value secondary crop (14-19 US$ m^{-2}).

This novel conceptual approach may not only provide a low-cost, value-added solution to the treatment of wastes from land-based intensive aquaculture but also offers a sustainable alternative for global sea cucumber production to meet the insatiable and growing demand from Asian markets. Where possible, it is recommended that future investments in the development of sea cucumber aquaculture be targeted at integration into existing land-based aquaculture activities to remediate waste streams, reduce environmental impacts and avoid placing additional pressure on natural resources. Furthermore, as the technology is transferable to other candidate deposit feeders, it may encourage aquaculture diversification to produce other high-value species such as polychaete worms that may be used for broodstock feeds or bait for anglers.

7.3 A new approach to closing the nitrogen loop

In the global nitrogen cycle, the size of the 'fixed' or 'reactive' nitrogen pool is determined by the balance of processes that fix nitrogen from the atmosphere (nitrogen fixation) and those that return nitrogen back to the atmosphere such as anaerobic oxidation of ammonia (anammox) and denitrification. This approach has been adopted in aquaculture bioremediation technologies such that processes that permanently remove nitrogen from the system, including nitrification, denitrification, and anammox are considered 'beneficial' while transformations that retain nitrogen in the system, including mineralisation; remineralisation and DNRA are considered 'detrimental' (Castine *et al.*, 2013a). The current paradigm to deal with the accumulation of inorganic nitrogen, is to close the loop in the nitrogen cycle, by converting reactive nitrogen back to its inert form as N_2 gas. Accordingly, technological developments in next generation RAS are focusing on permanently removing nitrogen from the culture system via conversion to dinitrogen (N_2) gas by denitrification or anaerobic oxidation of ammonia (Tal *et al.*, 2006; van Rijn *et al.*, 2006; Martins *et al.*, 2010).

In conventional RAS, removal of ammonia-nitrogen in the final biological filtration stage is based on nitrification, which requires the removal of particulate organic matter and high levels of aeration to deliver supplemental oxygen to transform inorganic nitrogen from its lowest oxidation state (-3) as NH_4^+ to its highest oxidation state (+5; Figure 7.1).

Furthermore, the reaction is inefficient, yielding limited autotrophic bacterial biomass, since only a very small fraction of the electron donor (6.2 %) is used for biosynthesis, with the remainder of the energy (93.8 %) used in the respiration of nitrite and nitrate (Ebeling *et al.*, 2006). In next generation RAS, separate filters are being developed to house the process of denitrification, that require exogenous carbon sources to reduce nitrate to dinitrogen gas (N_2), thus transforming inorganic nitrogen across an additional five oxidation states (Figure 7.1). The approach of using coupled nitrification-denitrification therefore transitions nitrogen through a total of 13 oxidation states and six different functional groups of bacteria (Figure 7.1).

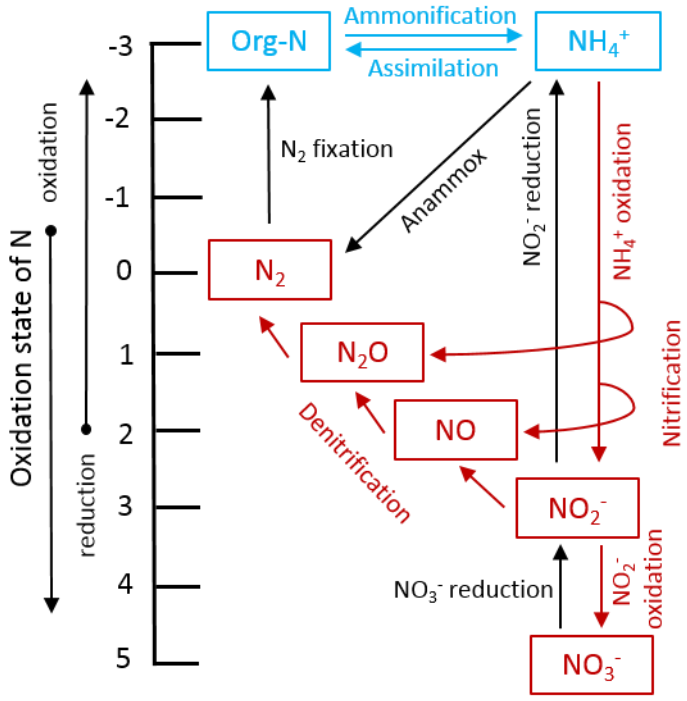

Figure 7.1. The microbial reduction-oxidation reactions in the global nitrogen cycle. Adapted from Capone (2000) and Kirchman (2012) to illustrate the different focal points of the nitrogen cycle (N_2 gas versus organic nitrogen). Nitrogen re-use by assimilation of NH_4^+ into organic nitrogen (Org-N; biomass) is illustrated in blue and permanent removal of nitrogen by conversion of NH_4^+ to dinitrogen gas (N_2; denitrification) is illustrated in red.

Reactive or 'fixed' nitrogen in its inorganic form (NO_3^-, NO_2^- and NH_4^+) constitutes less than 0.1 % of the total nitrogen in the Earth's biosphere and is one of the key nutrients limiting marine productivity (Thamdrup and Dalsgaard, 2008). The paradoxical situation is that nitrogen limitation, which constrains the growth and biomass production of most terrestrial and aquatic organisms, results from the intense re-use, recycling and retention of fixed nitrogen by microbes to meet their high nitrogen demands (Kirchman, 2012). In land-based intensive aquaculture, the accumulation of inorganic nitrogen, principally as NH_4^+ produced as the end result of protein catabolism in marine organisms reared on high protein formulated feeds, is considered to be one of the main threats due to its toxicity in its unionised

form. Furthermore, since feeds typically account for 40-60 % of the total production costs, and only 25 % of the nitrogen in feed is assimilated, this represents a substantial economic loss. When the source of protein used in the formulated feeds is acknowledged to be wild-caught fish, a finite resource, the environmental sustainability issues are even more clearly elucidated. After such expense of energy and consumption of finite resources, can the permanent removal of nitrogen, by conversion back to N_2 gas, be considered a sustainable approach to aquaculture technology development? Nitrogen is too valuable to be lost, rather than looking to promote technologies for permanent removal of nitrogen from culture systems, research should focus on recycling and re-use of waste in integrated aquaculture bioremediation and production systems.

Current technological developments in next generation RAS are trending towards the promotion of anaerobic heterotrophic pathways for wastewater treatment, including anaerobic digestors, denitrifying and anammox filters. The technology proposed in this thesis aligns with current trends, however it shifts the paradigm from considering N_2 as the final sink for nitrogen, to organic nitrogen (N_{org}) as high value biomass. This research advocates a transition from the reliance on pathways of permanent removal to closing the nitrogen loop, to assimilation of NH_4^+ into bacterial and deposit feeder biomass as an economically and environmentally attractive alternative. In the proposed system, dissimilatory pathways previously considered to be detrimental, (DNRA, mineralisation and remineralisation) are encouraged, since they lead to the transformation of nitrogen into ammonium — the most bioavailable form for bacteria. The approach advocated here is beautiful in its simplicity: it simply focuses on minimising the energy involved in nitrogen transformations by: 1) focusing on assimilatory reactions that are not redox reactions and therefore do not expend energy; and 2) maintaining N in its most bioavailable form as NH_4^+.

Only by rethinking our approach to the development of sustainable aquaculture bioremediation technologies, can we begin to adequately address the current inefficiencies of nitrogen use within the aquaculture production chain. Current inefficiencies of nitrogen use were highlighted in Chapter 1 at multiple levels within the aquaculture production chain, namely the use of wild caught fish for aquaculture production and the balance of 'fed' species versus extractive species leading to the net nitrogen loading in the global nitrogen cycle. When the balance of processes is viewed from a thermodynamic perspective, the alterative to closing the nitrogen loop advocated in this thesis is clear. In order to re-equilibrate the current inefficiency of nitrogen use in aquaculture, it is necessary to balance the dissimilatory process of protein catabolism with assimilatory processes that utilise NH_4^+ as the end product of protein catabolism to close the nitrogen and energy cycle (Figure 7.1). It is anticipated that

this research, illustrating the metabolic capacity of microbial communities within sediments and deposit feeder guts, that are primed at ecological and genomic levels, to respond to nitrogen, will provide a sound basis for the future development of ecologically-driven aquaculture bioremediation systems.

7.4 Future research

This thesis provides the initial scientific foundation for the future development of sediment-based aquaculture bioremediation systems; however, much research remains to be conducted to develop and validate the system *in situ.* Future research should focus on pilot testing deposit feeder sediment-based effluent treatment systems in conjunction with existing land-based aquaculture to test their ability to treat particulate organic waste originating from intensive land-based aquaculture systems. The research should aim to advance the findings detailed in this thesis by conducting multifactorial experiments to determine the optimum quality, quantity and frequency of addition of particulate organic aquaculture wastes to maximise biomass production of deposit feeders in sediment-based bioremediation systems. In these studies, the collection of baseline and time-series data for environmental, water quality and sediment quality parameters will be critical for the parameterisation of models in future system development and optimisation.

Since redox-stratified sediments − predominately reducing environments that approximate the natural habitat of *H. scabra* − have been shown to support significantly higher biomass densities, further studies on carbon addition are needed. As dissolved inorganic nutrients are assimilated simultaneously with the uptake of organic substrates, heterotrophic assimilation of NH_4^+ is encouraged, through the addition of supplementary carbon to enhance the metabolic demand for nitrogen (Fenchel and Blackburn, 1979; Church, 2008). Future studies should focus on utilisation of co-effluent streams from aquaculture (nitrogen-rich waste) and agriculture (carbon-rich waste), since many agricultural practices in temperate and tropical environments result in the production of complex carbohydrate wastes. Cellulose and starch-based polysaccharides such as bagasse, that have a slower degradation rate are likely to provide a more durable substrate for microbial utilisation (Chapter 4). This recommendation not only aligns with current priorities to promote nutrient recycling and encourage value-addition of waste streams in both primary industries, but is encouraged by the cost-benefit analysis demonstrating that economic benefits were only gained by utilising soluble starch as a carbon source. An intriguing option for the future development of downstream microbial-deposit feeder aquaculture bioremediation systems would be the planting of marine angiosperms for the *in situ* supply of autochonthous dissolved organic

carbon sources, as exudates from photosynthesis released by the roots of seagrasses could replace costly exogenous sources. This type of system would parallel constructed wetlands used for the remediation of freshwater aquaculture effluents (Turcios and Papenbrock, 2014). Furthermore, it has the potential to further increase the biomass yield of *H. scabra* by supplying exogenous sources of carbon and nitrogen via symbiotic bacteria in the rhizosphere of seagrasses (Welsh *et al.*, 1997).

Future studies should take a 'systems' based approach to the study of the system microbiome. The system microbiome incorporates all components of the culture system, including examination of microbial communities at all sediment depths, in the water column and in various locations within the sea cucumber. Sea cucumber sampling should extend beyond the digestive tract to other organs that play a role in nutrition, including the respiratory trees, the *rete mirabile* and epidermis, where microbial-host nutritional interactions may be present. Dual labelled stable isotope studies employing ^{13}C sources and ^{15}N–labelled particulate aquaculture waste, targeting multiple holothurian tissues and compartments within microbial sediment communities, would be crucial to elucidate the exact pathways underpinning the proximal causes of increased growth, as demonstrated by Jaeckle and Strathmann (2013).

The research conducted herein adopted an integrated multi-disciplinary approach, with the aim of understanding the benthic deposit feeder microbial recycling system and how the overall ecosystem function can be enhanced by the stimulation of endogenous microbial communities. The adoption of a fully integrated, systems-level approach to the study of 'aquaculture system microbiomes' will be essential to the further development of aquaculture bioremediation technologies. In this context, it is argued here that the concept of IMTA should be redefined and expanded beyond the limited 'macroscopic', species-specific definition of trophic levels, to a broader ecosystem approach that encompasses microorganisms (bacteria, protists, viruses, meiofauna) in recognition of their fundamental role in underpinning the biogeochemical reactions mediating aquaculture bioremediation.

Elemental stoichiometry will be central to the future understanding and performance of microbial-based aquaculture technologies, due to the close coupling between microbial community structure and function, biogeochemical cycling and deposit feeder growth. This is likely to extend well beyond the macromolecular level to the fundamental building blocks of genomes as microbes exhibit their adaptive capacity in response to key limiting nutrients. Hence, it is recommended that future studies extend their examination of the elemental cycling to include phosphorous (C:N:P), a key nutrient for nucleic acid synthesis and energy (ATP) production. It is anticipated that the adoption of techniques and principles

underpinning the emerging field of stochiogenomics will permit the further exploration of resource-driven DNA evolution in the context of nitrogen limitation and microbial—deposit feeder interactions and their impact on global biogeochemical cycles (Elser *et al.*, 2011).

7.5 Conclusion

This study has demonstrated the potential to harness the concerted actions of microbial communities and deposit feeders to process and upcycle nitrogen-rich effluent into high value biomass. As land-based intensive aquaculture production expands to meet the growing global demand for protein, the use of formulated feeds that underpin aquaculture intensification will lead to a proportionate increase in concentration of NH_4^+ and the volume of particulate organic waste requiring treatment. Technological developments in next generation RAS and zero exchange biofloc systems that aim to increase water re-use are further contributing to the volume of the solid waste that requires disposal. Marine bacteria, deposit feeding sea cucumbers and the tropical seagrass and mangrove ecosystems they inhabit, are all nitrogen-limited systems. Due to their complex pathways based on nitrogen re-use and re-cycling and their high capacity for nitrogen assimilation, marine sediment-based systems represent a natural basis for the development of aquaculture bioremediation systems. With their inherent genomic and metabolic capacity to respond to nitrogen, even in situations of high organic loading, the systems described herein may provide a viable option for ecologically-driven integrated aquaculture bioremediation and production systems. Furthermore, deposit feeder—microbial aquaculture bioremediation systems have the potential to rectify the current imbalance of inefficient nitrogen use in the aquaculture production chain by offering a more economically and environmentally sustainable alternative to closing the nitrogen cycle loop.

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Appendix A. Functional classification of sediment microbial communities under constrasting redox regimes

Table A1.1 Classification of the taxonomic biomarkers identified by linear discriminant effect size analysis (LEfSE) in oxic sediments according to their reduction-oxidation (redox) zone, oxygen-related ecophysiology and type of dissimilatory metabolism

Metabolism & redox zone			Phylum	Class	Order	Family	Genus	Relationship to O_2	Ecological role	Ref
Heterotophic	Aerobic respiration	Oxic	Bacteroidetes	Cytophagia	Cytophagales	*Flammeovirgaceae*		Aerobe	Oxidation: carbohydrates	[1]
			Bacteroidetes	[Rhodothermi]	[Rhodothermales]	*Rhodothermaceae*		Aerobe	Oxidation: sugars & amino acids	[2]
			Planctomycetes	OM190	CL500_15			Aerobe		
			Planctomycetes	Phycisphaerae	Phycisphaerales			Aerobe	Oxidation: sugars & sugar alcohols	[3]
			Planctomycetes	Planctomycetia	Pirellulales	*Pirellulaceae*		Aerobe		
			Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	*Planctomyces*	Aerobe or facultative anaerobe	Oxidation: carbohydrates	[4]
			Proteobacteria	δ-proteobacteriaγ	Myxococcales	*Nannocystaceae*	*Plesiocystis*	Obligate aerobe	Proteolytic/bacteriolytic	[5]
			Proteobacteria	γ-proteobacteria	Alteromonadales	*Alteromonadaceae*	*Alteromonas*	Aerobe	Oxidation: carbohydrates, alcohols, organic & amino acids.	[6]
			Proteobacteria	γ-proteobacteria	Alteromonadales	*Alteromonadaceae*	*nsmpVI18*	Aerobe	Degradation: ι-carrageenan, methylthiopropanoate	[7]
			Proteobacteria	γ-proteobacteria	Legionellales	*Coxiellaceae*		Aerobe	Obligate intracellular parasite	[8]
			Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	*Verrucomicrobiaceae*		Aerobe	Oxidation: mono- & disaccharides	[9]
	Fermentation	Anoxic	Chloroflexi	Anaerolineae	DRC31			Strict anaerobe	Chemoorganotrophic, non-photosynthetic	[10, 11]
			Proteobacteria	γ-proteobacteria	Vibrionales	*Vibrionaceae*	*Photobacterium*	Facultative anaerobe	D-glucose & D-mannose catabolism with the production of acid	[12]
Methylotrophs			Proteobacteria	γ-proteobacteria	Thiotrichales	*Piscirickettsiaceae*		Aerobe		[13]
Chemolithotrophic		Oxic	Nitrospirae	Nitrospira	Nitrospirales	*Nitrospiraceae*		Obligate aerobe	Nitrite oxidation	[14]
		Anoxic	Actinobacteria	Acidimicrobiia	Acidimicrobiales	*C111*			Ferrous iron (Fe^{2+}) oxidation	[15]
			Proteobacteria	γ-proteobacteria	Thiotrichales	*Thiotrichaceae*	*Thiopilula*		Sulphur oxidation	[16]
Phototrophic		Oxic	Cyanobacteria	Chloroplast	Stramenopiles			Aerobe	Oxygenic photosynthesis	
		Anoxic	Proteobacteria	α-proteobacteria	Rhodobacterales	*Rhodobacteraceae*		Anaerobe	Anoxygenic photosynthesis; oxidation of HS^- to S	[17]
			Proteobacteria	γ-proteobacteria	Chromatiales	*Ectothiorhodospiraceae*		Anaerobe	Oxidation of HS^- to S	[18]

Table A1.2. Classification of the taxonomic biomarkers identified by linear discriminant effect size analysis (LEfSE) in oxic-anoxic sediments according to reduction-oxidation (redox) zone, oxygen-related ecophysiology and type of dissimilatory metabolism.

Metabolism & redox zone			Phylum	Class	Order	Family	Genus	Relationship to O_2	Ecological role	Ref
Heteroptophic	Oxic	Aerobic respiration	Proteobacteria	ε-proteobacteria	Campylobacterales	*Helicobacteraceae*		Microaerophilic or anaerobic	Reduction: fumarate to succinate	[19]
			Spirochaetes	[Leptospirae]	[Leptospirales]	*Sediment-4*	*SJA-88*	Obligate aerobe/ microaerophile	Oxidise long-chain fatty acids or long-chain fatty alcohols	[20]
	Anoxic	Anaerobic respiration	Proteobacteria	δ-proteobacteria	Desulfobacterales	*Desulfobacteraceae*	*Desulfobacter*	Strict anaerobe	Reduction: sulphate, sulphite & thiosulphate to H_2S	[21]
			Proteobacteria	δ-proteobacteria	Desulfobacterales	*Desulfobulbaceae*		Strict anaerobe	Reduction: sulphate to sulphide	[22]
			Tenericutes	Mollicutes				Facultative/ obligate anaerobe	Commensals or parasites	[23]
		Fermentation	Bacteroidetes	Bacteroidia	Bacteroidales	*Marinilabiaceae*		Facultative anaerobe	Carbohydrate fermentation	[24]
			Bacteroidetes	Bacteroidia	Bacteroidales	*SB-1*		Anaerobe	Carbohydrate fermentation	[25]
			Chloroflexi	Anaerolineae	Anaerolineales	*Anaerolinaceae*		Strict anaerobe		[9, 10]
			Firmicutes	Clostridia	Clostridiales	*JTB215*		Anaerobic to aerotolerant	Cellulolytic	
			Fusobacteria	Fusobacteriia	Fusobacteriales	*Fusobacteriaceae*	*Propionigenium*	Strict anaerobe	Decarboxylation of succinate to propionate	[26]
			KSB3	MAT-CR-H3-D11				Strict anaerobe		[27]
			Planctomycetes	Phycisphaerae	AKAU3564			Facultative anaerobe	Fermentation of 5-C and 6-C sugars	[3]
			Spirochaetes	Spirochaetes	Spirochaetales	*Spirochaetaceae*	*Spirochaeta*	Obligate or facultative anaerobe	Carbohydrate fermentation	[28]
		Phototrophy	Chlorobi	OPB56				Obligate anaerobe	Anoxygenic photosynthesis; photoheterotroph	[29]

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Appendix B. Characterisation of the *Holothuria scabra* microbiome

Table B.1.1 Characterisation of the *Holothuria scabra* microbiome. Legend to interpret the table is detailed on pages 254-256.

Taxonomic assignment of the operational taxonomic units (OTUs)			**Relative abundance (%)**					**Ecophysiology, metabolism and habitat**							**Statistics**			
Phylum	**Order**	**Genus**	**S**	**FG**	**MG**	**HG**	**F**	**G**	**M**	**O_2**	**Func**	**Met**	**Nutrition**	**Habitat**	**Rel**	**Sed**	**Feed**	**Ref**
Acidobacteria	Gp10	*unclassified*	0.83	-	-	-	0.04	-						FL(me, te)	u	ns	FW< FF	
	Gp21	*unclassified*	0.13	0.02	-	-	0.08	-							u	ns	ns	
	Gp23	*unclassified*	0.17	0.18	0.34	0.20	0.13	-							u	SPS <DS	ns	
	Acanthopleuribacterales	*Acanthopleuribacter*	0.15	0.01	-	-	0.06	-	M	A_o	COH	R	Amino acids	HA(me, I an)	u	DS< SPS	ns	[1]
Actinobacteria	Actinomycetales	*Actinomyces*	-	-	0.59	0.13	-	+	N	AN_f	COH	F	Carbohydrates	HA(te, A & H; MM, O)	u	ns	ns	[2]
		Brevibacterium	-	-	0.05	-	-	+	N	A	COH	R	Proteolytic	FL(me, te); A, P	u	ns	ns	[3]
		Corynebacterium	-	-	0.47	0.04	-	+	N	A/A N_f	COH	F/R	Saccharolytic	FL;HA (fe,me,te) H,A	u	SPS <DS	FF< FW	[4]
		Kineosporia	-	-	0.11	-	-	+	M	A	COH	R	Wide range	FL(te: pl)	u	ns	ns	[5]
		unclassified	0.03	0.04	0.24	0.09	0.06	+							u	DS< SPS	ns	
	Bifidobacteriales	*Bifidobacterium*	0.09	0.07	2.72	0.38	-	+	N	ANf	COH	F	Saccharolytic	HA(te) H,A(I)	u	ns	ns	[6]
	Coriobacteriales	*Collinsella*	0.01	0.03	0.25	0.05	-	+	N	ANo	COH	F	Glucose	HA(te) H(I)	u	SPS <DS	FF< FW	[7]
		Enterorhabdus	-	0.07	0.20	-	-	+	N	AN	COH	F	Amino acid derivatives	HA(te); A(I)	u	ns	ns	[8]
Bacteroidetes	Prolixibacter	*unclassified*	0.12	0.06	-	-	0.05	+							n	ns	ns	
	Bacteroidales	*Bacteroides*	0.08	0.18	1.97	0.58	-	-	N	AN	COH	F	Saccharolytic	HA(te) H(I)	ns	ns	ns	[9]
		Odoribacter	-	-	0.11	-	-	-	N		COH	F	Saccharolytic	A	u	ns	ns	[10]
		Porphyromonas	-	-	0.06	-	-	-	N	AN	COH	F	Asaccharolytic	A & H	u	SPS <DS	ns	[11]
		Paraprevotella	-	-	0.05	-	-	-	N	AN	COH	F	Saccharolytic	HA(te); H(I)	u	ns	FW< FF	[12]

		Prevotella	0.02	0.44	1.97	0.50	0.02	-	N	AN	COH	F	Saccharolytic	HA(te) H,A(I)	u	ns	ns	[13]
		Alistipes	-	-	0.26	0.03	-	-	N	AN	COH	F	Saccharolytic	HA(te) H,A(I)	ns	ns	ns	[14]
	Cytophagales	*Cytophaga*	0.12	0.11	-	0.13	0.15	-	M	A	COH	R-O_2	Cellulolytic	FL(te)	ns	ns	ns	[15]
		Fabibacter	0.07	0.16	0.01	0.17	0.13	-	M	A	COH	R	Carbohydrates	FL(me: sed)	ns	ns	ns	[16]
		Marinoscillum	0.08	0.17	-	-	0.09	-	M	A	COH	R	Carbohydrates	FL(me: sed)	u	ns	FW< FF	[17, 18]
		Perexilibacter	-	0.03	-	0.06	0.28	-	M	A	COH	R	Carbohydrates	FL(me: sed)	ns	ns	ns	[17]
		Reichenbachiella	0.12	0.09	-	0.05	0.13	-	M	A	COH	R	Carbohydrate	FL(me: sw)	ns	ns	ns	[17]
		unclassified	1.42	1.04	0.07	0.60	2.39	-	M		COH			Wide range	u	ns	ns	[15]
	Flavobacteriales	*unclassified*	0.33	0.75	-	0.86	0.34	-		AN	COH	F	Fermentable carbohydrates	Wide range	u	ns	ns	[19]
		Actibacter	0.51	0.89	0.17	1.49	1.18	-	N	A	COH	R		FL(me: sed)	u	ns	ns	[20]
		Aureitalea	0.08	0.12	0.26	0.22	0.24	-	M	A	COH	R	Organic macromolecules	FL(me: sed, pl, an)	u	DS< SPS	ns	[21]
		Chryseobacterium	-	0.01	0.35	0.11	0.01	-	N	A	COH	R	Glucose degradation	FL(fe, me, te)	u	ns	ns	[22]
		Lutibacter	0.59	5.50	0.56	12.38	1.00	-	N	A	COH	R	Maltose	FL(me); SD	u	SPS <DS	ns	[23]
		Muricauda	0.10	0.18	0.05	-	0.07	-	N	A/FA	COH	R	Sugars and amino acids	FL(me)	ns	ns	ns	[22]
		Robiginitalea	0.59	1.18	1.06	1.96	0.95	-	N	A	COH	R	Starch degradation	FL(me)	u	SPS <DS	ns	[24]
		Tenacibaculum	0.22	1.32	0.01	-	0.15	-	M	A	COH	R		FL(me)	u	SPS <DS	FW< FF	[25]
		unclassified	0.71	1.48	0.01	1.66	0.59	-			COH	R			u	ns	ns	
	Sphingobacteriales	*Lewinella*	0.34	0.52	0.01	0.75	0.68	-	M	A	COH	R	Gelatin	FL(me)	ns	ns	ns	[26]
		unclassified	0.24	0.32	0.07	0.35	0.35	-		A				FL(fe, me)	u	ns	FF< FW	[27]
	unclassified	*unclassified*	1.88	3.91	0.16	4.67	3.39	-							u	ns	ns	
Chlamydiae	Chlamydiales	*Parachlamydia*	-	0.01	0.19	-	-	-	N	AN	COH			HA(te)	ns	ns	ns	[28]
		unclassified	0.03	0.19	1.70	0.70	0.01	-							u	ns	ns	
Chloroflexi	Anaerolineales	*unclassified*	0.25	0.05	-	0.02	0.15	-								DS< SPS	ns	
Firmicutes	Bacillales	*Gemella*	-	0.10	0.07	-	-	+	N	ANf	COH	F	Saccharolytic	HA(te);A;H(mm)	ns	ns	ns	[29]
		Staphylococcus	0.03	0.13	4.78	1.15	-	+	N	ANf	COH	R/F	CHO, AA	FL/HA(te) A	u	SPS	ns	[30]

																<DS		
	Lactobacillales	*unclassified*	-	-	0.21	-	-	+	N	ANf	COH	F		FL(te, me) A,H	ns	ns	ns	
		Lactobacillus	0.11	0.15	1.80	0.69	0.01	+	N	ANf	COH	F	Saccharolytic	HA(te) H,A	u	ns	FW< FF	[31]
		Streptococcus	0.02	0.20	1.86	0.61	0.01	+	N	ANf	COH	F	Carbohydrates	HA(te) H,A	ns	ns	ns	[32]
	Clostridiales	*Anaerococcus*	0.01	-	0.26	0.08	-	+	N	AN	COH	F	Peptone & AA	HA(te) H,A	u	ns	FW< FF	[33]
		Finegoldia	-	-	0.22	0.16	-	+	N	AN_o	COH	F	Peptone & AA	HA (te) H(GU,O)	u	ns	ns	[34]
		Blautia	0.03	0.04	0.67	0.02	-	+	N	AN_o	COH	F	Saccharolytic	HA(te), A,H(I)	u	SPS <DS	ns	[35]
		Clostridium_XlVa	-	0.01	0.26	-	-	+	M N						ns	ns	ns	[36]
		Coprococcus	-	0.02	0.61	0.17	0.01	+	N	AN_o	COH	F	Fermentable carbohydrates	HA(te) A,H(I)	u	DS< SPS	FW< FF	[37]
		Dorea	-	-	0.45	0.02	-	+	N	AN_o	COH	F	Fermentable carbohydrates	HA(te) H(I)	u	ns	ns	[38]
		Roseburia	0.04	0.09	0.81	0.40	-	+ -	N	AN	COH	R/F	Carbohydrates	HA(te) A,H(I)	u	ns	ns	[39]
		Ruminococcus2	-	0.01	0.51	0.03	-	+	M	AN	COH	F	Fermentable carbohydrates	HA(te) A,H(I)	u	ns	ns	[40]
		unclassified	-	0.04	1.63	0.03	-								u	ns	ns	
		Peptostreptococcus	-	-	0.22	-	-	+		AN	COH		Peptone and AA	HA(te) H(I)	ns	ns	ns	[41]
		Faecalibacterium	0.06	0.43	2.16	0.59	0.01	+	N	AN	COH	F	Sugars, starch. Acetate	HA(te) A,H(I)	u	ns	ns	[42]
		Flavonifractor	-	-	0.32	-	-	+	V	AN	COH	F	Asaccharolytic	HA(te) H(I)	u	DS< SPS	ns	[43]
		Oscillibacter	-	-	0.25	-	-	-	M	AN	COH	F	Carbohydrates	HA(me) A(I)	u	DS< SPS	ns	[44]
		Ruminococcus	0.01	0.09	0.73	0.03	-	+	M	AN	COH	F	Fermentable carbohydrates	HA(te) A,H(I)	u	ns	ns	[40]
		unclassified	0.12	0.07	0.68	0.23	0.08								u	ns	FW< FF	
	Erysipelotrichales	*Turicibacter*	0.01	-	0.17	-	0.02	+	N	AN	COH	F	Haemolytic	HA(te) A,H(I)	ns	ns	ns	[45]
	Selenomonadales	*Acidaminococcus*	0.01	-	0.26	-	-	-	N	AN	COH	F	Glutamate	HA(te) A,H(I)	u	DS< SPS	FW< FF	[46]
		Phascolarctobacterium	-	0.03	0.22	0.03	-	-	N	AN	COH	F	Succinate	HA(te) H(I)	u	DS< SPS	ns	[47]

		Dialister	-	-	0.13	-	-	-	N	AN_o	COH	R	Haemolytic	HA(te) H(O)	ns	ns	ns	[48]
		Veillonella	0.05	-	0.39	0.43	-	-	N	AN	COH	F	Organic acids	HA(te) A,H(I,O,R,GU)	ns	ns	ns	[49]
	unclassified	*unclassified*	-	-	0.22	-	-								u	ns	FW< FF	
Fusobacteria	Fusobacteriales	*Fusobacterium*	-	0.16	0.01	-	-	-	N	AN_o	COH	F	Peptone/CHO	FL/HA(te: I)	u	DS< SPS	ns	[50]
		unclassified	0.50	1.51	1.88	2.50	2.17	-	N	AN_o	COH	F	CHO/AA	FL/HA(me: sed) A(I)	ns	ns	ns	[51]
Planctomycetes	Planctomycetales	*Pirellula*	0.09	0.07	0.12	0.15	0.34	-	M	A_o	COH	R	Wide range	FL(me,te: sed, ad)	u	ns	ns	[52]
		Rhodopirellula	0.18	0.45	0.47	0.53	0.60	-	N	A_o	COH		Carbohydrates	FL(me)	ns	ns	ns	[53]
		unclassified	0.12	0.24	0.42	0.07	0.34	-	M	A/ AN_f	COH	R	Carbohydrates	FL(fe,me,te: soil, sew,I)	u	ns	ns	[54]
	unclassified	*unclassified*	0.12	0.06	-	-	0.05	-	M	A/ AN_f	COH	R	Carbohydrates	FL(fe,me,te: soil, sew,I)	u	ns	ns	[54]
α-proteobacteria	Magnetococcales	*Magnetococcus*	0.16	-	-	-	-	-	M	ME_o	CLA			FL(fe,me)	u	DS< SPS	FW< FF	[55]
	Rhizobiales	*Methylobacterium*	-	0.03	0.06	-	-	-	M	A_o	COH / Mf	$R\text{-}O_2$	Organic & C1 compounds		u	SPS <DS	FF< FW	[56]
		unclassified	-	0.01	0.06	-	-	-							u	ns	ns	
	Rhodobacterales	*Paracoccus*	-	-	1.07	-	-	-	N	Ao	COH / CLA	R	Organic/CO_2	FL(te,fe: soil, sew)	ns	ns	ns	[57]
		Rubellimicrobium	-	0.01	0.15	-	-	-	?	Ao	COH	?	Wide organic	FL(te)	u	ns	ns	[58]
		Shimia	0.13	0.19	0.40	0.76	0.30	-	M	Ao	COH		Sugars, AA	FL(me)	n	ns	ns	[59]
		unclassified	2.09	3.92	7.39	3.58	3.39	-							/	DS< SPS	FW< FF	
	Rhodospirillales	*unclassified*	0.05	0.04	0.04	0.05	0.24	-							ns	ns	ns	
	Sphingomonadales	*Sphingomonas*	-	0.01	0.07	-	-	-	M N	Ao	COH	$R\text{-}O_2$	Metabolically versatile	FL(te)	u	DS< SPS	FF< FW	[60]
		unclassified	0.03	0.01	0.74	0.02	-	-							u	SPS <DS	ns	
	unclassified	*unclassified*	0.11	0.10	0.07	-	0.04	-							u	DS< SPS	ns	
β-proteobacteria	Burkholderiales	*Alcaligenaceae – uncl*	-	0.02	1.16	0.13	0.01	-	M	A	COH	$R\text{-}O_2$	OA, AA	FL(te,fe: sew)	u	ns	ns	[61,

									N									62]
		Delftia	0.03	0.01	0.29	0.07	0.01	-	M	A	COH	R-O_2	Wide range	FL(fe,te: soil, sed)	u	ns	ns	[63, 64]
		Herbaspirillum	0.05	0.08	0.72	0.22	0.03	-							u	ns	FF< FW	
		unclassified	0.06	0.08	0.82	0.22	0.03	-							u	SPS <DS	ns	
		Parasutterella	-	-	0.12	-	-	-	N	ANo	COH	R	Asaccharolytic	FL(te Int-h)	u	SPS <DS	ns	[65]
	Neisseriales	*Neisseria*	0.03	0.15	0.23	0.27	-	-	N	A	COH		Saccharolytic/ haemolytic	HA; C(te: mm mammals)	u	SPS <DS	ns	[66]
	unclassified	*unclassified*	-	-	0.10	-	-	-							ns	ns	ns	
δ-proteobacteria	Bdellovibrionales	*Bacteriovorax*	0.06	0.32	-	0.15	0.05	-	M	Ao	COH	R-O_2	Bacteriolytic	FL/P/C(fe,me,te)	ns	ns	ns	[67]
		unclassified	0.14	0.38	-	-	0.08	-							ns	ns	ns	
		Vampirovibrio	0.01	-	0.09	-	0.02		N	Ao	COH	R	Chlorella	HA; P(fe: alg)	ns	ns	ns	[68]
	Desulfobacterales	*Desulfobacter*	0.18	0.03	-	0.05	0.27	-	M N	ANo	COH CA	R-SO_4^{2-}	Acetate/Sulfate/	FL(fe,me: sed)	u	DS< SPS	ns	[69]
		Desulfosarcina	0.16	0.04	-	0.01	0.02	-	M N	ANo	COH CA	F/R-SO2-4	Organic acids	FL(me: sed)	ns	ns	ns	[70]
		unclassified	0.82	0.56	0.11	0.35	1.52	-	M	ANo	COH CLA CLH	F/R -SO2-4	Simple compounds	FL(fe,me)	u	ns	ns	[71]
		Desulfocapsa	0.07	0.01	-	0.03	0.22	-	M	ANo	COH CLA	R -SO2-4	Simple compounds	FL(fe,me: sed)	u	ns	ns	[72]
		Desulfopila	0.29	0.38	0.19	0.51	0.22	-							u	ns	ns	
		unclassified	0.54	0.42	0.59	0.84	0.57	-	M	ANo	COH CLA CLH	F/R	Simple compounds	FL(fe,me)	u	ns	ns	[71]
	Desulfovibrionales	*Desulfovibrio*	0.07	0.33	-	0.52	0.07	-	N	ANo	COH	F/R-SO2-4		FL(fe,me: sed, Ian)	u	ns	ns	[73]
	Desulfuromonadales	*Pelobacter*	0.10	0.62	0.01	0.60	0.08	-	M N	ANo	COH	F	Simple	FL(fe,me: sed)	u	ns	FF< FW	[74]
		unclassified	0.48	0.53	-	0.47	0.64	-		ANo	COH	R	Wide range	FL(fe,me: sed)	/	ns	ns	[75]
	Myxococcales	*unclassified*	0.09	0.11	0.01	0.16	0.42	-	M	Ao	COH	R	Proteolytic/		ns	ns	ns	[76]

													bacteriolytic					
ε-proteobacteria	Campylobacterales	*Arcobacter*	0.10	0.64	-	0.10	0.61	-	M	ME	COH	R	AA	FL/P(fe,te: Ian)	u	DS< SPS	ns	[77]
		Sulfurovum	0.15	0.89	0.35	0.21	0.11	-	N	ANf	CLA	R-O_2/NO_3		FL(me: hv)	u	DS< SPS	ns	[78]
γ-proteobacteria	Alteromonadales	*Aestuariibacter*	0.21	0.19	-	0.13	0.44	-	M	Ao	COH	R-O_2	Hydrolytic	FL(me: sed)	u	ns	FF< FW	[79]
		Agarivorans	0.07	0.19	0.03	1.18	1.09	-	M	Ao	COH	R-O_2	Agarolytic	FL(me: Ian)	ns	ns	ns	[79, 80]
		Haliea	0.61	1.08	0.30	0.78	0.98	-	M	Ao	COH	R-O_2	Organic acids	FL(me: sw)	u	SPS <DS	ns	[79, 81]
		Unclassified	0.17	0.20	-	0.09	0.53	-	M	Ao	COH	R-O_2		FL(me)	n	SPS <DS	ns	[82]
		Thalassomonas	0.06	0.04	-	0.39	1.40	-	M	Ao	COH	R	Saccharolytic	FL(me: pl, an)	ns	ns	ns	[79, 83]
		Ferrimonas	0.04	0.05	-	1.07	0.81	-	M	Ao	COH	R-e-	Lactate oxidiser	FL(me: sed)	u	SPS <DS	ns	[84]
		Neiella	0.08	0.07	-	0.01	0.28	-	M	Ao	COH			FL(me: Isc)	u	ns	ns	[85]
		Pseudoalteromonas	0.08	0.11	0.02	0.09	0.44	-	M	Ao	COH	R-O_2	CHO, AA, OA	FL(me: sw, sed, pl, an)	ns	ns	ns	[86]
		Psychrosphaera	0.06	0.19	-	0.01	0.10	-	M	ANf	COH	F/R	CHO	FL(me: sw, sed)	ns	ns	ns	[87]
		unclassified	0.05	0.03	-	0.06	0.68	-	M	ANf/ Ao	COH	F/R		FL(me)	u	SPS <DS	FW< FF	[82]
	Incertae sedis	*Marinicella*	0.16	0.03	-	-	0.01	-	N	A	COH	R	Hydrolytic (DNA, gelatin)	FL(me)	u	ns	FW< FF	[88, 89]
		Thiohalomonas	0.12	0.88	1.22	1.17	0.26	-	N	ANf	CLA	R-O_2, NO_3	Sulphur-oxidising	FL(se)	ns	ns	ns	[90]
		Thioprofundum	0.17	0.12	0.01	0.04	0.06	-	N	ANf	CLA		Sulphur oxidation/CO_2 fixation	FL(me,se)	ns	ns	ns	[91, 92]
		Unclassified	0.74	1.67	1.23	7.22	1.61	-							/	ns	ns	
	Legionellales	*Coxiella*	0.11	0.06	0.91	0.16	0.01	-	N	ANf	COH			HA; IE(te)	u	SPS <DS	ns	[93]
		Legionella	0.06	0.09	1.64	0.48	0.01	-	M	Ao	COH	R	Amino acids	FL(te, fe: I)	ns	ns	ns	[94]
	Oceanospirillales	*Reinekea*	0.22	0.31	-	0.05	0.18	-	M	ANf	COH	R	Carbohydrates	FL(me: sed)	u	DS< SPS	FW< FF	[95]

	Pasteurellales	*unclassified*	0.04	0.14	1.05	0.09	-	-	N	A/ME/Anf	COH	F/R	Carbohydrates	HA; P(I)	ns	ns	ns	
	Pseudomonadales	*Acinetobacter*	0.22	0.07	1.00	0.36	0.01	-	N	A	COH	R-O_2	Saprophytic	FL(te: D,I)	u	ns	ns	[96]
		Moraxella	0.01	0.06	1.65	0.21	-	-	N	A	COH	R		P(te: I)	u	SPS <DS	ns	[97]
		Pseudomonas	0.51	0.80	11.39	2.25	0.10	-	M	A		R-O_2		FL/P(te: I)	u	ns	ns	[98]
	unclassified	*unclassified*	2.43	4.64	4.82	8.25	4.56	-							u	ns	FF< FW	
	Vibrionales	*Photobacterium*	0.15	0.86	0.02	0.48	0.61	-	M	ANf	COH	F/R	7-22 C compounds	FL(me: sed,sw,I)	u	ns	ns	[99]
		unclassified	0.13	0.06	-	0.06	0.30	-	M	ANf	COH	F/R-O_2	Carbohydrates	FL/P(fe,me: pl, an)	n	DS< SPS	ns	[100]
		Vibrio	0.58	1.59	0.39	2.27	3.45	-	M	ANf	COH	F/R-O_2	Carbohydrates	HA; C(me) A(I)	ns	ns	ns	[101]
	Xanthomonadales	*Luteimonas*	-	-	0.06	-	-	-	N	Ao	COH	R-O_2		FL(te)	u	ns	ns	[102]
Proteobacteria	unclassified	*unclassified*	0.13	0.38	0.18	0.08	0.11	-							u	SPS <DS	ns	
Spirochaetes	Spirochaetales	*Spirochaeta*	0.06	0.08	-	0.27	0.21	-	M	ANf/o	COH	F	Saccharolytic	FL/C(fe,me: Ian)	ns	ns	ns	[103]
unclassified	unclassified	*unclassified*	3.91	18.29	5.37	3.79	3.62								u	ns	ns	
Verrucomicrobia	Puniceicoccales	*Pelagicoccus*	0.07	0.01	-	0.07	0.21	-	M	Ao/ANf	COH		Saccharolytic	FL(me)	ns	ns	ns	[104]

Legend to interpret Table B.1.1.

Taxonomic assignment of the operational taxonomic unit (OTU)

The number of reads for each OTU in each sampling location was converted to relative abundance and after assigning the taxonomy to genus level where possible, the list was filtered and subtotalled to combine OTUs with the same taxonomic assignment.

Relative abundance in each sampling location

S = sediment; FG = foregut; MG = midgut; HG = hindgut; F = Faeces.

Eco-physiology, metabolism and habitat

G = gram stain:

+ = gram positive;

- = gram negative.

M = motility:

M = motile;

N = non-motile.

O_2 = oxygen-related eco-physiology:

A = aerobic;

AN = anaerobic;

ME = microaerobic growth or growth in a CO_2-enriched atmosphere;

$_f$ = facultative;

$_o$ = obligate.

Func = functional group:

COH = chemoorganoheterotroph;

CA = chemoautotroph;

CLA = chemolithoautotroph;

CLH = chemolithoheterotroph.

Met = metabolism:

R = respiratory;

F = fermentative;

$R\text{-}O_2$ = aerobic respiration with oxygen as the terminal electron acceptor;

$R\text{-}NO_3^-$ = anaerobic respiration with nitrate as the terminal electron acceptor;

$R\text{-}SO_4^{2-}$ = anaerobic respiration with sulphate as the terminal electron acceptor.

Nutrition = enzymatic capacity of the bacteria i.e. saccharolytic, or the main carbon or energy source utilised:

CHO = carbohydrates; AA = amino acids; OA = organic acids.

Nature of symbiotic relationship:

FL = free-living; HA = host-associated; C = commensal; P, parasitic; S, saprophytic; IE –Intracellular endosymbiont

Environment:

fe = freshwater environment; me = marine environment; se = saline environment; te = terrestrial environment.

Host:

H = human; A = animal

Habitat:

I = intestine; O = oral cavity; R = respiratory tract; GU = genitourinary tract

D = food associated; MM = mucosal membrane

ad = anaerobic digester; alg = algae; an = animal; hv = hydrothermal vent; mm = mucosal membrane; pl = plant; sc = sea cucumber; sed = sediment; sew = sewerage works; soil = soil; sw = seawater.

Statistics

Rel = Nature of the relationship of the OTU with passage along the sea cucumber feeding chain from the sediment (S) to the faeces (F):

u = negative quadratic relationship i.e. decreases from high relative abundance in the sediment to lowest relative abundance in the midgut, then increases to high relative abundance in the faeces;

n = positive quadratic relationship i.e. increases from low relative abundance in the

sediment to highest relative abundance in the midgut, then decreases to low relative abundance in the faeces;

/ = positive linear relationship i.e. relative abundance increases from sediment through the gut to highest relative abundance in the faeces

\ = negative linear relationship i.e. relative abundance decreases from sediment through the gut to lowest relative abundance in the faeces

Sed = sediment type. This column indicates whether the experimental factor of 'sediment type' had a significant effect on the relative abundance of the OTU.

SPS > DS indicates that the relative abundance of the OTU was higher in the shrimp pond sediment (SPS) treatment compared to the dune sand (DS) treatment.

DS > SPS indicates that the relative abundance of the OTU was higher in the dune sand (DS) treatment compared to the shrimp pond sediment (SPS) treatment.

Feed = feed type. This column indicates whether the experimental factor of 'feed type' had a significant effect on the relative abundance of the OTU.

FW > FF indicates that the relative abundance of the OTU was higher in the fish waste (FW) treatment compared to the formulated feed (FF) treatment.

FF > FW indicates that the relative abundance of the OTU was higher in the formulated feed (FF) treatment compared to the fish waste (FW) treatment.

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