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The role of the seagrass leaf microbiome in assisting nitrogen uptake by the Western Australian seagrass, Posidonia sinuosa

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The role of the seagrass leaf microbiome in assisting nitrogen uptake by the Western Australian seagrass, *Posidonia sinuosa*

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USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

ABSTRACT

Microorganisms play a key role in facilitating the cycling of several elements in coastal environments, including nitrogen (N). N is a key component for maintaining high seagrass productivity and is often the limiting nutrient in marine environments. Seagrasses harbour an abundant and diverse microbial community (the 'microbiome'), however their ecological and functional roles related to the seagrass host are still poorly understood, in particular regarding N cycling. Microorganisms capable of mineralising dissolved organic nitrogen (DON) may play a pivotal role in enhancing N availability in coastal environments such as seagrass meadows. Thus, the overall aim of my thesis was to enhance current understanding of abundance and diversity of the microbial community associated with seagrass meadows and their ecological role, with specific focus on N cycling. This was achieved by using molecular techniques together with ^{15}N-enrichment experiments and nanoscale imaging techniques.

Firstly, I reviewed the literature on the potential effects that microorganisms associated with both the above- and belowground seagrass tissue may have on plant fitness and the relevance of the seagrass microbiome and I have highlighted literature gaps.

For my second chapter, I determined the abundance and community composition of bacteria and archaea associated with seagrass *Posidonia sinuosa* meadows in Marmion Marine Park, southwestern Australia. Data were collected from different seagrass meadows and meadow 'microenvironments', i.e. seagrass leaf surface, sediment and water column. I performed the quantitative polymerase chain reaction (q-PCR) targeting a series of bacterial and archaeal genes: 16S rRNA, ammonia oxidation genes (*amoA*) and genes involved in mineralisation of DON, via the urease enzyme (*ureC*). High-throughput sequencing was applied to 16S rRNA and *amoA* genes, to explore the diversity of these microbial assemblages related to *P. sinuosa* meadow microenvironments. Results from this chapter show that the *P.*

sinuosa leaf biofilm represents a favourable habitat for microorganisms, as it hosts a significantly higher microbial abundance compared to the sediment and water. Moreover, 16S rRNA and *amoA* sequencing data indicate a high degree of compartmentalisation of functional microbial communities between the microenvironments of the seagrass meadow (leaf, sediment and water column), pointing towards the existence of a core seagrass leaf microbiome that could have specific interactions with the plant.

For my third chapter I determined the role that microorganisms inhabiting *P. sinuosa* seagrass leaves may play in the recycling of DON, and subsequent transfer of inorganic N (DIN) into plant tissues. To achieve this, I performed an experiment whereby seagrass leaves with and without microorganisms were incubated with $DO^{15}N$, and I traced the fine-scale uptake and assimilation of microbially processed N into seagrass cells, using nanoscale secondary ion mass spectrometry (NanoSIMS). Results from this chapter show for the first time that seagrass leaf epiphytic microorganisms facilitated the uptake of ^{15}N from DON, which was unavailable to the plant in the absence of epiphytes. This indicates that seagrass leaves have limited to no ability to take up DON, and the seagrass leaf microbiome could therefore play a much more significant role than previously thought in enhancing plant health and productivity.

Finally, I determined the net nitrification rates associated with ammonia-oxidising microorganisms (AOM) inhabiting *P. sinuosa* leaf surfaces, and explored whether AOM facilitated, or competed for, the plant's N uptake. My findings show that AOM may compete with seagrasses for NH_4^+ uptake, but that their potential to outcompete seagrass epiphytic algae for DIN uptake indicates that AOM on seagrass leaves may serve as a 'biocontrol' over excess epiphytic algal growth.

In summary, the present thesis represents a significant advance in our understanding of the seagrass leaf-microbiome relationship and transformations of N within seagrass meadows. Moreover, it opens up new questions for future research not only on seagrass-microbiome interactions but other macrophytes in aquatic systems that may benefit from the presence of specific N-cycling microorganisms.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

incorporate without acknowledgment, any material previously submitted for a degree or diploma in any institution of higher education;
contain any material previously published or written by another person except where due reference is made in the text of this thesis;
contain any defamatory material; or
contain data that have not been collected in a manner consistent with ethics approval.

Flavia Tarquinio

August 2017

This Thesis is Dedicated to My Family

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There are many people that helped me through this journey and that without them I would not have completed this thesis. First, I would like to thank my family for all the support. So probably noone will get offended if I write a couple of sentences in Italian! Grazie mille per tutto quello che avete fatto e farete per me, per sostenermi anche se siete lontani e per l'amore che continuo a ricevere, mi sembrate cosi vicini!!!Grazie a mia mamma mio padre e mio fratello vi voglio bene! Anche alle nonne, cosi non si offende nessuno!

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LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

This thesis is presented as a series of manuscripts that are to be submitted for publication in international journals. I am the first author with a contribution of more than 50% to each publication. The thesis conforms to the “thesis with publication” style of Edith Cowan University.

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CHAPTER ONE: GENERAL INTRODUCTION

1.1. Seagrass and the role of seagrass meadows in the marine ecosystem

Seagrasses are marine flowering plants that form benthic habitats along the coastal zones of every continent, except Antarctica (Green, 2003; Douglas et al., 2008). Since seagrass survival is dependent on light intensity in order to produce enough carbohydrates to meet their growth and respiration requirements (Dennison 1987; Fourqurean & Zieman, 1991; York et al., 2013), they are restricted to the littoral zone of coastal waters to a maximum depth of 40m depending on seagrass species and water quality such as clarity and light attenuation (Kenworthy & Haunert, 1991; Duarte, 1991, Dennison et al., 1993; Saunders et al., 2013). Seagrass meadows represent one of the most complex and highly productive marine coastal ecosystems (Duarte & Chiscano, 1999; Orth et al., 2006; Hyndes et al., 2013; Hyndes et al., 2014) and, despite their restricted distribution within the aquatic environment (<0.2% of the area of the world's oceans; Duarte, 2002; Fourqurean et al., 2012), they provide a variety of pivotal ecosystem services (Coles et al., 1987; Lanyon et al., 1989; Beck et al., 2001; Duarte et al,. 2013). These services include: i) coastal protection from erosion by attenuating wave energy and turbulence intensity (Gambi et al., 1990; Ackerman & Okubo, 1993; Barbier et al., 2011); ii) improvement of water quality by increasing sedimentation and reducing resuspension by stabilizing the sediment through their roots and rhizomes (Ward et al., 1984; Gacia et al., 1999; Terrados & Duarte, 2000; Gacia & Duarte, 2001); iii) sequestration of carbon dioxide (which accounts for ca 15% of carbon stored in the oceans), thus mitigating the effects of ocean acidification (Nellemann et al., 2009; Fourqurean & Duarte, 2012; Kennedy & Bjork, 2012; Duarte et al., 2013), and, iv) habitat and source of food for many macro- and micro-organisms, both resident (e.g. sea urchins, fish, gastropods, bivalves, epiphytic algae and microorganisms) and transient (e.g. dugongs, manta ray, turtles and migratory waterfowl; Fig. 1.1; Valentine & Heck, 1999; Valentine & Duffy, 2006). A number of studies provide evidence for the importance of seagrass meadows as nursery habitat for juveniles of economically important species including fish (e.g. snappers, spinfish and groupers; Dorenbosch et al., 2004; Heck et al., 2015), shrimp (Murphey & Fonseca, 1995; Erhardt et al., 2001), crabs (Thresher et al., 1992) and lobsters (De Lestang et al., 2006).

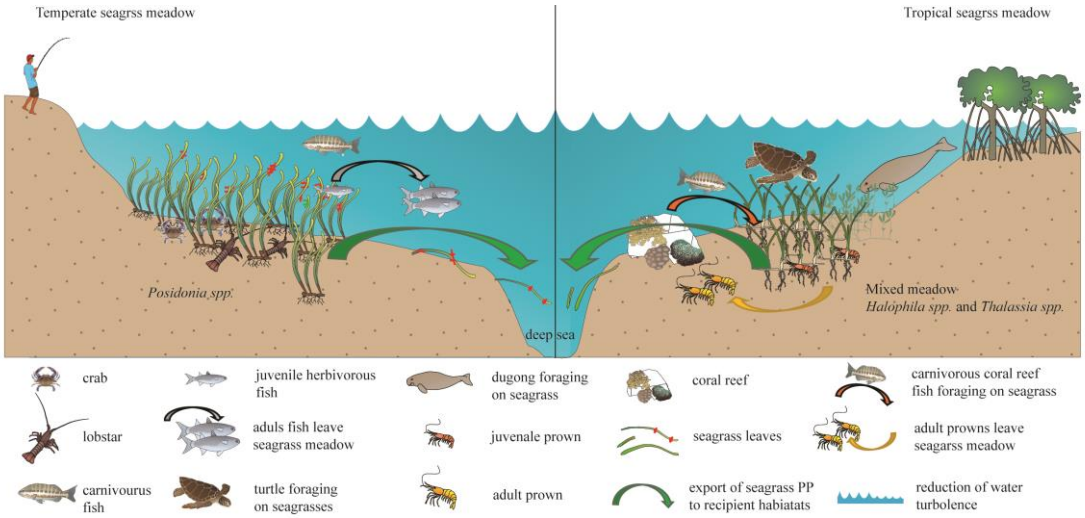


Fig. 1.1 –Ecosystem services provided by seagrasses. Seagrass meadows: i) attenuate wave and water turbulence, thus protecting the coastal line (Barbier et al., 2011), ii) provide habitat and nutrients for transient (turtles, dugongs and manta rays) and resident animals (sea urchins, crabs, lobster, fish) (Valentine & Duffy, 2006), iii) nursery habitat for juveniles of economically important species (Thresher et al., 1992; Erhardt et al., 2001; De Lestang et al., 2006; Heck et al., 2015) and, iv) export a high amount of their primary production to other ecosystems (Heck et al., 2008).

Seagrass meadows are also linked to different ecosystems through the export of a high proportion of their production (Wolff, 1980; Suchanek et al., 1985). Canopy primary production (PP), has been estimated to be as high as coral reefs and mangroves (ca 0.5 Kg C $m^{-2}y^{-1}$; Hyndes et al., 2014), of which about 70% is exported in the form of leaves, particulate and dissolved organic matter to a variety of ecosystems (Cebrián, 2000) via passive movement on currents (Fig. 1.1; Robertson & Lucas, 1983; Slim et al., 1994). The flow of seagrass organic matter to recipient habitats is highly influenced by microbial activities (Säwström et al., 2016) as microorganisms drive the regeneration and transformation of organic material within the environment (Azam & Malfatti, 2007; Koho et al., 2013, Säwström et al., 2016). Organic matter released from seagrass ecosystems significantly enhances food web structure and productivity of recipient habitats (Vetter, 1994; Vetter, 1998; Hyndes et al., 2014) that may be characterised by low primary production and limited habitat structure (Kirkman & Kendrick, 1997; Heck et al., 2008). Large amounts of leaves and particulate matter can be deposited on beaches (Mateo e al.,

2003; Mateo et al., 2006) where the leached organic material enhance bacterial biomass that become consumed by ciliates and flagellates (Robertson et al., 1982). Moreover, seagrass wracks represent a nutrient source for shore birds, terrestrial rodents (such as ground squirrels, capybara and nutria; Kantrud, 1991) and crabs (Gunter, 1967). Seagrass detritus is also commonly found in deep sea canyons where it subsidises low autochthonous productivity (Vetter, 1994; Vetter, 1998; Polis & Strong, 1996). Indeed, tropical and temperate regions provide a multitude of examples of trophic transfer and faunal interactions between seagrass meadows and adjacent habitats. The value of seagrass meadows for the provision of these pivotal ecological services has been estimated to vary from ca US$29,000 per ha per year (for nutrient cycling solely; Costanza et al., 2014) up to AU$230,000 per ha per year (for enhanced fishery production in southern Australia; Blandon & zu Ermgassen, 2014). Despite the extremely different monetary values attributed to seagrass meadows, these estimates highlight the key importance of seagrass habitats.

1.2 Seagrass morphology and physiological adaptation to a nutrient poor environment

Seagrasses colonised the marine environment about 100 millions of years ago from terrestrial plant ancestors (Den Hartog, 1970; Larkum et al., 2006) and developed many characteristics to live and reproduce submerged in water (Hemminga & Duarte, 2000). Seagrass shoots are connected and stabilised through rhizomes and roots. Seagrass leaf tissue consists of an enlarged aerenchyma system for translocation of oxygen derived from photosynthesis into below-ground tissues (Borum et al., 2007), and a high concentration of chloroplasts are present within the epidermal cells (Ferreira et al., 2013). Above the epidermis, a very thin cuticle is present (<0.5µm; Kuo, 1993), thus providing seagrasses with an advantageous opportunity to uptake nutrients through the whole leaf surface. Nutrient uptake, in fact, occurs from the water column through the leaves and from the sediment through the roots (Borum et al., 1989; Penhale & Thayer, 1980). Transport of nutrients throughout the plant occurs through the vascular system, enabling the physiological interaction between shoots and roots via the rhizome (Libes & Boudouresque, 1987; Marbà et al., 2002).

The high primary production levels of seagrassesimplies a high nutrient demand (Hillman et al., 1989) and, as in all photosynthetically driven ecosystems, nitrogen (N) and phosphorous are two important nutrients that are considered to limit seagrass growth and primary production (Short, 1987; Zimmerman et al., 1987; (Powell et al., 1989; Williams, 1990; Kenworthy & Fonseca, 1992; Touchette & Burkholder, 2000). In general, seagrasses that grow in temperate areas are N-limited, whereas tropical seagrasses are P-limited due to the tendency for phosphorous to become adsorbed to carbonate sediments (Short et al., 1985; Williams, 1990; Kenworthy & Fonseca, 1992). In some cases, seagrass growth may be co-limited by both N and P (Thursby, 1984; Agawin et al., 1996; Udy & Dennison, 1997; Touchette & Burkholder, 2000). Inorganic N (DIN), ammonium in particular, is the preferred source of nitrogen taken up by seagrasses (Terrados & Williams, 1997; Lee & Dunton, 1999; Touchette & Burkholder, 2000), however, low environmental concentrations of DIN are common in coastal waters (e.g. to 0.4μM for NH_4^+, and <0.05 to 1.7μM for NO_{2-}^- and NO_3; Touchette & Burkholder, 2000; Gobert et al., 2002; Barron & Duarte, 2009).

In order to survive in a nutrient poor environment, seagrasses have developed different adaptations to optimize N uptake. For example, the growth of *Posidonia* spp. leaves varies seasonally to optimize nutrient uptake and reduce leaf damage (Gobert et al., 2002). Seagrass leaves of *Posidonia* spp. reach maximum length in summer, resulting in the meadow forming a barrier that encloses the water and its nutrients in the canopy (Gacia et al., 1999; Gobert et al., 2002; Lepoint et al., 2002). However, the most significant adaptation of seagrasses is the capacity of the canopy to assimilate nutrients by both leaves and roots (Iizumi & Hattori, 1982; Thursby & Harlin, 1982; Stapel et al., 1996). In general, inorganic N uptake rates are higher for leaves than roots (Short & McRoy, 1984; Touchette & Burkholder, 2000 Alexandre et al., 2010; Alexandre et al, 2014), with a few exceptions such as *Zostera marina* and *Thalassia testudinum* whose N uptake by roots exceeds the substrate taken up by above ground tissue (Lee & Dunton, 1999; Thursby & Harlin, 1982). Nitrogen absorption through seagrass leaves can provide 40 to 74% of the annual N requirement for plant growth (Touchette & Burkholder, 2000; Lepoint et al., 2002). Since the uptake of nutrients by roots is primarily limited by substrate diffusion from pore water to seagrass tissue (Stapel et al., 1996; Lee & Dunton, 1999), the mean

annual nutrient fluxes across the sediment only provide up to 20% of N required for plant growth and development (Gobert et al., 2002).

1.3 Importance of allochthonous material for seagrass N subsidy and uptake of organic nitrogen

The imbalance between low concentrations of inorganic-N typically detected in seagrass meadows (Gobert et al., 2002) and seagrass N demand, has led to the idea that seagrass adaptations, while important, are not sufficient to explain seagrass high productivity against oligotrophic environment (Touchette & Burkholder, 2000; Gobert et al., 2002; Barron & Duarte, 2009). Recent studies have demonstrated that significant biomass of allochthonous materials, for example seston (Barron & Duarte, 2009) and kelp (Kirkman & Kendrick, 1997; Wernberg et al., 2006; de Bettignies et al., 2013), is transported into seagrass meadows (Fig. 1.2). By reducing water flow, seagrass leaves force suspended particles to fall into the canopy, thus enhancing nutrient deposition and accumulation within the meadow (Agawin & Duarte, 2002). Seston contribution to the organic matter pool in seagrass sediments has been estimated between 50 and 300 g C m^{-2} yr^{-1} (Kenworthy & Thayer, 1984), which is equivalent to ca 7 to 45 g N m^{-2} yr^{-1} (Hemminga et al., 1991). Detrital macrophytes also represent a significant constituent of the allochthonous material pool trapped within seagrass meadows. Kelps are among the most productive components of benthic marine ecosystems (Mann, 1973; Cambridge & Hocking, 1997), and a large biomass of kelp detached from rocky reefs can be transported into seagrass meadows as entire kelp thalli or smaller-sized particulate organic matter (POM; Fig. 1.2) (Wernberg et al., 2006). Since seagrass meadows can lose from 1% up to 77% of their biomass through leaves/nutrient export (Heck et al., 2008), including large amounts of their N, macroalgal material could subsidise marine plants with an extra source of available N (Cambridge & Hocking, 1997; Lourey & Kirkman, 2009; Hyndes et al., 2012).

Dissolved organic nitrogen (DON) represents the main form of nitrogen lost during leaching of POM and, Hyndes and colleagues (2012) showed that freshly detached kelp thalli and few weeks old thalli are able to exude a high amount of DON, but low DIN, in a relative short time (24hours). Leached DON is considered to be highly labile (Blum & Mills, 1991), and therefore, readily available for

bacterioplankton and residential microorganisms of recipient habitats in which they deposit. Microorganisms play a pivotal role by mineralizing organic material, which deposit in seagrass beds that would otherwise be unavailable for seagrass uptake. Nonetheless, extremely low microbial mineralisation rates of annual sedimentary N inputs have been detected in seagrass beds (Holmer et al., 2001; Gacia et al., 2002), suggesting an alternative path for seagrass nitrogen uptake.

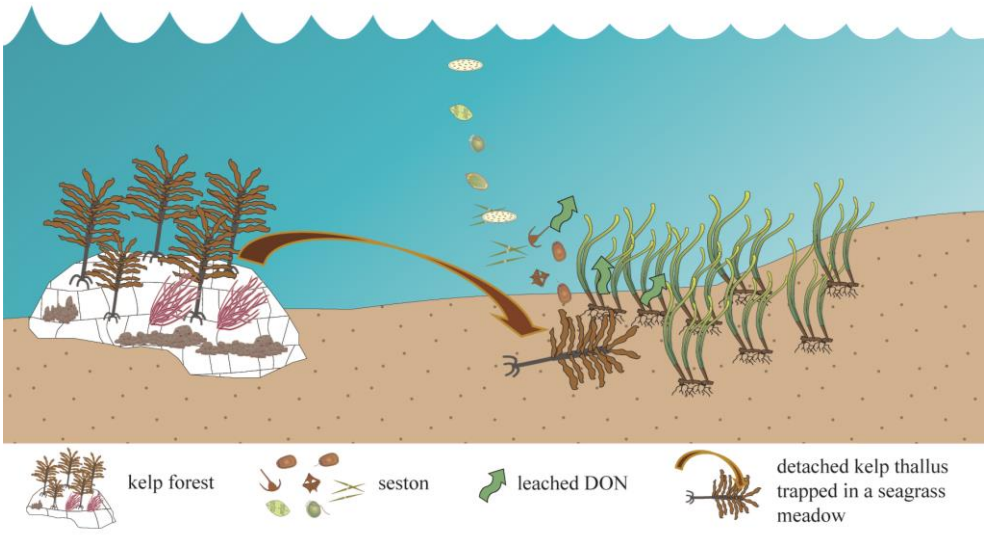


Fig. 1.2 – Fate of allochthonous nutrient sources in a seagrass meadow in South-Western Australia, with a focus on nitrogen. Kelp whole thallus, kelp-fragments and planktonic-derived organic matter can be trapped into seagrass meadows where they enhance nitrogen availability for seagrass uptake through leaching DON.

Vonk and colleagues (2008) were the first to hypothesise and demonstrate that uptake of DON by seagrass (*Thalassia hemprichii*, *Halodule uninervis* and *Cymodocea rotundata*) leaves and roots may represent a significant mechanism to supply N to seagrasses in a low inorganic-N environment. By incubating marine plants with enriched ^{15}N-amino acids and urea, the appearance of enriched ^{15}N within seagrasses suggested the existence of N-acquisition mechanisms that allow direct uptake by plants of DON. In this experiment, seagrass leaves exhibited a high uptake affinity for DIN (ammonium and nitrate) and urea and a lower affinity for amino acids. Roots had a higher uptake affinity for ammonium and amino acids and a lower affinity for nitrate and urea. In comparison, the above-ground tissue of *Zostera noltii*, *Cymodocea nodosa* and *Caulerpa prolifera* had a higher uptake affinity for urea, glycine and leucine than below-ground tissue (Van Engeland et al.,

2013). Alexandre and colleagues (2015) presented evidence for the uptake of alanine and trialanine by *Zostera marina* leaf tissue, even if amino acids concentrations were significantly lower than the values reported for roots, and it has been demonstrated that in low-nutrient environments, *Z. noltii* takes any form of nitrogen that is available (La Nefie at al., 2014). Although, a variety of studies have been performed with enriched $DO^{15}N$ and different seagrass species, doubts exist on the capability of seagrass to utilise DON directly. In fact, in these studies, the role of seagrass leaf epiphytic microorganisms in DON mineralisation have never been considered as microorganisms present on seagrass leaf surface were never removed. Given the possibility that heterotrophic microorganisms (capable of mineralising DON) may be part of the seagrass leaf community, their presence needs careful consideration as their metabolism may enhance DIN availability for seagrass uptake. Indeed, Hyndes and colleagues (2012) were the first to hypothesise that the appearance of kelp-derived DON within seagrass tissue during a field experiment was linked to the microbial transformation of organic matter within the canopy water. In this study, Hyndes and colleagues characterised the kelp leachate in a laboratory aquarium and detected low DIN concentration but high DON and urea. Because kelp is unlikely to release urea they linked the presence of this product to the microbial catabolisation of large N compounds (Berg & Jørgensen, 2006) suggesting a link between mineralisation of leached DON and seagrass leaf uptake.

1.4 The importance of microorganisms for organic nitrogen cycling in seagrass meadows

Significant seagrass-microbial interactions are likely because seagrass harbour diverse and abundant microbial communities on the surface of their roots and leaves (Weidner et al., 2000; Uku & Björk, 2001; Uku et al., 2007; Borowitzka, Lavery, & van Keulen, 2007; Piazzi et al., 2007; Crump & Koch, 2008; Hamsi et al., 2013). Seagrasses are able to influence their leaf and root epiphytic microbial community through changes in physical-chemical conditions on tissue surface by exuding oxygen (Larkum et al., 2007; Borum et al., 2007; Duarte & Chiscano, 1999), nutrients (Wetzel & Penhale, 1979; Moriarty et al., 1986), and antimicrobial substances (Reichelt et al., 1984; Engel et al., 2006; Bushmann & Ailstock, 2006). Studies on seagrass epiphytic community diversity indicate that: (i) plant-attached

microorganisms are different from the microbial community present in the surrounding environments (e.g. sediments and water column) (Pasmore & Costerton, 2003; Bhadury & Wright, 2004); (ii) leaf- and root-attached microorganisms are different to each other, which is not surprising due to the different environmental conditions provided by leaves and roots (Haglund et al., 2002; Touchette, 2007); and (iii) leaf-attached microbial community diversity seems to be related to the seagrass species rather than location (Crump & Koch, 2008). Since microorganisms are key players in the recycling of nitrogen (Henriksen, 1988; Glibert & Bronk, 1994; Arrigo, 2005; Papadimitriou et al., 2005; Uku, et al., 2007), seagrasses may depend on specific N cycling processes performed by microorganisms within their meadows, one of which could be the mineralisation of DON. Microbial mineralisation of DON can take place within or outside the microbial cell by intracellular or extracellular enzymes that break down dissolved organic nitrogen (Berman, 2003). The path utilised by microorganisms to mineralise DON depends on substrate molecular weight (Chrost, 1991; Stepanauskas et al., 1999). Low molecular weight (LMW) molecules (<1KDa) can be transported within microbial cells, while high molecular weight (HMW) molecules need to be converted into LMW molecules by extracellular enzymes, including hydrolases, oxidases, deaminases and lyases (Halemejko & Chrost 1986, Amy et al., 1987; Geisseler et al., 2010). The final product of mineralisation of DON is ammonium (Berman & Bronk 2003; Solomon et al., 2010; Kirchman, 2012), which can be released into the environment and taken up by the seagrass itself or used by different microorganisms to satisfy their nutritional requirements.

Most of the studies of seagrass microbiology have focused on the role that bacteria within seagrass sediments play in the regeneration of nutrients (Jørgensen, 1982; Shieh & Yang, 1997; Säwström et al, 2016). These studies indicate that microbial mineralisation dominates over the immobilisation of PON and DON in the seagrass rhizosphere. For example, the bacterial community associated with the rhizosphere of different seagrass species (*Thalassia hemprichii, Halodule uninervis, H. pinifolia, Halophila ovalis* and *Syringodium isoetifolium*) responded to the addition of ^{15}N-labeled phytodetritus within a short timeframe suggesting that plant ^{15}N enrichment was due to the microbial mineralisation of DON and/or ^{15}N inorganic became available to seagrass uptake once microorganisms died and their cell contents were released within the sediment (Evrard et al., 2005). In particular,

sulphate-reducing bacteria (SRB) represent the main drivers of nutrient mineralisation in coastal sediments (Duarte, 1990) and SRB abundance within seagrass beds is dramatically higher compared to bare sediments due to the ability of seagrasses to trap nutrients within their beds (Shieh & Yang, 1997). The most abundant group of SRB identified in seagrass-colonised sediments belongs to the Family *Desulfobacteraceae* (*Deltaproteobacteria*), which are able to oxidise organic nutrients in order to produce CO_2. Microbial mineralisation of OM occurs through a number of electron acceptors (such as oxygen; Holmer et al., 2001; Kristensen & Holmer, 2001), and it is, therefore, largely supported by the photosynthetic oxygen released from seagrass roots (Larkum et al., 2007). While these studies suggests that mineralised DON would be promptly available for seagrass uptake, annual budgets on nitrogen inputs in seagrass sediments show that it is usually insufficient to satisfy the seagrass N demand. For example, N mineralisation in the sediments of *Cymodocea rotundata* and *Thalassia hemprichii* could only supply between 6 and 22% of ammonium annually taken up by roots (Holmer et al., 2001).

While little information is available on N cycling by microorganisms associated with the seagrass rhizosphere, even less is known of the nitrogen processes performed by heterotrophic microorganisms associated with seagrass leaves and their possible contribution to the whole plant nitrogen budget. Plant leaf microorganisms, as well as strains present within the sediment, may be able to mineralise DON by using photosynthetic oxygen lost through leaf surface as electron acceptor, thus linking the recycle of N through heterotrophic metabolism.

The process of DON mineralisation can be linked to other microbial processes by such as nitrification (in presence of oxygen) and denitrification or annamox in suboxic and anoxic conditions (Zehr et al., 2002; Galloway et al., 2008). Ammonia-oxidizing microorganisms (AOM), both bacteria (AOB) and archaea (AOA), are key players in nitrogen cycling in marine systems, since they link the process of N mineralisation to nitrification (Kowalchuk & Stephen, 2001; Francis et al., 2007), thus avoiding the loss of ammonium from the system. Ammonium oxidation is typically thought to be an obligatory aerobic, chemoautotrophic process restricted to just a few groups of bacteria (belonging to *Proteobacteria*) and archaea (belonging to *Thaumarchaeota;* Kowalchuk & Stephen, 2001). AOM perform the first step of nitrification: the conversion of ammonium (NH_4^+) to nitrite (NO_2^-) through ammonia monooxygenase (encoded by the gene *amoA*) (Francis et al., 2007). Recent studies

have shown (Alonso-Sáez et al., 2012) that some autotrophic archaea and bacteria may be able to link the process of mineralisation of organic nitrogen, in particular urea, to nitrification. Among DON compounds, urea has long been recognised as an important N source in many marine coastal environments (Antia et al., 1991, Crandall & Teece, 2012) which can derive from zooplankton and fish excretion (Miller & Glibert, 1998; Conover & Gustavson, 1999), bacterial regeneration (Cho & Azam, 1995) and release from the sediment (Cho et al., 1996; Fig.1.3). These microorganisms assimilate urea into the cell and break it down using urease (encoded by the gene *ureC*) with production of CO_2 and NH_4^+. CO_2 is fixed by the cell, whereas the ammonium is oxidised into nitrite by ammonia monooxygenase (Francis et al., 2007) and released into the environment. Nitrite released by AOMs can be taken up by different bacterial groups (i.e. nitrifying bacteria) that perform the second step of nitrification: the oxidation of nitrite to nitrate (NO_3^-), which is then released by microbial cells (Francis et al., 2007; Hong & Keith, 2016; Fig. 1.3).

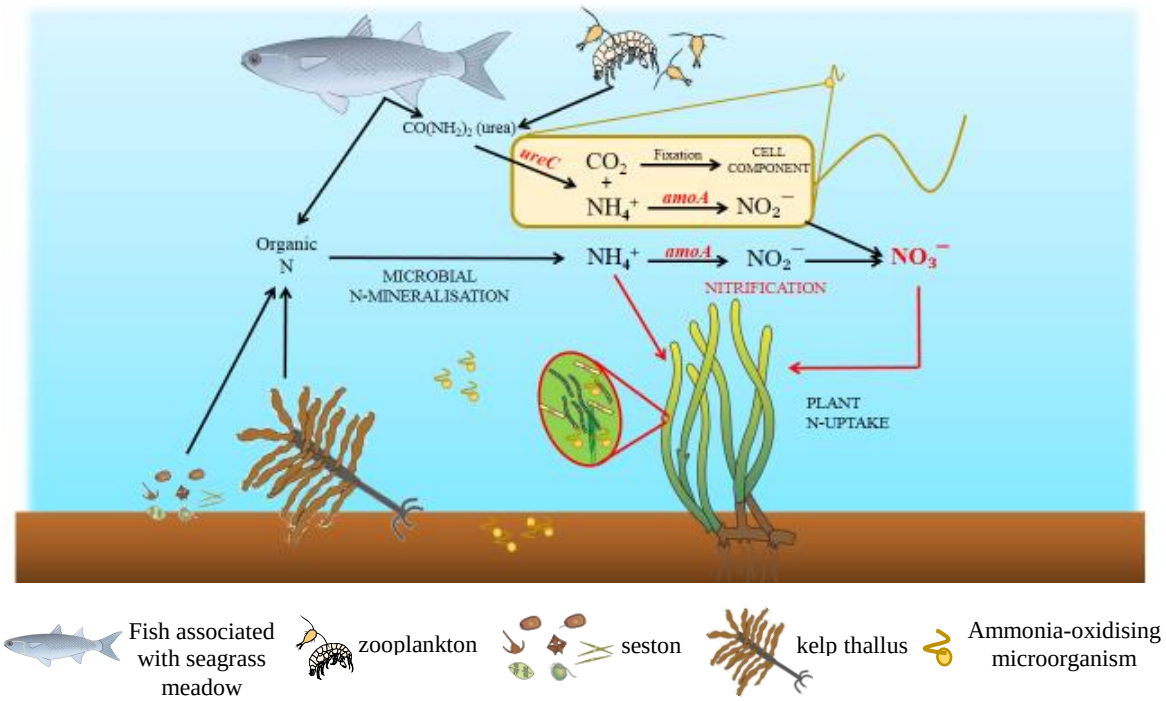


Fig. 1.3 – Process of ammonia-oxidisation in a seagrass meadow in South Western Australia. Kelp (*Eklonia radiata*) and seston trapped within the meadow leach highly labile DON mineralised by microorganisms with the production of ammonium. Ammonium can be taken up by seagrass leaves and/or AOM. Nitrification is performed in two steps: oxidation of ammonium to nitrite through the use of monooxygenase (encoded by *amoA*) and oxidation of nitrite to nitrate. Ureolytic AOM are able to link the process of DON mineralisation and nitrification. In particular, ureolytic AOM can use urea (usually excreted by fish and zooplankton) which is mineralised through urease (encoded by *ureC*) to produce CO_2 and NH_4^+. CO_2 is fixed by the cell, whereas the ammonium is oxidised into nitrite by ammonia monooxygenase (Francis et al., 2007). The final product of nitrification, nitrate, can be released by microbial cells and it may be hypothetically absorbed by seagrass leaves. DON mineralisation and nitrification may take place within the sediment, water column and on the surface of seagrass leaves.

AOMs have been found associated with a variety of marine organisms such as sponges, corals and macroalgae, but they can also be found in the water column or marine sediments such as mangrove and seagrass beds. Using quantitative-PCR, Trias and colleagues (2012) found a great abundance of AOM on macroalgae, especially AOB (1% of total bacteria) compared to that previously demonstrated for other marine habitats [i.e. 0.1% for marine sponges (Bayer et al., 2008)]. They hypothesised that the process of ammonium oxidation is largely performed by microorganisms inhabiting the macroalgae surface using photosynthetic oxygen produced and exuded by algal surface. The fact that a similar environment to that of macroalgae is found on seagrass leaves, which constantly release oxygen during the

photosyntetic process (Larkum et al., 2007; Borum et al., 2007), suggests that seagrass leaves may represent and ideal habitat for AOMs. In reality, nothing is known about the relationship between AOM and seagrasses in terms of competition for or provision of nitrogen. Effectively, their relationship seems controversial since seagrass may actively compete with AOM for ammonium uptake (Sousa et al., 2012; Soana & Bartoli, 2013), however, marine plants may still benefit from the release of the final product of nitrification, NO_3^-. In this context, the ecological significance of AOM on marine autotrophs needs further attention.

1.5 Study Area

South-western Australia hosts the largest and the most diverse seagrass meadows in the world, covering an extensive area of about 2200sq km, which is similar to the surface occupied by rainforest in the entire continent (Kirkman & Walker, 1989). The generally high productivity of seagrasses, which should be paralleled by a high nutrient demand, has attracted attention for this area due to the oligotrophic nature of these waters because of the influence of the Leeuwin Current. The Leeuwin Current is a tropical current that transports warm, low salinity and low nutrient water along the Western Australian coast from north-west to south-west (Thompson, 1984; Holloway & Nye, 1985). Interestingly, despite the extremely low nutrient concentration of these waters, extensive *Posidonia* sp. meadows persist and flourish in this area. Eight *Posidonia* species are found in the south-western coast of Western Australia comprising *P. robertsoniae, P. kirkmanii, P. coriacea P. denhartogii, P. australis, P. angustifoli*a and *P. sinuosa.* Each species has distinct habitat requirements such as minimum light demands and water hydrodynamics. In particular, *P. sinuosa* is the dominant species from north of Perth (Western Australia) to Lacepede Bay (South Australia; Kirkman & Walker, 1989). It usually colonises depths of approximately 2 to 10 m in continuous and dense meadows (Kirkman & Kuo, 1990) that can be monospecific or mixed meadows with *P. australis* (Cambridge & Kuo, 1982).

North of Perth, offshore reefs and islands, tombolas and the shore form many lagoons, an example is the sheltered Marmion Marine lagoon (the sampling site for this study) which is characterised by high density meadows of *P. sinuosa*. This is a key representative area characterised by the presence of rich and diverse marine

habitats, including kelp forests and seagrass beds that contribute to nutrient and energy flows in coastal ecosystems and stabilise sandbanks. In this area, *P. sinuosa* meadows and rocky reefs exist in close proximity. The common brown kelp *Ecklonia radiata,* participates in forming the highest benthic biomass of the rocky reef communities with an average density of 20 to 80 plants m^{-2} in the region (Kendrick et al., 1998). Large meadows of *P. sinuosa* (Cambridge & Kuo, 1979) occur inshore of these reefs, where leaf and epiphyte production range from 600 to 900 and 133 to 161 g dry wt m^{-2} yr^{-1}, respectively (Cambridge & Hocking, 1997). Flow of material across different habitats in this region appear evident and it has been estimated that between 1270 and 7800 t dry wt km^{-2} of detached kelp passes through seagrass meadows (Kirkman & Kendrick, 1997), resulting in significant increase of nutrients being released into the meadows. Whether *P. sinuosa* would be able to uptake leached DON directly through leaves or leaf-associated microorganisms mediate seagrass N uptake remains a knowledge gap.

1.6 Aims and Objectives

In addition to seagrass, their epiphytes, and microphytobenthos, allochthonous inputs can contribute significantly to the organic matter pool of seagrass ecosystems. These sources are likely to be essential for the health of seagrass meadows in oligotrophic environments, particularly in supplying nitrogen, which is considered a major limiting nutrient for seagrass productivity. Hence, microbial (bacterial and archaeal) nutrient demands in seagrass ecosystems can potentially depend on one or a variety of nutrient sources. In general, benthic microbial incorporation of organic matter into seagrass ecosystems is influenced by: (i) the organic matter concentrations in the environment (Bouillon & Boschker, 2006); (ii) the microbial community's location in the ecosystem; and (iii) the microbial community proximity to autochthonous and allochthonous organic matter inputs (Boschker et al., 2005; Williams et al., 2009). While there has been considerable focus on the role of sedimentary microorganisms in incorporating organic matter into seagrasses, there is limited understanding of the role of microbes associated with seagrass leaves.

The aim of this study is twofold:

To determine the importance of microorganisms associated with seagrass leaves and their role in enhancing nitrogen availability and uptake in seagrasses,

To verify and quantify microbial processing of allochthonous N sources in seagrass meadows.
The research can be outlined in four specific objectives:

1. Review the literature on the current knowledge of seagrass microbiome diversity, interactions and potential effects that microorganisms associated with both the above- and belowground seagrass tissue may have on plant fitness, highlighting literature gaps.

2. Quantify and compare the abundance of bacterial and archaeal 16SrRNA genes, and genes involved in nitrogen cycle (*amoA* and *ureC*) and diversity of 16SrRNA and *amoA* genes in the water column above the canopy, and within 3 micro-habitats of seagrass meadows (canopy water, leaves surface and sediment). To achieve this, I determined whether patterns in those parameters are variable according to the microhabitat examined and time.

3. Determine whether seagrass leaves are capable of taking up DON intact or if the uptake is mediated by microbial mineralisation of organic matter. To achieve this, I traced the fine-scale uptake and assimilation of microbial processed N into seagrass cells, by using the innovative Nanoscale secondary ion mass spectrometry (NanoSIMS).

4. Determine the nitrification rates associated with AOMs inhabiting *P. sinuosa* leaf surface and the role of AOMs in enhancing, or competing with, seagrass N uptake. Hence, I performed a series of experiments using a mixture of simple ^{15}N-algal amino acids and a more complex organic ^{15}N-compounds kelp derived, in order to mimic two common allochthonous nitrogen sources for seagrass meadows of South-Western Australia.

CHAPTER TWO: THE SEAGRASS HOLOBIONT AND THE ROLE OF MICROORGANISMS IN SUPPORTING AQUATIC ANGISOSPERMS

2.1 Introduction

Seagrasses are marine flowering plants which colonised the aquatic environment about 100 million years ago (den Hartog, 1970; Les et al., 1997; Orth et al., 2006), and are present in coastal areas of all continents except Antarctica (Hemminga & Duarte, 2000; Green, 2003). Seagrasses are important benthic ecosystem engineers and, where canopy meadows are present, provide a variety of ecological services (Costanza et al., 1997; Orth et al., 2006). They represent an important source of food and a habitat for a number of organisms (Valentine & Heck, 1999; Beck et al., 2001, Heck et al., 2003; Valentine & Duffy, 2006), support different ecosystems through the export of their primary production (Wolff, 1980; Suchanek et al., 1985; Duarte & Cebrian, 1996; Heck et al., 2008), and are extremely important for coastal protection due to their capacity to reduce wave intensity and turbulence (Gambi et al., 1990; Ackerman & Okubo, 1993). Seagrasses also harbour diverse communities of epi- and endophytic bacteria associated with their leaves and roots (Weidner et al., 2000; Krutz et al., 2003; Jensen et al., 2007; Uku et al., 2007; Crump & Koch, 2008; Hamisi et al., 2010; Garcias-Bonet et al., 2012; Garcias-Bonet et al., 2016).

From our knowledge of terrestrial plant-microbiota interactions, it has become clear that the presence of a specific set of microorganisms can have great consequences for plant growth, health and productivity (Compant et al., 2005; Rodríguez et al., 2006; Hayat et al., 2010). There is substantial field and laboratory-based evidence showing that seagrasses, while supporting their epiphytic microbial communities through the exudation of nutrients (Brylinski, 1971; Kirchman, 1984; Wang et al., 2014), obtain a number of advantages in return from these communities (McClung et al., 1983; Wirsen et al., 2002; Jensen et al., 2007). The close association between marine plants and microorganisms (Kirchman, 1984; Welsh, 2000; Holmer et al., 2001; Kurilenko et al., 2007; Cole & McGlathery, 2011) supports the idea that seagrasses and seagrass microbiota constitute a 'holobiont' (Thompson et al., 2014; Bordenstein & Theis, 2015), where the holobiont is defined as an organism (biont) that has/have symbiotic relationships with a variety of other organisms, including prokaryotes and eukaryotes (Margulis, 1991). The organism

and its symbiotic partners, together (holo, meaning whole) represent an integrated community that support each member for the success of survival (Margulis, 1991).

In this chapter, an overview is given of the diversity of epiphytic bacteria among seagrass species and environmental communities, underlining the presence of species-specific microbiota. I review the various positive and negative interactions that occur between marine plants and colonising bacteria that will shape the final seagrass epiphytic community, and the advantages that seagrass obtain from their epi- and endophytes. Finally, I discuss the mode of transmission of the microbiome across seagrass generations, highlighting the literature gaps.

2.2 Seagrass microbiota specificity

Seagrass tissues provide a physical substrate for a great variety of epiphytic organisms belonging to the prokaryotic and eukaryotic domains, including autotrophs (diatoms, cyanobacteria, encrusting algae) (Borowitzka, 1989; Borowitzka et al., 1990; Uku & Björk 2001; Uku et al., 2007; Borowitzka et al., 2007; Piazzi et al., 2007; Hamsi et al., 2013), and heterotrophs (microorganisms, fungi, invertebrates) (Weidner et al., 2000; Devarajan et al., 2002; Krutz et al., 2003; Jensen et al., 2007; Crump & Koch, 2008a; Sakayaroj et al., 2010). Both the above- and below-ground tissues host a high abundance of microorganisms, ranging between 1 x 10^6 and 8.5 x 10^6 cells cm^{-2} for leaves (Kirchman, et al., 1984), and between 10^5 and 10^6 cells cm^{-2} for root and rhizome tissue (Blaabjerg & Finster, 1998). Seagrasses seem to possess a core microbiome of epiphytic microorganisms, which differ from the microbial strains present in the surrounding environments (e.g. the SAR11 cluster of the water column; Weidner et al., 2000). Additionally, bacterial communities associated with various seagrasses present similar patterns among individuals of the same species sampled at different locations, rather than among individuals of different species taken from the same environment, indicating that seagrasses may support species-specific microbiota (Crump & Koch, 2008; Uku et al., 2007). For example, although Stuckenia pectinata and Potomogeton perfoliatus were collected from meadows located in close proximity, their epiphytic communities were highly divergent between the two species (Crump & Koch, 2008). Epiphytic cyanobacteria of *C. serrulata* showed similarities among plants collected

at different sites (dominated by the genus Lyngbya and Cyanosarcina), and distinct differences between *C. serrulata* and *T. hemprichii* cyanobacterial populations (the most abundant cyanobacterial genus of T. hemprichii being Synechococcus), suggesting that specific interactions between seagrass and microbiome may be involved in the establishment of these associations (Uku at al., 2007). For each plant species (*P. perfoliatus, Vallisneria americana, Zostera marina* and *S. pectinata*) analysed by Crump & Koch (2008), a few strains were present on all replicates. However, they were generally the most abundant microorganisms associated with each seagrass species, suggesting species-specific dominance of a few bacterial taxa. Two bacterial taxa from *Z. marina* roots and leaves collected at Chesapeake Bay (USA) were closely related to taxa associated with *Z. marina* roots in a study conducted in Denmark (Europe), suggesting the presence of a cosmopolitan *Z. marina*-associated microbial community (Crump & Koch, 2008). Yet, the possibility that generalist strains may be associated with more seagrass species exists. In fact, Crump and Koch (2008) identified 12 members of the Proteobacteria, Spirochaetes, and Bacteroidetes that were associated with more than one plant species. They hypothesised that these ubiquitous populations comprise unique, adapted, and potentially mutualistic communities of plant-attached bacteria. Several studies also highlight significant differences between the microbial communities inhabiting different sections of a plant, implying that, for example, the 'leaf' microbiome may differ significantly from the 'root' microbiome. In the case of *Vallisneria americana*, the most significant difference between portions of the plant was due to the dominance of Gram negative (Gram-) bacteria isolated from the root surface, compared to the high abundance of Gram positive (Gram+) isolated from the rhizosphere (Krutz et al., 2003). Also, Epsilonproteobacteria, Gammaproteobacteria and Actinobacteria seem important colonisers of *Z. marina* roots, while Deltaproteobacteria appears to dominate the rhizosphere (Jensen et al., 2007).

Microorganisms can also live endophytically in between and inside root epidermal, exodermal and cortex cells (Kuo 1993; Kusel et al., 1999, Kristensen 2005), and within leaf tissues (Garcias-Bonet et al., 2012). Few studies have investigated the diversity of seagrass leaf and root endophytes. For Posidonia oceanica, the low number of bacterial operational taxonomic units (OTUs) found in internal tissues suggests a high level of specialisation to an endophytic life (Garcias-

Bonet et al., 2016). For example, *Posidonia oceanica* represents a source of *Marinomonas* species associated with its microbiota (Lucas- Elío et al., 2012). In comparison, *Marinomonas* strains have not been found associated with internal tissue of *Z. Marina* or *C. serrulata* and only one bacterium related to *M. vaga* was found as an epiphyte on *Halophila stipulacea* leaves (Weidner et al., 2000).

2.3 Seagrass-bacterial interactions are both positive and negative

The specificity of seagrass microbiota depends on the ability of plants to shape their epiphytic microbial communities through a series of positive (such as the release of nutrients from plant surface; McRoy et al., 1972; McRoy & Goering, 1974; Kirchman et al., 1984) and negative (the production of antimicrobial defences) interactions (Zidorn, 2016). Seagrass leaves and roots exude nutrients that can attract bacteria to the plant surface (Wood & Hayasaka 1981; Perry & Dennison, 1999), and bacteria isolated from the roots of *Z. marina* demonstrate chemotaxis toward root exudates; in particular toward amino acids (Wood & Hayasaka, 1981; Krutz et al., 2003). In terms of plant tissue colonization, it seems that seagrass epiphytic bacteria may adhere selectivily to substrates released by the plant (Lucas-Elío et al., 2012). The results obtained by Kurilenko and colleagues (2007) support this evidence: during their experiment, two epiphytes isolated from *Z. marina* leaves attached exclusively to the seagrass in preference to abiotic surfaces, and remained viable for the duration of the experiment (60 hours). Moreover, they showed that free-living bacteria that settled on seagrass leaves were considered not viable 30 hours after settlement, suggesting that plant defences and/or competition with resident bacteria may have affected their viability (Kurilenko et al., 2007). Vascular plants are able to recognise microbe-derived compounds and adjust their defence and growth responses according to the type of microorganism encountered (Bais et al., 2004) and a recent study has shown that similar receptors exist in seagrasses (e.g. *T. testudinum*) that perceive the presence of bacterial lipopolysaccharides (Loucks et al., 2013).

2.3.1 Seagrass defences:

Seagrass defences include secondary metabolites and reactive oxygen species (ROS). Plant secondary metabolites can be divided into three chemically distinct groups: terpenes (e.g. terpenoids), phenolics (e.g. flavonoids and phlorotannins), and nitrogen-containing compounds (e.g. alkaloids) (Fig. 2.1; Zidorn, 2016). Sieburth and Conover (1965) were among the first to demonstrate that phlorotannins from two macroalgae inhibit two species of fouling bacteria. Subsequent studies have shown that extracts and purified metabolites from marine plants can inhibit microorganisms associated with biofilm formation (Bernard & Pesando, 1989; Sundberg et al., 1997; Steinberg & De Nys 2002; Xu et al., 2005; Engel et al., 2006; Newby et al., 2006), and help plants in resisting decomposition by saprophytes and pathogens (Zapata & McMillan, 1979; Engel et al., 2006; Kannan et al., 2010). For example, antibacterial activity in the leaves of *Posidonia australis* was detected against the Gram+ bacteria *Streptococcus pyogenes* and *S. pneumoniae* but not against Gram- bacteria (Reichelt et al., 1984). Extracts from *Halodule beaudettei, Syringodium filiforme, Enhalus acorioides* and *Halophila minor* were active at different levels against *Pseudoalteromonas bacteriolytica* and the stramenopiles *Halophytophthora spinosa* and *Schizochytrium aggregatum* (Engel et al., 2006). Additionally, three species of estuarine seagrass (*Potamogeton pectinatus, Potamogeton perfoliatus* and *Ruppia maritima*) had antimicrobial effects on 12 strains of Gram+ and a few species of Gram- bacteria (Bushmann & Ailstock, 2006).

Seagrass defences through secondary metabolites are activated when membrane receptors (encoded by resistance R-genes) recognise and bind specific molecules (elicitors) originating from pathogens, and thus alert the plant of their presence (Bent, 1996; Boller & Felix, G. (2009). Subsequently, a process of local defence responses begins with the production of reactive oxygen species and secondary metabolites at the site of the attempted attack (Fig. 2.1; Bent, 1996). Although the mechanism of action of many secondary metabolites has not been characterised yet, it seems that some molecules are able to interfere with bacterial quorum sensing (QS). Many plant pathogenic bacteria are dependent on QS to invoke disease, due to the fact that some virulence-related traits are induced only when the bacterial population reaches a specific density threshold (Andersen et al.,

2010; Barnard et al., 2007; de Kievit and Iglewski, 2000; von Bodman et al., 2003). The most studied signalling molecules of Gram- bacteria are the acyl homoserine lactones (AHLs) (Williams 2007), and their concentrations in biofilm can be up to 1,000-fold higher than in the environment (Stoodley et al., 2002; Dobretsov et al., 2009). In regards to plant defence, the mechanism of interference with QS is achieved by producing AHL-degrading enzymes (Fig. 2.1; Manefield et al., 2002; Bauer & Mathesius, 2004). One class of seagrass secondary metabolites with promising properties against AHLs is the flavones, which showed a minimum inhibitory concentration at 8-31 µg ml^{-1} towards a bacterial density of 5 x 10^5 colony forming bacteria ml^{-1}.

Reactive oxygen species, generated in response to a stressor by NADPH oxidase in the infected area (Torres et al., 2006), include different compounds (such as superoxide anion radical, O_2^-, hydrogen peroxide, H_2O_2, and the highly reactive hydroxyl radicals, ·OH) that target DNA, RNA, proteins and lipids of many bacteria (Cabiscol Català et al., 2000; Zhao & Drlica, 2014). To protect themselves, resident bacteria can express a variety of antioxidant enzymes (i.e. peroxidase, catalase and other oxidases) that degrade ROS and reduce the damage. Moreover, it seems that some of the bacteria that successfully settle on seagrass tissue produce oxidising-enzymes that may enhance the plant's tolerance to oxidative stress (Stajner et al., 1997, Sanchez-Amat et al., 2010). Following the production of ROS in response to a pathogenic attack, the seagrass has a need to protect itself from oxidative stress through the additional production of several enzymes that express antioxidant activity. For example, Costa and colleagues (2015) found a higher antioxidant scavenging capacity within the leaves of *P. oceanica* covered with epiphytes, when compared to leaves that were not colonised by epiphytes: the epiphytes caused a higher production of ROS in *P oceanica* leaves. Among the cultivable microbiota of *P. oceanica* leaves, the bacterium *Marinomonas mediterranea* MMB-1 has proven to be an excellent source of oxidative enzymes, including tyrosinase, which produces melanin from L-tyrosine (Fig. 2.1; Sanchez-Amat et al., 2010). Since melanins are known free radical scavengers, they could protect the producer strains from oxidative stress and, in case of cellular death with consequent release of intracellular molecules, melanins may protect the plant itself, acting as a sink for extracellular oxidative radicals (Geng et al., 2008; Sanchez-Amat et al., 2010).

2.3.2 Support of microbiota by seagrass

Bacteria that successfully colonise the seagrass surface must be able to withstand interactions with the plant's defence and competition with other members of the microbiome (Egan et al., 2013). In turn, they will benefit from the release of nutrients and dissolved organic nutrients from plant tissues (Kirchmanl, 1984; Penhal & Thayer, 1980). Bacterial epiphytes of *Z. marina* obtain carbon (C), nitrogen (N) and phosphorus (P) from low molecular weight organic compounds lost through leaching from the leaves (Fig. 2.1; Brylinski, 1971; Kirchmanl 1984). By placing *Z. marina* roots in a solution containing enriched substrates [e.g. $(^{15}NH_4)_2{}^{14}CO_3$)], it was found that nutrients were absorbed from the solution by plant roots, transferred to leaves and consumed by leaf-associated epiphytes (Kirchmanl 1984). Seagrass roots and rhizomes also exude 2-11% of the organic carbon produced during photosynthesis, which can be used by bacteria to satisfy their C demand (Fig. 2.1; Moriarty et al., 1986; Pollard & Moriarty, 1991; Holmer et al., 2001). Importantly, seagrasses may also represent a source of vitamins, iron (Thomas et al., 2008; Burke et al., 2011b; Fernandes et al., 2011) and sulphur for their microbiota (Bhararthi et al., 2016). For example, ferric iron represents a key molecule for cell-cell communication of some bacterial species in biofilms, including *Bacillus subtilis* (Vlamakis et al., 2013), a common component of seagrass and macrophytal microbiota (Ravikumar et al., 2012). Since in terrestrial plants, *B. subtilis* is responsible for the plant's defence against pathogens (by inhibiting the growth of plant pathogens such as *Pseudomonas syringae*; Chen et al., 2012), the exudation of these molecules by its seagrass host may be pivotal to the recruitment of the 'right microbiota' from the environment (Vlamakis et al., 2013). Members of the seagrass microbiome are also able to use dimethylsulfoniopropionate (DMSP) excreted by seagrasses (Jean et al., 2006; Borges & Champenois, 2015) as a source of sulfur, which can then be incorporated into bacterial proteins (Kiene et al., 2000). Genetic evidence indicates that strains of *Roseobacter,* commonly found on macroalgae (Brinkhoff et al., 2008), have the capacity to utilise DMSP produced by the algae (Egan et al., 2013). Since populations of *Roseobacter* are also commonly detected on the leaves of *Z. marina, P. perfoliatus, and S. pectinata* (Crump & Koch, 2008), these bacteria may also be able to use DMSP produced by the seagrass. Indeed, information from whole genome sequencing of *Marinomonas sp.*MWYL1A,

isolated from the root surface of the salt marsh grass *Spartina anglica*, and its relative *M. posidonica* IVIA-Po-181T isolated from *P. oceanica*, indicate that these *Marinomonas* strains share the capability to catabolise DMSP (Lucas-Elío et al., 2012).

2.4 Role of epiphytic and endophytic microorganisms in maintaining plant fitness

Vascular plants support a variety of epi- and endophytic microorganisms, which may profoundly influence plant health and productivity. Bacteria can enhance plant growth through the increase of nutrient availability (e.g. through nitrogen fixation or by mineralising organic compounds; Welsh 2000; Evrard et al., 2005; Cole & McGlathery 2011), produce or modulate plant hormones, thus influencing shoot and root development (Kamper et al., 1975; Werner et al., 2009; Kurtz et al., 2003), and confer immunity against a variety of plant pathogens by producing antibacterial compounds (Armstrong et al., 2001; Burja et al., 2001; Wu et al., 2012; Vlamakis et al., 2013). Many plant growth-promoting bacteria (PGPB) associated with terrestrial plants have been identified (Bashan & Holguin, 1998; Vessey, 2003), and the use of PGPB to increase crop yields has become a common practice in the field of agriculture (Ortíz-Castro et al., 2009). Similar to terrestrial ecosystems, bacteria associated with seagrasses may play a pivotal role in supporting their host fitness, as outlined below.

2.4.1 Microorganisms provide nutrients to seagrass

Experimental evidence for nutrient limitation of seagrass growth in tropical and temperate regions is provided by a number of studies, and marine plants may depend on their associated bacteria for enhanced nutrient availability (Donnelly & Herbert 1999; Hansen et al., 2000; Welsh, 2000; Cole & McGlathery, 2011). Nitrogen and phosphorous are two essential nutrients that can limit seagrass growth and primary production (Zimmerman, et al., 1987; Williams, 1990; Touchette & Burkholder, 2000). Thus, most studies on bacterial-seagrass interactions have focused on nitrogen (N) fixation and mineralisation of organic N and phosphorous (P). A significant portion of the seagrass plant's nitrogen requirement may be

fulfilled through nitrogen fixation on the leaves by cyanobacteria, and on the roots by sulphate-reducing bacteria (SRB) (Fig. 2.1; Welsh et al., 1996; Welsh, 2000; Nielsen et al., 2001; Pereg-Greg, 2002; Hamisi et al., 2009). In reality, direct evidence of nitrogen exchange between microbial hosts and seagrass tissue itself does not exist, however, indirect evidence has been reported. For example, Uku and colleagues (2007) found that cyanobacterial abundance was higher on seagrasses sampled from oligotrophic waters when compared to the same species from a less oligotrophic site, suggesting that a mutualistic symbiosis may exist between seagrasses and cyanobacteria, and that seagrasses may benefit from the diazotrophic nature of their epiphytic community in terms of N supply. Additionally, considerable nitrogenase activity has been identified in cyanobacterial populations associated with various seagrass species at different locations (Welsh, 2000; Hamisi et al., 2009; Cole et al., 2012). For sulphate-reducing bacteria, quantification of the contribution of anaerobic nitrogen fixation to seagrass productivity shows that it may represent a substantial input of nitrogen, especially for seagrasses located in tropical and subtropical regions, where up to 65% of N requirement could be provided by N-fixation (Hansen et al., 2000). *Z. capricorni* roots incubated with $^{15}N_2$ showed the appearance of about 50% of the total ^{15}N within seagrass tissue after 6 hours of incubation (Donohue & Moriarty 1990). These results suggest: (i) the existence of direct exchange of N between the bacteria and the plant itself; or (ii) the possibility that fixed nitrogen may become available to the plant once the bacteria die and the cell components are mineralised (Donohue & Moriarty 1990). In fact, mineralisation of organic nutrients by seagrass microbiota may also increase the availability of N and P for uptake by seagrasses (Smith et al., 1984a,b; Evrard et al., 2005). In field-based experiments, after marine sediment was fertilised with organic matter, inorganic nutrients were provided by microbial mineralisation, and seagrasses (e.g. *Thalassia* and *Halodule*) showed significant growth responses to nutrient additions (Powell et al., 1989). It also seems possible that seagrass leaf epiphytes, such as cyanobacteria *Anabena sp., Calothrix braunii,* and *Nostoc sp.* (Uku et al., 2007; Hamisi et al., 2013), may increase P availability for seagrass uptake by being able to solubilise phosphorous compounds to inorganic forms (Sharma et al., 2013).

The internal cells of seagrass roots support a diverse community of bacteria, which may have beneficial effects on the plant by expressing protease activity and

being able to solubilise inorganic phosphorus from insoluble compounds (Jose, et al., 2014), thus increasing nutrient availability. Endophytic bacteria (including *Kocuria sp., Vibrio sp., Saccharomonaspora sp., Bacillus sp., Desulfovibrio zosterae* and *Celerinantimonas diazotrophicus*) isolated from the root tissues of *C. serrulata, P. oceanica* and *Z. marina* are also able to fix nitrogen (Nielsen et al., 1999a, Ivanova et al., 2004, Werner et al., 2009, Garcias-Bonet et al., 2016). Nutrient translocation experiments using labelled nitrogen isotopes have shown that nitrogen is not only translocated in the same seagrass shoot from roots to leaves and vice versa, but also between shoots of the clonal plant up to tens of centimeters (Marbà et al 2002). This clonal connectivity suggests that, although endophytic bacteria may be present only in a few shoots of the clone (such as in the case of *P. oceanica* endophytic N-fixing bacteria), the whole plant could benefit from their activity (Garcias-Bonet et al., 2016).

2.4.2 Microorganisms protect seagrass from toxic compounds

Seagrasses often grow in suboxic or anoxic sediments (Goodman et al., 1995; Borum et al., 2005). Anaerobic mineralisation of organic matter in coastal marine sediments is due to the activity of sulphate-reducing bacteria (SRB, Jørgensen, 1982), and a high abundance and activity of SRB has been reported in the seagrass rhizosphere compared with non-vegetated sediments (Shieh & Yang 1997), probably due to the increased organic carbon availability surrounding the plant's below-ground tissue (Fig. 2.1; Holmer & Nielsen, 1997). An endophytic *Sulfitobacter* and *Desulfovibrio zosterae*, both isolated from *Z. marina* roots, are capable of mineralising organic nutrients by reducing sulphate (Nielsen et al 1999; Ivanova et al 2004). This mineralisation process supplies nutrients for seagrass growth (Holmer et al., 2001), but also results in the accumulation of highly toxic compounds, especially hydrogen sulphides (Bagarinao, 1992), and sulphide poisoning has been linked to recent die-back events of seagrasses worldwide (Koch & Erskine, 2001; Borum et al., 2005; Holmer et al., 2006). Seagrasses can respond to this potential phytotoxin by translocating photosynthetically produced oxygen from leaves into the roots, with consequent leaching of oxygen into the sediment to enhance sulphide oxidation (Armstrong & Armstrong, 2005). Slow, spontaneous sulphide oxidation can be accelerated by intervention of some of the seagrass epiphytic bacteria that metabolise

toxic substances. For example, nitrate-reducing sulphide-oxidising bacteria have been found associated with *H. wrightii* roots (Küsel et al., 2006). Sulphide-oxidising bacteria may be partly responsible for the oxidation of sulphide that has been observed during daytime in seagrass-inhabited sediments (Lee & Dunton, 2000) and *Spartina alterniflora* roots (Lee et al., 1999), with consequent relief of sulphite toxic effects on plants.

2.4.3 Microorganisms enhance seagrass growth

Plant hormones, also known as phytohormones, regulate plant growth and affect seed germination, time of flowering and fruit production (Miransari & Smith, 2014; Davies, 2010). Five major classes of plant hormones exist: abscisic acid, auxins, cytokinins, ethylene and gibberellins (Gaspar et al., 1996; Kende & Seevaart, 1997; Davies, 2010). In terrestrial plants, root-associated microorganisms are able to synthesise and release hormones as secondary metabolites, enhancing plant development and root proliferation (Kampert et al., 1975; Ortíz-Castro et al., 2009). It is commonly thought that bacterial production of plant hormones, which do not have the same function in microbial cells, may have evolved in bacteria because of their importance for the bacterium-plant relationship (Bacon et al., 2011). Of these growth regulating substances, indole-3-acetic acid (IAA) represents the primary active auxin in most plants, and both IAA and cytokinins affect cell division and stimulate growth of plant roots and shoots (Woodward & Bartel, 2005). Bacteria with plant growth-promoting traits have been found associated with a number of seagrasses. For example, the strains of *Actinobacteria kocuria sp.* and *Vibrio sp.* that were isolated from *C. serrulata* internal root cells are both involved in the production of IAA (Werner et al., 2009). Epiphytic *Methylophilus sp.*, associated with *H. stipulacea* leaves and *V. americana* internal root tissue (Kurtz et al., 2003) can produce cytokinins with similar growth-promoting activity to that found in terrestrial plants (Ryu et al., 2006). A Proteobacterium isolated from *H. stipulacea* leaves is closely related to *Alteromonas macleodii*, which has also been found to produce plant growth-promoting oligosaccharides from alginate, a polymer commonly found in algal cells (Natsume et al., 1994; Ferrier et al., 2002). PGPB belonging to *Marinomonas sp.* have already been shown to assist the differentiation and growth of macroalgae from the genus *Ulva* (Ravindra et al., 2011; Witchard,

2015), while *M. posidonica*, a member of the *P. oceanica* leaf endophytic microbiome (Lucas-Elío et al., 2012; Celdran et al., 2012; Goecke & Imhoff, 2016), is suspected to play a similar role in the development of seagrass shoots (Celdran et al., 2012). Cedran and colleagues (2012) grew *P. oceanica* shoots from sterilised seeds and performed experiments by inoculating germinated seeds with and without *M. posidonica*. The low growth rates of 2-week-old *P. oceanica* shoots cultured in sterilised media confirmed that inoculation with *M. posidonica* significantly enhanced shoot growth and development, suggesting that this bacterium may have profound implications for the development of *P. oceanica* during its early life-history stages (Cedran et al., 2012).

2.4.4 Microorganisms protect seagrass from pathogens and fouling organisms

Bacteria associated with seagrass above- and below-ground tissue represent a rich source of bioactive metabolites, and some microorganisms may play a protective role, releasing chemicals that protect their host from pathogens and biofouling by other organisms (Armstrong et al., 2001). The epiphytic bacterium *M. mediterranea* MMB-1 on *P. oceanica* synthesises an antibacterial agent, marinocine, with activity against both Gram+ and Gram- bacteria, such as *E. coli* and *P. aeruginosa* (Lucas-Elío et al., 2006). Seagrass epiphytic cyanobacteria play a pivotal role in plant protection from pathogens (e.g. *Staphylococcus epidermis;* Jaki et al., 1999) by producing antimicrobial and antifungal molecules, such as majusculamide A-D, malyngolide, laxaphycin A-B and diterpenoids (Burja et al., 2001). Representatives of the genus *Bacillus* are usually found in high abundance as seagrass leaf epi- and endophytes (*E. acoroides* and *T. Hemprichii*), and show incredibly high activity against biofilm-forming bacteria (Marhaeni et al., 2010). Interestingly, Nijland and colleagues (2010) found that *Bacillus licheniformis* can use an extracellular DNase (NucB), in addition to AHL-degrading enzymes, to rapidly break up the biofilms of both Gram+ and Gram- bacteria. *Bacillus licheniformis* has been isolated recently from the seagrass *T. hemprichii* (Achadi Nugraheni et al., 2010), and it may use this strategy against its bacterial competitors to obtain a selective advantage over them: thus providing its eukaryotic host with a powerful tool to control bacterial biofouling (Fig. 2.1).

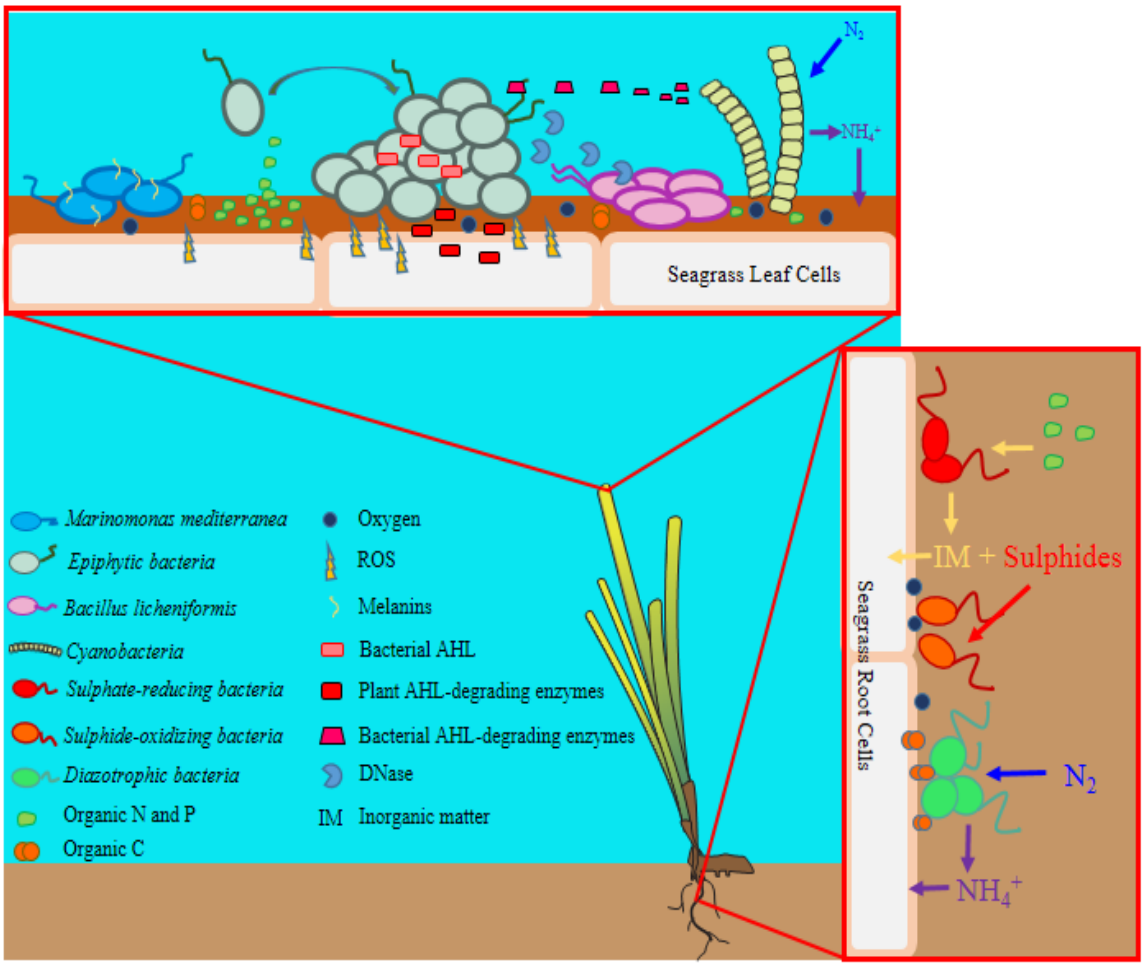


Fig. 2.1. – A conceptual diagram of the functions played by bacteria on the leaves and roots of seagrasses. On the leaf: The leaf surface attracts bacteria through the exudation of nutrients. Once a bacterial population reaches a specific density threshold, it may induce some virulence-related traits. Plants can activate a defence system, which includes the production of reactive oxygen species (ROS) and acyl homoserine lactones (AHL)-degrading enzymes at the attached site. Under high stress conditions, ROS concentrations may exceed the plant antioxidant compensation capacity, leading to oxidative stress. The presence of specific bacteria, such as *Marinomonas spp*., which represent a source of oxidative enzymes, may enhance plant tolerance to oxidative stress. Some common epiphytes, such as *Bacillus spp*. and some cyanobacteril taxa, represent a source of antibacterial and antifouling molecules, which may protect the plant from pathogenic attacks. These molecules include lactonase, which degrades Gram- quorum sensing (QS) chemicals by hydrolysing the AHL ring and an extracellular DNase (NucB) used to rapidly break up the biofilms of both Gram+ and Gram—bacteria. Cyanobacteria can also enhance nitrogen availability through nitrogen fixation. On the root: Sulphate-reducing bacteria can enhance nutrient availability by mineralising organic matter; however, free sulphides, one of the products of anaerobic mineralisation, are toxic to eukaryotes. Epiphytic sulphide-oxidising bacteria are able to use the oxygen leached by seagrass roots to oxidise sulphides and reduce their toxic effects on plants. Diazotrophic bacteria support seagrass nitrogen demand through nitrogen fixation, fueled by organic carbon exuded through seagrass roots.

Seagrass endophytic bacteria such as *Actinobacteria*, which are commonly associated with the seagrass internal root tissue (e.g. *Z. marina, T. hemprichii*; Jensen et al., 2007), are also able to synthesise a broad spectrum of antiviral, antiparasitic and antibacterial compounds, which are active against several pathogens (e.g. *Vibrio sp., Aeromonas hydrophyla* and *A. sobria;* Wu et al., 2012).

The presence of specific epiphytic bacteria also seems to influence the structure of epiphytic algal assemblages of marine plants (Cedran et al., 2012). The ability of seagrass microbiota to influence algal fouling depends upon the production of a variety of compounds with antifouling activity (Dahms et al., 2006). In the specific case of *P. oceanica*, algal epiphytes can be present in high abundance (up to 30% of the seagrass biomass; Mazsella & Ott, 1984), supporting a substantial community of grazers (Orth & Van Montfrans, 1984), and displaying high species diversity (Boero, 1981; Mazsella et al., 1989). Although antifouling molecules produced by *M. posidonica* have not been characterised yet, Cedran and colleagues (2012) showed that this endophytic bacterium plays an important role in regulating algal settlement. Patterns in the epiphytic algal community were driven mainly by changes in the abundances of red crustose, filamentous and red corticated algae, which were higher on *P. oceanica* leaves where the bacterium *M. posidonica* was present, in comparison to leaves that did not contain the bacterium (Cedran et al., 2012). Among seagrass epiphytes, cyanobacteria show a strong antifouling activity by producing a variety of antialgal compounds, such as fischerellin, aponin and galactosyldiacylglycerol, with the most studied compound being cyanobacterin (Gleason & Paulson, 1984; Borowitzka, 1995; Papke et al., 1997; Jaki et al., 1999; Ghasemi et al., 2003), which inhibits the growth of the common fouling diatom *Nitzschia pusilla* (Gleason & Paulson, 1984; Dahms, Ying & Pfeiffer 2006; Bhadury & Wright, 2004; Mazard,et al., 2016). Bioactivity against green algae was also detected in cyanobacterial species commonly associated with seagrass leaves (e.g. *Fischerella, Nostoc, Calothrix* and *Oscillatoria;* Bagchi et al., 1990; Schlegel et al., 1998, Mazard et al., 2016).

2.5 Potential for vertical transmission of microbial communities between plant generations

In terrestrial plants, the acquisition of microbial endophytes can occur both horizontally (i.e. mature plants and seeds are colonised by strains present in the environment) and vertically, whereby microorganisms are transmitted within seeds from parental plants (Truyens et al., 2014). In the terrestrial sphere, the dynamics of horizontal tissue colonisation by endophytic bacteria begins with an infection of the root tissue, which allows the bacteria to reach the root xylem vessels, and subsequently migrate upwards into the stem base, leaf base and leaves James et al., 2002, Compant et al., 2005b, 2008a. Although no direct evidence exists for a similar pattern of colonisation of marine plants, it seems that some endophytic bacteria of seagrass roots (*Thalassia hemprichii, Cymodocea serrulata, Halodule uninervis, Syringodium isoetifoliu*) originally belong to strains present within the rhizosphere, which eventually penetrate inside seagrass tissue (Kusel et al., 1999). Interestingly, eight out of the nine endophytic bacteria (*Kocuria spp., Bacillus spp., Vibrio spp., Saccharomonospora, Photobacterium*) isolated from *C. serrulata* roots are able to carry out cellulase activity (Jose et al., 2014), which may be useful in penetrating inside the plant tissue. *Streptomyces spp.* and *Bacillus spp.*, often found as leaf endophytes, present cellulase activity, but it is still unknown whether they use these enzymes to colonise seagrass aboveground tissues (Larkum, 2006).

During vertical transmission in terrestrial plants, microorganisms are passed into the seeds from parental plants directly through gametes (Agarwal & Sinclair, 1996; Madmony et al., 2005; Malfanova et al., 2013), or via vascular connections (Rand & Cash, 1933; Samish & Etinger-Tulczynska, 1963; Agarwal & Sinclair, 1996; Truyens et al., 2014). Most seagrass genera are dioecious with separate male and female clones and, usually in spring, pollen is released to fertilise the female flower and produce seeds. Surprisingly, there are no currently published studies regarding the transmission of microorganisms from seagrass parental plants to their offspring. Some authors have speculated that *M. posidonica* IVIA-Po-181^{T}, characterised by an unusually small genome size (3.9 Mb versus 4.6 Mb and 5.1 Mb of *M. mediterranea* and *Marinomonas sp*. MWYL1, respectively), might be transmitted across generations (Lucas-Elío et al., 2012). They inferred that the

smaller size of the *M. posidonica* genome could be a consequence of a close and stable relationship with *P. oceanica*, which makes a high genomic potential for the adaptation to different environments unnecessary (Lucas-Elío et al., 2012).

The seagrass genera *Amphibolis* and *Thalassodendron* are uniquely viviparous, with seedlings developing while still attached to the mother plant from which they receive nutrients (Kuo & Kikman, 1990). These seeds lack the hypocotyl designated for nutrient storage and develop footing tissue that enable them to attach to the maternal plant. In both genera, the footing tissue presents similar characteristics and contains a high number of transfer cells in the outermost layers (Kuo & Kirkman, 1990). Transfer cells are cells characterised by regions of wall ingrowths, considered to be involved in short-distance transport of nutrients. Although direct evidence for the transport of nutrients through transfer cells is lacking in seagrasses, such proof exists for terrestrial plants (Gunning et al., 1968; Browning & Gunning, 1979**),** suggesting that these cells may have a similar role in marine plants (Kuo & Kirkman, 1990). Briefly, nutrients are transported through the maternal vascular system and unloaded at the interface of fruit tegument and epidermal cells of the 'footing' tissue, which attaches the seedling to the maternal plant (Kuo & Kirkman, 1990). At this interface, nutrients are absorbed apoplastically by wall ingrowths of epidermal cells, and loaded into the footing vascular system, from where they travel to reach the developing seedling (Kuo & Kirkman, 1990). Since seagrass restoration efforts for these species may depend upon the facilitation of seedling recruitment *in situ* (Irving et al., 2013; Tanner, 2015), it would be beneficial to understand whether their seedlings acquire not only nutrients but also endophytic microorganisms from maternal plants, as has been shown for terrestrial plants (Puente et al., 2009b). However, further research is needed on the acquisition and mode of transmission of seagrass-associated microbiota

2.6 Concluding remarks and future perspectives for microbial implications in seagrass management

Studies on microbial ecology in the marine environment have been extensively improved through the use of culture-independent techniques, yet information on the diversity and function of epiphytic and endophytic microorganisms associated with

above- and belowground tissue of seagrass is limited when compared to terrestrial plants or analogous marine systems (e.g. corals and sponges). In terms of bacterial diversity, it seems that the seagrass epiphytic community may comprise both generalist and specialist strains (Crump & Koch 2008; Lucas-Elío et al, 2012). Some bacteria are well-adapted to live in association with plants and are common epiphytes of several seagrass species, whereas others (e.g. *Marinomonas spp*.) appear to be part of the microbiota of specific seagrasses (*Posidonia spp.*). Bacteria and their secondary metabolites appear to provide important cues for the development of their host, since seed germination highlights a key role for bacteria in the early life stages of seagrass (Celdran et al., 2012). However, while the field has moved a long way from the first observations that microbiota can assist in the morphological development of seagrasses (Celdranet al., 2012), there is clearly more work to be carried out, probably most critically in relation to the mechanisms of recruitment of microorganisms from the environment, and whether seagrass are able to pass some strains to their offspring. The possible existence of vertical transmission of bacteria is intriguing, as it would enable a plant with an established endophytic community to pass bacteria with beneficial characteristics to the offspring. Since it has been suggested that a link exists between the endophytic content of plant seeds and seed quality, this is especially important in terms of management. The potential to control the quality of seeds by inoculating them with specific beneficial strains would be of dramatic consequence with respect to efforts at seagrass restoration. Finally, most of these studies have investigated only bacteria-seagrass associations, overlooking the diversity and role that archaea and other microorganisms may play in maintaining plant fitness. Indeed, it is crucial to understand the mechanisms that regulate these specific interactions and their ecological significance.

CHAPTER THREE: AMMONIA-OXIDISING AND UREOLYTIC MICROBIAL COMMUNITIES OF SEAGRASS MEADOWS (*POSIDONIA SINUOSA)* IN SOUTH WESTERN AUSTRALIA

3.1 Abstract

Seagrass meadows are important coastal engineer ecosystems, and the uptake of nitrogen (N) is essential for maintaining seagrass growth and primary production. Field and experimental studies show that seagrasses preferentially uptake inorganic nitrogen (DIN) and small amino acids, however, the majority of the N pool of coastal waters is composed of complex organic nitrogen molecules (DON) and urea. Microorganisms in various ecosystems are responsible for nutrient cycling via a series of enzyme-mediated transformations and microbial recycling of N may be key processes contributing to the high seagrass productivity. Under oxic conditions, the mineralisation of bioavailabile DON is linked to nitrification which consists of two-step oxidation of ammonium to nitrite and nitrate by ammoni-oxidising microorganisms (AOM) mediated by ammonia monooxygenase (encoded by the amoA operon) or hydrolysis of urea mediated by urease (encoded by the ureC operon).

In this study, I determined the abundance and diversity of bacteria and archaea (using the 16S rRNA gene) and AOM (using the bacterial and archaeal *amoA* gene, AOB and AOA, respectively) and, the abundance of ureolytic ammonia-oxidising bacteria (using the *ureC* gene), associated with four microenvironments of *Posidonia sinuosa* meadows (surface water, canopy water, sediment and leaves) in two sampling occasions. In general, bacteria outnumbered archaea 10:1 in all the microenvironments and times. Among the four microenvironments, *P. sinuosa* leaves always hosted the highest abundance of microorganisms (varying between 10^7 to 10^{10}) compared to other microenvironments (10^4 to 10^7). Furthermore, 16S rRNA phylogenetic analyses suggested the presence of a *P. sinuosa* 'core microbiota' that differed from the surrounding environment. In the specific case of AOM, AOB abundance was up to 2 orders of magnitude greater than AOA on *P. sinuosa* leaves, however, AOA showed a higher host specificity than AOB, indicating a closer relationship between the plant and epiphytic AOA. Within the AOB community, ureolytic genes were detected in high abundance, presenting AOB with the ability to switch from autotrophy to mixotrophy ammonia concentrations are low and use small organic compounds, such as urea, to fuel the reaction of nitrification.

3.2 Introduction

Nitrogen (N) is one of the most important elements required for the success of all living organisms, as it is a component of amino acids, proteins and a variety of organic compounds (Vitousek et al., 1991; Liu et al., 2010; Hill et al., 2011). Recycling of N in seagrass meadows occurs in different ways. Seagrass canopies, by reducing water velocity and turbulence (Gacia et al. 1999, Koch et al 2006), enable the formation of a nutrient rich water layer (Gobert et al. 2002, Agawin & Duarte, 2002) so that N can be directly taken up and retained within seagrass tissue (Vonk et al. 2008, Van Engeland et al. 2011) or recycled by microroganisms present in the system (Duarte, 1990; Shieh & Yang, 1997; Evrard et al., 2005; Säwström et al, 2016).

The microbial regeneration of bioavailable N can occur via N fixation and the subsequent mineralisation of organic N. Under oxic conditions, the final product of microbial mineralisation of organic N is ammonium (NH_4^+). Ammonium is further transformed to nitrite (NO_2^-) and nitrate (NO_3^-) via nitrification, which links organic matter degradation with the production of dissolved inorganic N (DIN) (Francis et al., 2005, Špela et al., 2012). The conversion of NH_4^+ to NO_2^- represents the first and rate-limiting step of nitrification, which is mediated by ammonia monooxygenase, a membrane-bound multiple subunit enzyme encoded by the *amo* operon. This operon consists of at least three genes, *amoC*, *amoA*, and a*moB*; the *amoA* gene encodes the alpha subunit containing the putative enzyme active site (Norton et al., 2002). As it is highly conserved, the *amoA* gene has been extensively used as a molecular marker for identifying potential nitrifying microorganisms (AOMs; Francis et al., 2005; Junier et al., 2010). Ammonia oxidation is restricted to bacteria belonging to Gamma and Betaproteobacteria (Teske et al., 1994; Purkhold, 2000) and archaea, classified within the new phylum Thaumarchaeota (Könnek et al., 2005; Brochier-Armanet et al., 2008, Pester et al., 2011). The presence and activity of ammonia-oxidising bacteria (AOB) and archaea (AOA) has been extensively researched in several terrestrial, freshwater and marine environments (Ward, 2005; Gonzalez et al., 2012; Peralta et al., 2014), as they can live free in the water, in the sediment, or on the surface of different substrates (e.g. macroalgae) (Francis et al., 2005, Santoro & Casciotti, 2011; Trias et al., 2012).

Within coastal environments, DON often represents the largest N reserve, contributing up to 85% of the total N pool (Voss et al., 2013). Evidence suggests potential mixotrophy for ammonia-oxidising microorganisms (Ingalls et al., 2006) and the ability to break down urea, an organic N-containing molecule with two molecules of ammonia and one of carbonic acid (Koper et al., 2004; Solomon et al.; 2010; Alonso-Saez et al.; 2012; Lu et al., 2012). Degradation of urea may satisfy microbial carbon demand, while the two molecules of ammonia could be used in turn to fuel ammonia oxidation (Alonso-Saez et al., 2012). Among DON compounds, urea has long been recognised as an important N source in tropical, subtropical, and temperate marine environments (Antia et al., 1991; Crandall & Teece 2012), where it is primarily derived from zooplankton and fish excretion (Bidigare, 1983; Miller & Glibert, 1998; Conover & Gustavson, 1999), bacterial regeneration (Cho & Azam, 1995; Walsh et al., 2000) and release from the sediment (Cho et al., 1996). The hydrolysis of urea is catalysed by the urease enzyme, releasing ammonia via three genes: *ureA*, *ureB* and *ureC*. The *ureC* gene encodes for the alpha subunit of urease, and is highly conserved in all known ureolityc genes (Koper et al., 2004).

Inorganic N can be a limiting nutrient in the marine environment, and the ability of using small organic compounds, such as urea, to fuel the process of nitrification may therefore represent an ecological advantage for AOMs in times of ammonia depletion. However, while AOMs containing the *ureC* gene have been investigated in a range of environments such as soils, estuarine and open-ocean communities and sponges (Fujita et al., 2008; Alonso-Saez et al., 2012; Levičnik-Höfferle et al.; 2012, Lu et al., 2012), nothing is known about these microorganisms within seagrass meadows, nor their role in N cycling. Seagrass meadows are important benthic habitats spread along the coastal areas of all continents except Antarctica (Moncreiff et al., 1992; Duarte & Chiscano, 1999; Barron & Duarte, 2009; Hyndes et al., 2014). Since microorganisms are abundant and diverse in seagrass ecosystems (Kirchman, et al., 1984; Blaabjerg & Finster, 1998; Uku et al., 2007; Crump & Koch, 2008), and they have been shown to play a critical role in DON recycling other aquatic systems (e.g. mangroves; Alongi 1994), seagrass-associated microorganisms may play a pivotal role in the recycling of N within these coastal habitats. In particular, AOMs capable of mineralising urea may enhance N

availability within seagrass meadows, with profound implications for N regeneration in coastal waters.

In this study, I explored the abundance and diversity of bacteria and archaea in four different microenvironments of three *P. sinuosa* meadows: surface water above the canopy, water within the canopy, biofilm on seagrass leaves, and sediment in the meadow. Additionally, I investigated the presence of microorganisms involved in two N cycling processes that have potentially strong links to each other, and to the plant's N-uptake: the hydrolysis of urea, and ammonia-oxidation. By using functional genes involved in ammonia oxidation and ureolysis as markers, I compared the abundances and diversity of AOMs and abundances of ureolytic bacteria within *P. sinuosa* meadows on two sampling occasions.

3.3 Materials and Methods

3.3.1 Sample collection and processing

The Marmion Marine Park in South Western Australia is characterised by oligotrophic waters because of the influence of the Leeuwin Current that transports warm, low salinity and low nutrient water along the Western Australian coast from north-west to south-west (Thompson 1984; Holloway & Nye, 1985). Despite the low level of environmental nutrient concentrations, extensive and dense meadows of seagrass *Posidonia sinuosa* are found in this area, in close proximity of rocky reefs dominated by brown kelps, order *Laminariales*. Inorganic nitrogen concentrations are often low in the area but higher DON values are detected (Table 5.2 Chapter 5). For this study, samples were collected from three *Posidonia sinuosa* meadows in Marmion Marine Park (31°48'49.21''S 115°42'41.23''E; 31°48'08.50''S 115°43'07.29''E; 31°48'55.85''S 115°43'38.39''E), south-western Australia. For each meadow, samples were collected from four microenvironments [surface water (50 cm depth), canopy water (3-4 m depth), sediment (0-2 cm) and seagrass leaves] on two separate sampling occasions (December 2013 and August 2014), to determine if any patterns observed in microbes across the different microenvironments were consistent across two times. I collected six replicate samples of sediment and seagrass shoots, and triplicate 1 L water samples from both

the surface and canopy water. Sediment samples were collected within each meadow at a distance of 1mt along a transect by using sterile 5 mL glass vials which penetrated the first 2 cm of the oxygenic sediment layer.

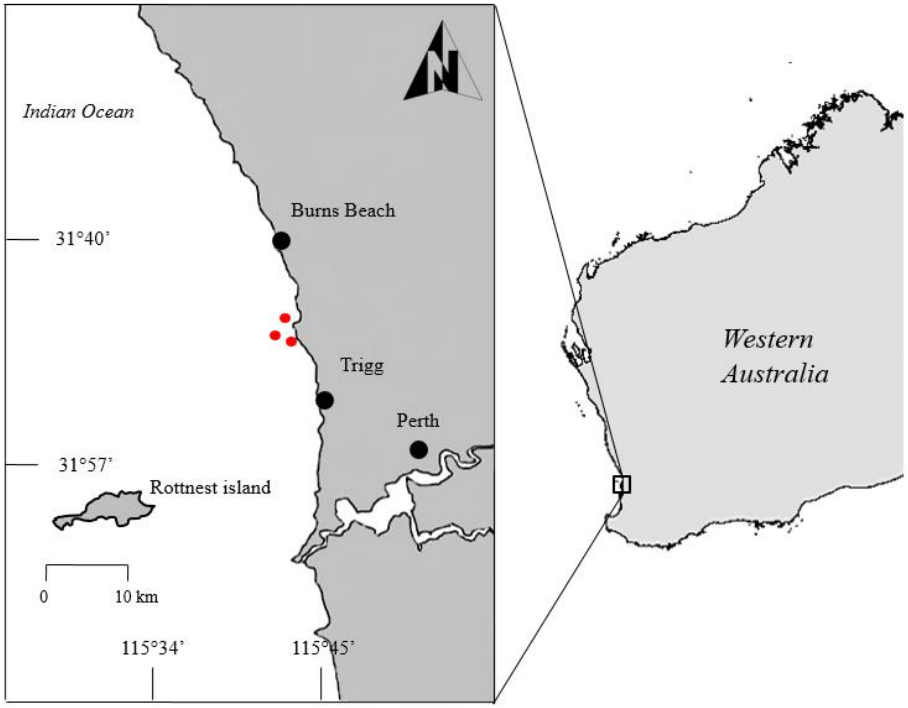


Fig. 3.1- Map of the *Posidonia sinuosa* meadows (red dots) chosen for the present study in the Marmion Marine Park, offshore Perth, South Western Australia.

3.3.2 DNA isolation

For sediment and seagrass biofilm samples, DNA was isolated using the PowerSoil and PowerBiofilm DNA Isolation Kits (Mo-Bio, USA), respectively, according to the manufacturer's instructions. DNA from water samples was manually extracted following the method of Gilbert and colleagues (2011). DNA was eluted in nuclease free water (QIAGEN, USA), quantified using the Qubit dsDNA HS Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, California, USA), and stored at -20 °C.

3.3.3 PCR amplification and preparation of q-PCR standards

To confirm the presence/absence of each gene (16S rRNA, *amoA*, and *ureC*) in each seagrass meadow compartment (surface water, canopy water, sediment, biofilm), PCRs were performed on every extracted DNA sample. Primer pairs are listed in Table 3.1 for each gene, and PCR conditions are detailed in Table 3.2. For bacterial and archaeal 16S rRNA and bacterial *ureC*, the final PCR volume reaction was 25 µl, while for bacterial and archaeal *amoA*, the PCR final volume reaction was 50 µl. Positive amplification for each reaction was confirmed using gel electrophoresis with a 1.5% agarose gel. Duplicate PCR products were combined, and the resulting bands were isolated from the gel using the PCR product purification kit Isolate II (Bioline, London, UK), following the manufacturer's instructions, except for archaeal 16S rRNA, which was purified using the Agencourt AMPure XP PCR Purification kit (Beckman Coulter, California, USA). Purified PCR products were then quantified using the Qubit® 2.0 Fluorometer (Invitrogen).

Cloning of purified amplicons was performed using the pGEM T Easy Vector System II (Promega, Wisconsin, USA) following the manufacturer's instructions. Transformed cells were plated on Luria-Bertani agar plates containing ampicillin (100 µg ml^{-1}), X-gal (40 µg ml^{-1}) and IPTG (1 mM). For each gene, white colonies containing the vector were screened using PCR with the M13 primers (Table 3.1) and DNA fragment size was checked. For all the genes, 1 µl of template DNA was used in a 25 µl final reaction volume, containing 12.5 µl of GoTaq Master Mix (Promgea), primers M13F and M13R (final concentration reported in Table 3.1). PCR cycle conditions are reported in Table 3.2. The fragment size was confirmed using gel electrophoresis, and purified using the Agencourt AMPure XP – PCR Purification (Beckman Coulter, California, USA), according to the manufacturer's instructions. Purified PCR products were sequenced at the Australian Genome Research Facility (AGRF, Perth). Taxonomy was inferred using the Basic Local Alignment Search Tool nucleotide (BLASTn; Madden et al., 2002; Boratyn, et al., 2013) on the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to confirm that PCR clones contained the expected genes. Purified clones were then quantified using the Qubit® 2.0 Fluorometer (Invitrogen) and used to construct q-PCR standard curves, ranging from 10^3 to 10^7

copies μl^{-1} for all the genes and seagrass meadow microenvironments, except for archaeal *amoA* in the water column and canopy water (10^4 to 10^8 copies μl^{-1}).

3.3.4 The quantitative polymerase chain reaction (q-PCR)

The quantitative polymerase chain reaction (q-PCR) was used to assess the abundance of bacterial and archaeal 16S rRNA genes, as well as genes representing bacterial and archaeal ammonia oxidisers (*amoA*) and ureolytic bacteria (*ureC*). Primer pairs are listed for each gene in Table 3.1, and the slope (r^2) and efficiency (E%) of each q-PCR reaction is reported in the Table 3.3. All q-PCR reactions were performed on an iCycler iQ 5 thermal cycler (Bio-Rad, California, USA). Standard curves for each gene were constructed using serial dilutions of environmental DNA, amplified and cloned as described above. Real-time PCR were performed in a 15 µl reaction mixture made of 7.5 µl of SensiFAST Sybr No-Rox Kit (Bioline, London, UK), 1 µl of DNA template, and primers at the final concentrations reported in Table 3.1 All reactions were run in technical triplicate for each sample and gene. Conditions of q-PCR are reported for each gene in Table 3.2.

Table 3.1 Primer concentrations used for polymerase chain reaction (PCR), quantitative polymerase chain reaction (q-PCR) and clone library construction for production of DNA standards.

Target gene	Target organism	Primer pair	Primer sequence, 5' - 3'	Final primer concentration in 25/50 µl PCR reaction	Final primer concentration in 15 µl PCR reaction	Fragment size, bp	Reference
16S rRNA	Bacteria	Bact 1369F Prok 1492R ("BACT2")	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	300	300	123	Suzuki et al., 2000
16S rRNA	Archaea	Parch519f ARC915r	CAGCCGCCGCGGTAA GTGCTCCCCCGCCAATTCCT	300	300	396	Coolen et al., 2004
amoA	Bacteria ammonia oxidisers	amoA1F amoA2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	500	500	490	Rotthauwe et al., 1997
amoA	Archaea ammonia oxidisers	Arch-amoA-F Arch-amoA-R	STAATGGTSTGGCTTAGACG GCGCCATCCATCTGTATGT	400	400	630	Francis et al., 2005
ureC	Bacteria ammonia oxidisers	L2F 733R	ATHGGYAARGCNGGNAAYCC GTBGHDCCCCARTCYTCRT	700	700	389	Fujita et al., 2008
ureC	Archaea ammonia oxidisers	Thaum-UreC-F Thaum-UreC-R	ATGCAATYTGTAATGGAACWACWAC AGTTGTYCCCCAATCTTCATGTAATTTTA	800		390	Alonso-Sáez et al., 2012
ureC	Archaea	CRUR_F155 CRUR_R1420	GTHTTTGGKGGVGGVAARAGY GCRCCAAACATDGGBCKRTA	800		1265	Yakimov et al., 2011
Cloning		M13F M13R	CGCCAGGGTTTTCCCAGTCACGAC TCACACAGGAAACAGCTATGAC	200			

Table 3.2 PCR and q-PCR conditions for each functional gene analysed in this study.

Target gene/organisms	Den. Step	Num. cycles	Den. Step	Ann. Step	Ext. Step	Num. cycles	Den. Step	Ann. Step	Ext. Step
PCRs conditions									
B 16S rRNA	94 °Cx2 min					30	94 °Cx1min	55 °Cx1 min	72 °Cx2 min
A 16S rRNA	94 °Cx4 min					30	94 °Cx30 sec	53°Cx1 min	72 °Cx2 min
B amoA	94 °Cx7 min	10	94 °Cx1 min	60->50 °C x 40 sec	72 °Cx90 sec	30	94 °Cx1 min	50 °Cx40 sec	72 °Cx90 sec
A amoA	94 °Cx7 min	10	94 °Cx1 min	60->50 °C x 40 sec	72 °Cx90 sec	30	95 °Cx40 sec	56 °Cx30 sec	72 °Cx1 min
B ureC	94 °Cx5 min					30	94 °Cx1 min	54 °Cx90 sec	72 °Cx2 min
A ureC	94 °Cx5 min					30	94 °Cx1 min	54 °Cx90 sec	72 °Cx2 min
A ureC	94 °Cx5 min					35	94 °Cx1 min	50 °Cx1 min	72 °Cx2 min
B and *A Cloning*	94 °Cx2 min					30	94 °Cx1 min	55 °Cx1 min	72 °Cx2 min

Q-PCRs conditions						
Target gene/organisms	**Denaturation Step**	**Number of cycles**	**Denaturation Step**	**Annealing Step**	**Extension Step**	**Ref**
B 16S rRNA	95 °Cx10 min	45	95 °Cx15 sec	62 °Cx5 sec	72 °Cx15 sec	Suzuki et al., 2000
A 16S rRNA	94 °Cx5 min	40	94 °C30 sec	63°Cx40 sec	72 °Cx40 sec	Coolen et al., 2004
B ureC	95 °Cx10 min	45	95 °Cx15 sec	54 °Cx90 sec	62 °Cx45 sec and 78 °Cx15 sec	Fujita et al., 2008
B amoA	95 °Cx3 min	40	95 °Cx40 sec	55 °Cx30 sec	72 °Cx1 min and 78 °Cx20 sec	Rotthauwe et al., 1997
A amoA	95 °Cx3 min	40	95 °Cx40 sec	56 °Cx30 sec	72 °Cx1 min and 76 °Cx20 sec	Francis et al., 2005

Table 3.3 The slope (r2) and efficiency (E%) of each q-PCR reaction is reported for every DNA sample analysed and sampling time.

Target	Compartment	r^2 Summer	E% Summer	r^2 Winter	E% Winter
Bacterial 16S rRNA	Water Column and Canopy Water	0.999	89.6	0.999	82.9
	Biofilm	0.999	88.0	0.999	92.8
	Sediment	0.999	88.0	0.998	87.6
Archaeal 16S rRNA	Water Column and Canopy Water	0.999	87.4	0.999	94.8
	Biofilm	0.998	94.6	1.000	95.8
	Sediment	0.999	82.0	0.999	92.8
Bacterial *amoA*	Water Column and Canopy Water	0.998	86.2	0.993	91.3
	Biofilm	0.998	83.8	0.994	78.4
	Sediment	1.000	84.7	0.998	85.1
Archaeal *amoA*	Water Column and Canopy Water	1.000	93.1	0.999	95.6
	Biofilm	0.999	92.3	1.000	74.5
	Sediment	1.000	97.8	0.999	95.3
Bacterial *ureC*	Water Column and Canopy Water	0.999	79.7	0.999	82.8
	Biofilm	0.999	77.2	0.999	77.4
	Sediment	0.999	76.2	0.999	80.0

3.3.5 16S rRNA and amoA sequencing

For both sediment and biofilm samples, the six replicates of extracted DNA for each time point and from each meadow were pooled together, so that each meadow and each time point was represented by a single sample. The same was done for the three replicates from each compartment (surface water and canopy water), except the two microenvironments were pooled, so that a single sample represented the water column for each meadow and each time point.

The bacterial and archaeal 16S rRNA genes were amplified and subjected to paired-end sequencing using Illumina MiSeq 2500, according to the manufacturer's instructions (Murdoch University, Perth, Western Australia). The V4 hypervariable region of the 16S rRNA gene was amplified with the modified versions (Apprill et al., 2015) of the 515F 5'– GTGCCAGCMGCCGCGGTAA -3' and 806R 5' – GGACTACHVGGGTWTCTAAT - 3'primers (Caporaso et al. 2012), following the the "16S Metagenomic Sequencing Library Preparation" Part# 15044223 Rev.B (http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Briefly, for each DNA sample, PCR was carried out in triplicate, in a 25 µl total volume including 2.5 µl of normalised total genomic DNA (5 ng μl^{-1}), 0.2 µM (final concentration) of each forward and reverse primer and 12.5 µl of 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems, USA). Extraction blanks and no template controls were always included in all PCR amplification. For each sample or control, the PCR products from the three replicates were then pooled, checked by gel electrophoresis and purified using AMPure XP beads (Beckman Coulter, USA), prior to the index PCR using the Nextera XT Index Kit indexes (Illumina, USA). The index PCR was performed as described above; the PCR products were purified again using AMPure XP beads (Beckman Coulter, USA) and quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). For each sample, 1 µl of 1:50 diluted PCR product was run on a Bioanalyzer DNA 1000 chip (Agilent 2100) to verify the fragment size (~630 bp). All the PCR products were then pooled at equimolar ratio. The pool was then further concentrated, purified by a Quiaquick PCR Purification Kit (Qiagen, USA) and quantified by using the Qubit dsDNA HS Assay Kit prior to dilution to 4 nM and

paired-end sequencing. Sequencing was then performed on a 2 x 300 bp/600 cycle V3 kit (Illumina, USA).

The bacterial and archaeal *amoA* genes were amplified and subjected to 300 bp paired-end sequencing using an Illumina MiSeq 2500 (Molecular Research MrDNA, Texas, USA). The primer pairs used for sequencing were: bac*amoA*1F 5'-GGGGTTTCTACTGGTGGT-3', bac*amoA*2R 5'-CCCCTCKGSAAAGCCTTCTTC-3'and Arch-*amoA*F 5'-STAATGGTCTGGCTTAGACG-3', Arch-*amoA*R 5'-GCGGCCATCCATCTGTATGT-3'.

3.3.6 Bioinformatics analysis

An in-house AOA and AOB database was constructed by collecting environmental *amoA* sequences (546 and 528 for bacteria and archaea, respectively) from the National Center for Biotechnology Information (NCBI), covering a range of marine habitats including seawater, sediment, corals, sponges and macroalgae. The database was constructed in MOTHUR (version 1.11.0 for Windows; Department of Microbiology and Immunology, The University of Michigan [http://www.mothur.org/]) using the Standard Operating Procedure (SOP) (Schloss et al., 2013), and sequences failing quality criteria were removed: I excluded any sequence with an ambiguous base and/or containing a homopolymer stretch longer than eight bases. I also screened sequences for the presence of chimeric reads using "chimera uchime" (UCHIME; Edgar et al., 2011). The sequences were then aligned in MEGA 6.0 (Tamura et al., 2013), and used as a database against which to assign taxonomy to my samples.

The environmental sequences obtained for this study screened in MOTHUR, according to the SOP for MiSeq analyses (Schloss et al., 2013). 16S rRNA gene sequences were aligned using against reference sequences from Silva (Yilmaz et al., 2014). Following alignment, sequences were preclustered for further error reduction and 'chimera uchime' (Edgar et al., 2011) was used for de novo removal of chimeric reads. Screening for contaminant sequences (mitochondria, chloroplasts and Eukarya) was performed by using the 'remove lineage command'. To compare bacterial diversity among samples, chimera-free and error-free sequences were used

to build a pairwise distance matrix using the 'dist.seqs' and 'cluster' command. Sequences were finally clustered at 97% nucleotide identity. Where possible, operational taxonomic units (OTUs) were identified to genus level using the Silva reference taxonomy database (Wang et al., 2007).

For *amoA* genes, sequences were analysed using the MOTHUR pipeline, as described above. Sequences were aligned to the in-house database and screened for poor quality and chimeric reads. Cleaned sequences were imported into Geneious (v8.0.5; http://www.geneious.com Kearse et al., 2012) and translated into protein amino acids. Sequences presenting stop codons were manually removed, and OTUs defined at 97% nucleotide identity.

For 16S rRNA and both bacterial and archaeal *amoA*, OTUs were used to estimate the depth of sequencing [through cover percentage estimator (Good 1953) and rarefaction curves built in MOTHUR), richness (Chao1; Chao, 1984) and calculate Simpson (1949) index of diversity. For *amoA*, representative sequences for each OTU were identified as the sequences having the maximum average similarity to the other sequences in the same OTU, and were used to construct a phylogenetic tree using maximum likelihood and 1000 bootstrap replicates in MEGA6.0. Phylogenetic trees were then imported into the website iTOL: Interactive Tree of Life (http://itol.embl.de/).

3.3.7 Statistical Analyses

Statistical analyses on gene abundances and sequencing data were performed in PRIMER 6 (PRIMER-E Ltd, Plymouth, UK; Clarke 1993) and MOTHUR (Schloss et al., 2013). Euclidean similarity matrices were calculated from $\log_{10}$-transformed abundance data to meet the requirements of homogeneity of variance. Differences in abundance among genes (16S rRNA, *amoA* and *ureC*) related to seagrass microenvironments (surface water, canopy water, sediment and leaves), within meadows and sampling times (December 2013 and August 2014) were tested in PRIMER 6 (PRIMER-E; Clarke 1993). The factors 'gene', 'compartment' and 'sampling time' were fixed factors, while 'meadow' was a random factor nested. Sorenson (Bray-Curtis) distance was constructed on square root-transformed MiSeq sequencing data and principal coordinates analysis (PCoA) was used to visualise

patterns in microbial community structure (PRIMER-E; Clarke 1993). Similarity percentage (SIMPER) analysis was used to identify OTUs that were driving patterns of separation between groups (PRIMER-E; Clarke 1993). Venn diagrams were created in MOTHUR (Schloss et al., 2013) to visualise patterns in the distribution of AOA and AOB between different seagrass meadow microenvironments. For 16S rRNA and *amoA* genes, comparisons of the community structure were performed using the weighted and unweighted UniFrac algorithm (Lozupone & Knight, 2005) to construct phylogenetic trees. While unweighted UniFrac only infers community diversity without accounting for differences in abundance, the weighted UniFrac approach is used to also account for changes in relative abundance of individual OTUs. Microbial community structure results were analysed by using the 'thetayc' calculator (Yue & Clayton, 2005) in MOTHUR (Schloss et al., 2013).

3.4 Results

3.4.1 Differences in microbial gene abundances among seagrass meadow microenvironments

Regardless of the primer pairs used, no *ureC* gene associated with archaea was found in this study. Consequently, only the results for bacterial and archaeal 16S rRNA, *amoA* and bacterial *ureC* are described in this chapter. Based on q-PCR results, gene abundances differed among gene type, compartment, and meadow within compartment but not between sampling times. However, there were significant two-way interactions between gene type and compartment, meadow and time, and between compartment and time and meadow and time, and a three-way interaction among gene type, compartment and time (Table 3.4, $p<0.05$).

In general, Bacterial abundance was about one order of magnitude higher than archaeal abundance in all the seagrass microenvironments and sampling time (Fig. 3.2 A & B). The abundances of the target genes were significantly greater within the seagrass biofilm compared to sediment and both water microenvironments on both sampling times ($F=17.9$ $p<0.001$, Fig. 3.2). Moreover, *ureC* gene abundance was between 100 and 500 times greater in the seagrass biofilm than in sediment (during December 2013 and August 2014, respectively) ($p<0.001$), and up to five orders of

magnitude higher than in both water microenvironments ($p<0.001$, Fig. 3.2 C). AOB abundance was 40 to 280 times greater in the biofilm than in sediment (in August 2014 and December 2013, respectively) ($p<0.001$), and four orders of magnitude higher than in both water microenvironments ($p<0.001$, Fig. 3.2 D). AOA abundance within the biofilm was 70 times greater compared to the sediment compartment ($p<0.001$), and four orders of magnitude higher than both water column microenvironments ($p<0.001$, Fig. 3.2 E). Bacterial *ureC* abundance dominated over AOB in all the microenvironments and sampling times. However, a significantly greater abundance of bacterial *amoA* was observed in all the microenvironments and time points when compared to AOA ($p<0.05$) with the only exception for the AOA biofilm in the first meadow (Fig. 3.2 E). The abundances of bacterial and archaeal 16S rRNA and bacterial *ureC* in each compartment did not show significant differences between sampling times (Fig. 3.2 A & C). Bacterial *amoA* showed an increase in abundance within sediment during winter ($p<0.05$), but no variations for the biofilm and water microenvironments (Fig. 3.2 D). The same pattern was followed by archaeal *amoA* abundance for sediment ($p<0.05$), with no variation between sampling occasions for the biofilm compartment, but a decrease in abundance in the surface and canopy water at the second sampling time ($p<0.05$, Fig. 3.2 E).

Table 3.4 PERMANOVA test on gene abundances within four seagrass microenvironments (water column, canopy water, sediment and biofilm) in two sampling seasons. Permanova test shows variations in the abundance of functional genes between microenvironments (first column), meadows and sampling times. For each functional gene there are significant differences in the abundance within the different microenvironments, but not among meadows. Significant differences in AOB and AOA abundances are also related to different time points. Significant values ($p < 0.05$) are shown in bold.

Factor	**df**	**Pseudo-F**	**P**
Gene type (Ge)	4	740.3	**0.0001**
Compartment (Co)	3	204.4	**0.0001**
Sampling Time (Ti)	1	0.81	0.39
Meadow (Me) in (Co)	8	2.48	**0.0081**
Ge x Co	12	27.8	**0.0001**
Ge x Ti	4	10.9	**0.0001**
Co x Ti	3	11.7	**0.0021**
Ge x Me (Co)	32	1.1	0.31
Ti x Me(Co)	8	3.7	**0.0002**
Ge x Co x Ti	12	17.9	**0.0001**
Ge x Ti x Me (Co)	32	0.9	0.69
Total	735		

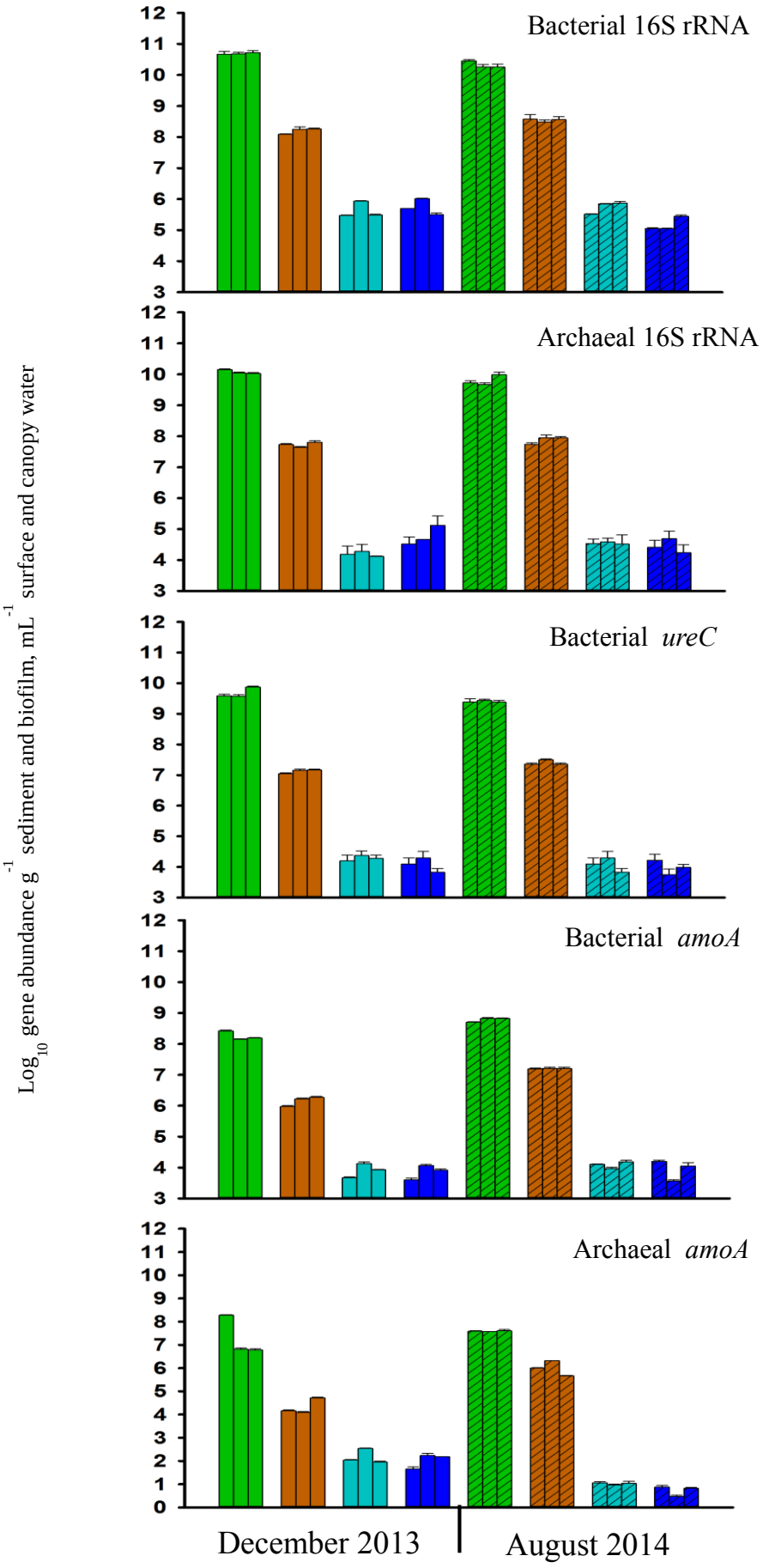

Bacterial 16S rRNA
Archaeal 16S rRNA
Bacterial ureC
Bacterial amoA
Archaeal amoA
Log10 gene abundance g-1 sediment and biofilm, mL-1 surface and canopy water
December 2013
August 2014
Biofilm Dec
Sediment Dec
Surface Water Dec
Water Column Dec
Biofilm Aug
Sediment Aug
Surface Water Aug
Water Column Aug

Fig. 3.2. - Mean abundances (+1 SE) of bacterial 16S rRNA, archaeal 16S rRNA, bacterial *ureC*, bacterial *amoA*, and archaeal *amoA* within four microenvironments (biofilm, sediment, surface water, canopy water) and two separate sampling times (solid colour for December 2013 and diagonal stripes for August 2014). In order to compare abundance patterns between all genes, $\log_{10}$ abundances are plotted on each *y* axis, varying from 10^3 to 10^{12} for all functional genes, except AOA (10^1 to 10^{12}). The four microenvironments are plotted on the *x* axis, whereby each group of three bars represents the three replicate seagrass meadows sampled. Error bars indicate standard error, calculated from six replicates for biofilm and sediment, and three replicates for surface and canopy water. Gene abundances were calculated from standard curves using the *r*2, *y* intercept and efficiency values given in Table 3.3.

3.4.2 Changes in bacterial and archaeal community diversity between different seagrass meadow microenvironments and seasons

For this study, 20,684 bacterial and 20,979 archaeal 16S rRNA high quality sequences were retrieved from 18 environmental samples which led to 574 bacterial and 579 archaeal 16S rRNA OTUs. Richness estimator (Chao1) revealed that sampling effort was sufficient for both the bacterial and archaeal 16S rRNA in all the samples analysed (Table 3.5). For bacteria 16S rRNA, the sediment harboured the highest number of OTUs (204 OTUs out 574 obtained from 8504 sequences), followed by water column (190 OTUs out 574 obtained from 5489 sequences) and biofilm (180 OTUs out 574 obtained from 6691 sequences). A similar result was found for the archaeal 16S rRNA sequencing, whereby the highest number of OTUs were associated with the sediment (252 out 579 retrieved from 8992 sequences), followed by biofilm (203 out 579 retrieved from 6728) and water column (171 out 579 retrieved from 5259).

To analyse how well each microenvironments was sampled, sequence coverage (%) was calculated. The analysed sequence coverage highlighted the depth of sequencing by varying from 90% up to 99% for both bacteria and archaea (Table 3.5) and supported the results obtained from the rarefaction curves (reported in Appendix A). Simpson index showed that diversity was well covered within the samples analysed, as shown in Table 3.5. A high diversity of the bacterial community was detected in almost every sample analysed, varying between 0.02 and 0.09 among all the seagrass meadow microenvironments and sampling seasons, with a lower diversity detected for the biofilm in meadow1 and meadow3 and water column meadow3 in summer (0.14, 0.17 and 0.17, respectively).

For archaeal 16S rRNA, Simpson index indicated that the highest community diversity was related to the microorganisms associated with *P. sinuosa* biofilm (varying between 0.02 and 0.11 with the only exception of meadow2 in winter, 0.71). The archaeal community in the water column revealed the lowest diversity, varying between 0.13 to 0.25 and, interestingly, the archaeal sediment community revealed a relatively low diversity (0.06 to 0.41), regardless of the high sequencing effort (7428 16S rRNA sequences).

Table 3.5 Bacterial and archaeal 16S rRNA cover percentage (%), community richness (Chao1) and diversity (Simpson) indices.

Bacterial 16S rRNA				Richness estimator	Diversity index
Group	Nums seqs observed	OTUs obtained	(%) Coverage	Chao1	Simpson
Biofilm M1_summer	637	25	90	32	0.14
Biofilm M2_summer	921	27	98	34	0.09
Biofilm M3_summer	2139	44	94	52	0.17
Biofilm M1_winter	1814	39	92	47	0.06
Biofilm M2_winter	749	17	98	32	0.02
Biofilm M3_winter	831	28	97	34	0.03
Sediment M1_summer	606	18	95	32	0.03
Sediment M2_summer	2717	57	99	58	0.05
Sediment M3_summer	1053	29	96	38	0.03
Sediment M1_winter	883	19	92	32	0.07
Sediment M2_winter	1288	35	94	47	0.04
Sediment M3_winter	2157	46	97	48	0.03
Water M1_summer	933	28	95	39	0.08
Water M2_summer	794	25	98	29	0.05
Water M3_summer	917	36	93	36	0.17
Water M1_winter	729	28	95	31	0.04
Water M2_winter	956	31	94	38	0.05
Water M3_winter	1160	42	96	44	0.05

Archaeal 16S rRNA

Biofilm M1_summer	1976	49	96	52	0.11
Biofilm M2_summer	480	21	92	25	0.04
Biofilm M3_summer	880	34	97	48	0.02
Biofilm M1_winter	1480	35	91	44	0.05
Biofilm M2_winter	936	29	93	41	0.71
Biofilm M3_winter	976	31	96	39	0.05
Sediment M1_summer	2528	52	98	68	0.14
Sediment M2_summer	944	26	95	35	0.11
Sediment M3_summer	1136	31	97	44	0.18
Sediment M1_winter	2944	51	99	83	0.06
Sediment M2_winter	680	28	93	37	0.41
Sediment M3_winter	760	37	96	39	0.20
Water M1_summer	556	21	92	37	0.23
Water M2_summer	524	25	90	33	0.25
Water M3_summer	836	27	95	41	0.19
Water M1_winter	1184	29	96	48	0.17
Water M2_winter	1197	32	98	57	0.14
Water M3_winter	962	20	94	39	0.23

Bacterial 16S rRNA OTUs fell into six major lineages: Proteobacteria, Planctomycetales, Verrucomicrobiales, Bacteroidetes, Firmicutes and Actinobacteria (Fig. 3.3 A). Among all of the different microenvironments, most of the sequences (241 OTUs, equal to 41.6%) were of proteobacterial origin and, of all the proteobacterial sequences, the majority fell into the Betaproteobacteria (43.9%), followed by Gammaproteobacteria (26%), Alphaproteobacteria (21.2%), Deltaproteobacteria (6.3%) and Epsilonproteobacteria (1.6%). Planctomycetes, Bacteroidetes and Verrucomicrobiales were relatively abundant (11.5%, 11.1% and 8.7%, respectively), while Actinobacteria and Firmicutes were less abundant (6.7% and 2.5%, respectively) (Fig. 3.3 A). For the biofilm, the Betaproteobacteria, Verrucomicrobiales and Bacteroidetes represented the most abundant bacterial lineages, while Deltaproteobacteria and Epsilonproteobacteria were not present on the *P. sinuosa* leaf biofilm during either sampling time. For the water column, differences were clear depending on sampling season. In particular, Alpaproteobacteria and Deltaproteobacteria were absent in summer but present in winter, while Firmicutes were absent in winter but present in summer, and Planctomycetes and Verrucomicrobia were absent from the water column during both sampling times.

For archaeal 16S rRNA, sediment samples were clearly dominated by Euryarchaeota (58.4%), while the water column harboured a greater abundance of Crenarchaeota (60.8%), and the biofilm hosted a higher abundance of Thaumarchaeota (42.9%) (Fig. 3.3 B).

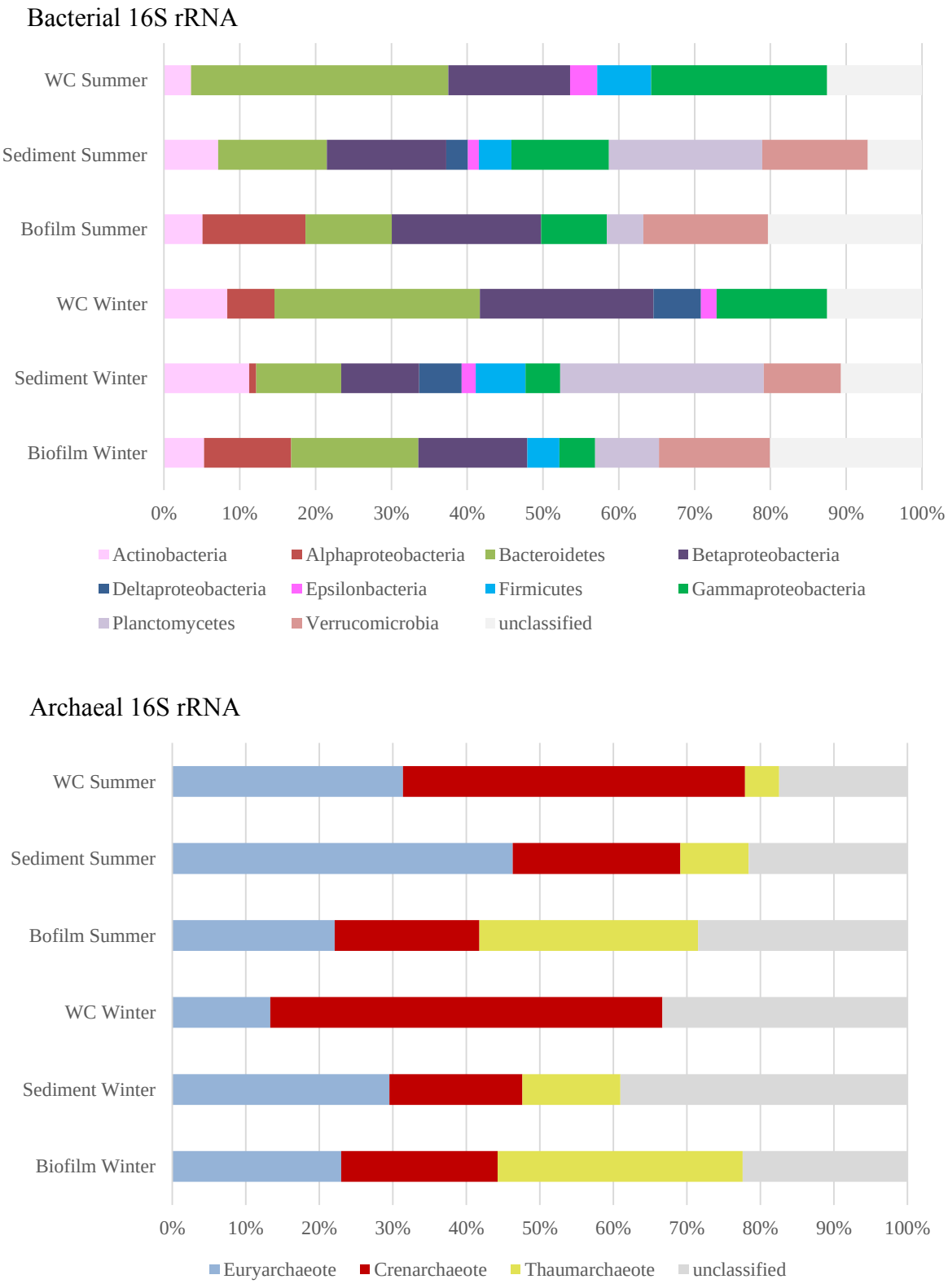


Fig. 3.3 – Percentage contributions of 16S rRNA sequences for Bacteria and Archaea retrieved from water column (WC), sediment and biofilm microenvironments during two sampling times (summer, December 2013, and winter, August 2014) from the three *P. sinuosa* meadows in Marmion Marine Park, Western Australia.

The 20 most abundant OTUs obtained from bacterial 16S rRNA belonged to Proteobacteria, in particular Betaproteobacteria (comprising four OTUs) and Gammaproteobacteria (comprising six OTUs): Bacteroidetes (four OTUs), Verrucomicrobia (OTU 8) and Actinobacteria (OTU 20), while three OTUs (OTU 14, OTU 16 and OTU 17) were 'uncultured bacteria' (Fig. 3.4).

Almost every OTU matched with known sequences obtained from studies performed in different environments [i.e. marine waters (Flammeovirgaceae, Nitrosomonadales and Actinobacteria), environmental biofilm (uncultured marine bacterium), plant biofilm/surface (Flavobacteriaceae and *Haloferula* sp.), associated with marine organisms such as sponges (*Haliea* sp., *Vibrio* sp., marine sponge symbionts), corals (Gammaproteobacteria, Bacteroidetes and OTU 16), mollusc gut (*Maritimimonas rapanae*) and soils (Methylophilus)], with the only exception of OTU 12 associated with *P. sinuosa* biofilm that matched only at 92% with *Luteolibacter cuticulihirudinis*. The archaeal 16S rRNA community was mainly composed of Thaumarchaeota, Marine Group II Euryarchaeota and Marine Group I Crenarchaeota (Fig. 3.5**)**. Also for the archaea, the 20 most abundant OTUs matched with sequences found in studies performed within the marine environment, in particular were related to organisms found in the water column form the Gulf of Mexico and Adriatic Sea, hydrothermal vents and surface microlayer of corals. Only three OTUs were not related to known sequences: two OTUs matched at 96% with organisms retrieved from marine sediment (collected from Japan, OTU 20) and surface microlayer of tropical corals (OTU 8), the third OTU matched at 92% with an archaea retrieved from the water column in South Pacific (OTU 12) (Fig. 3.4**)**.

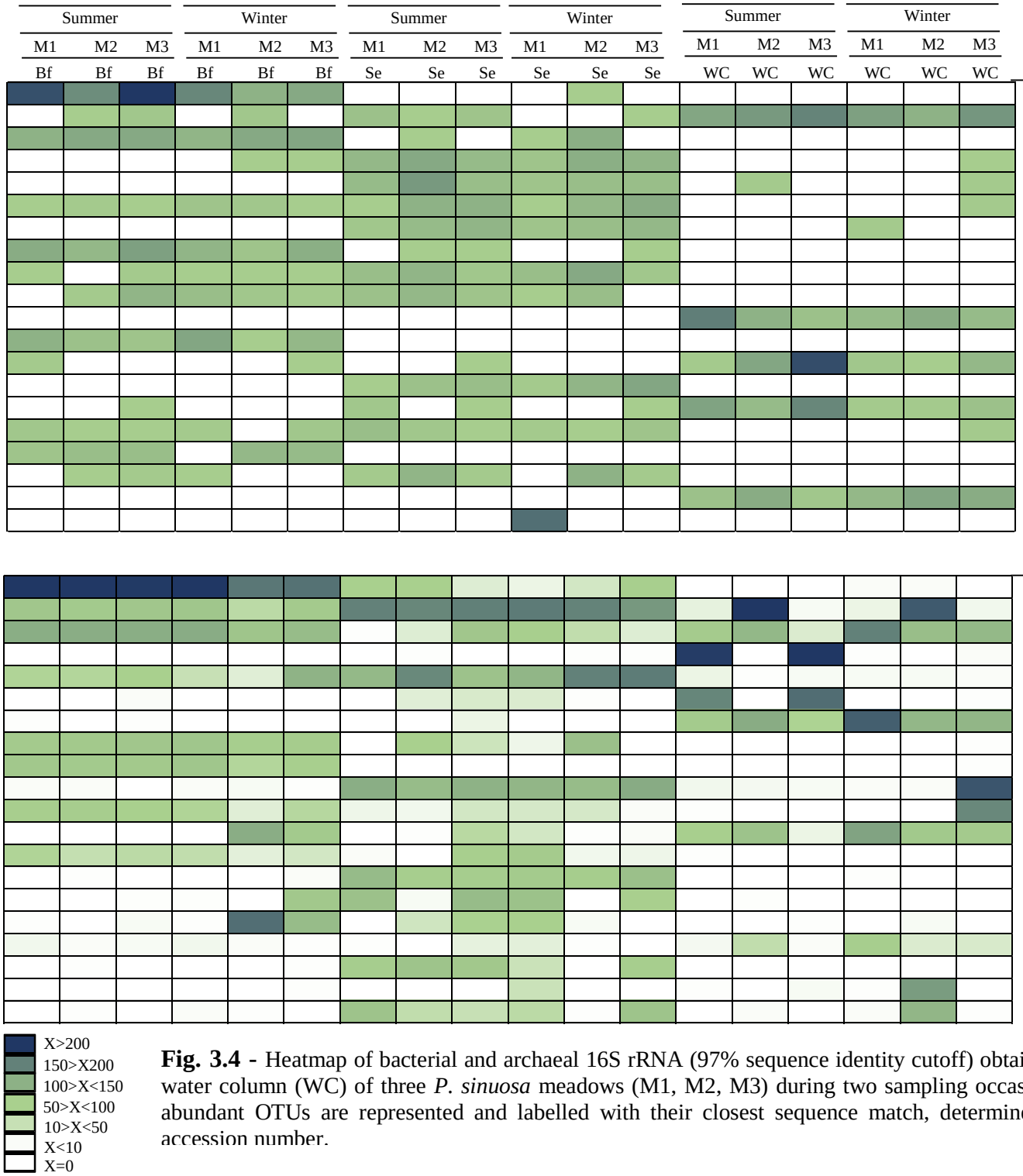


Fig. 3.4 - Heatmap of bacterial and archaeal 16S rRNA (97% sequence identity cutoff) obtained from biofilm (Bf), sediment (Se) and water column (WC) of three *P. sinuosa* meadows (M1, M2, M3) during two sampling occasions (Summer and Winter). The 20 most abundant OTUs are represented and labelled with their closest sequence match, determined using BLAST, and its corresponding accession number.

PCoA comparison of bacterial and archaeal diversity associated with the three microenvironments of *P. sinuosa* meadows revealed clear partitioning of the communities belonging to the water, sediment and biofilm (Fig. 3.5), with the first two axes explaining 55.2% and 49.6% of bacterial and archaeal variability, respectively. Regarding to the biofilm, the bacterial 16S rRNA community structure was mainly influenced by OTU 1 (*Hydrogenolus hirschii*), OTU 3 (*Vibrio harveyi*), OTU 12 (*Luteolibacter cuticulihirudinis*) and OTU 17 (uncultured marine bacterium) (average dissimilarity between biofilm and sediment was 86%, SIMPER analysis). OTU 4, OTU 6, OTU 14 (uncultured marine bacterium), OTU 5 (*Halelia mediterranea*) and OTU 16 (*Fabibacter* p.) determined the cluster of the sediment community (average dissimilarity between sediment and water was 94%, SIMPER analysis), while the water column bacterial community structure was mainly influenced by the presence of *Vibrio sp* (OTU 13) and uncultured Flammeovirgaceae (OTU 18) (average dissimilarity between biofilm and water was 97%, SIMPER analysis). Changes in the archaeal community structure relative to different seagrass microenvironments were mainly driven by OTU 1 (*Nitrosopumilus sp.*), OTU 9 (uncultured archaeon) and OTU 11 (Euryarchaeota) for the biofilm, OTU 2 and 14 (Euryarchaeota), OTU 18 (Thaumarchaeota), OTU 20 (uncultured archaeon) for the sediment and OTU 7 (uncultured archaeon) for the water column.

For both bacterial and archaeal 16S rRNA, the observed differences in community composition among microenvironments were confirmed by the UniFrac analysis (Table 3.6). Both the weighted and unweighted UniFrac measures showed significant differences ($p<0.001$ and $p<0.05$; Table 3.6) in the bacterial community composition among the three microenvironments, except for biofilm *vs*. sediment (Table 3.6). Archaeal community composition followed a similar pattern, whereby significant differences among the microenvironments were evident using both the unweighted and weighted UniFrac measures, except for the biofilm *vs.* sediment in the unweighted UniFrac (Table 3.6). Patterns across microenvironments using Weighted UniFrac were similar across sampling times (Table 3.6).

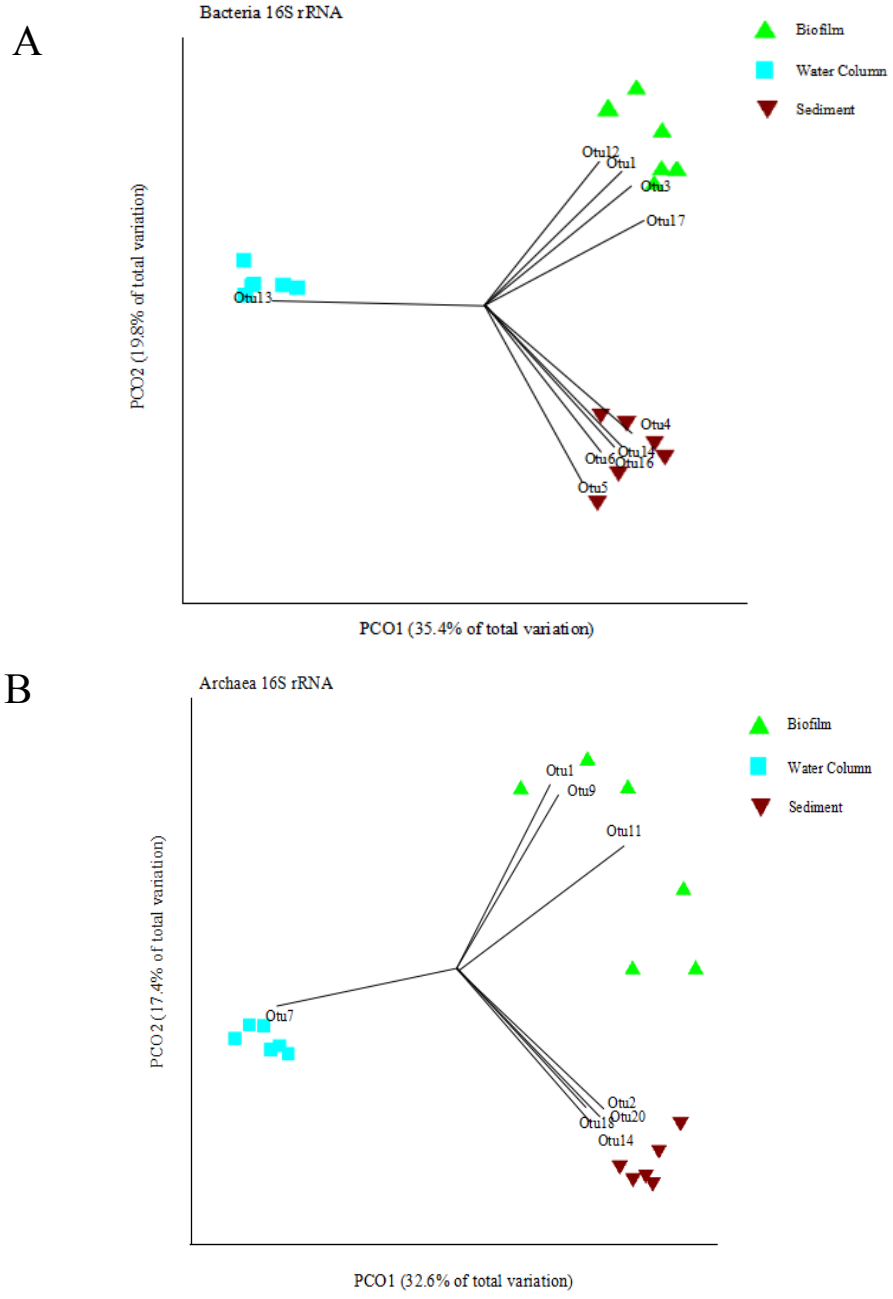


Fig. 3.5 – Principal coordinates analysis plot (PCoA) of bacterial and archaeal 16S rRNA OTUs from the water column, sediment and biofilm during two sampling occasions. Vectors are overlaid, and represent the OTUs which contributed mostly to the diversity of these communities.

Table 3.6 Weighted and unweighted UniFrac measures of differences in the bacterial and archaeal community composition between different microenvironments of *P. sinuosa* meadows (biofilm, sediment and water column) and two different seasons (summer, December 2013, and winter, August 2014).

UniFrac Test	Groups Compared	Score	P
Bacterial 16S rRNA Weighted	biofilm-sediment	0.92	<0.001
	sediment-water	1	<0.001
	biofilm-water	1	<0.001
Bacterial 16S rRNA Unweighted	biofilm-sediment	1	0.044
	sediment-water	1	0.043
	biofilm-water	1	0.021
Bacterial 16S rRNA Weighted	biofilm_summer-biofilm_winter	0.90	0.001
	sediment_summer-sediment_winter	0.70	0.014
	water_summer-water_winter	0.90	<0.001
Archaeal 16S rRNA Weighted	biofilm-sediment	0.86	<0.001
	sediment-water	1	<0.001
	Biofilm-water	0.92	<0.001
Archaeal 16S rRNA Unweighted	biofilm-sediment	0.99	0.096
	sediment-water	1	0.043
	biofilm-water	1	<0.001
Archaeal 16S rRNA Weighted	biofilm_summer-biofilm_winter	0.81	0.001
	sediment_summer-sediment_winter	0.83	0.014
	water_summer-water_winter	0.90	<0.001

3.4.3 Differences in the diversity of the ammonia-oxidising community between different seagrass meadow microenvironments and seasons

A total of 173 archaeal and 178 bacterial OTUs were retrieved from 18 samples. Richness estimator (Chao1) and Good's sequence coverage (together with the rarefaction curves reported in Appendix A) revealed that sampling effort was sufficient for both the bacterial and archaeal *amoA* sequenced in all the samples analysed (Table 3.7), however based on the Simpson index, the AOB community exhibited a higher diversity than the AOA community (Table 3.7). Moreover, Simpson measure showed a higher diversity associated with marine sediments than both biofilm and water (Table 3.7).

Seagrass sediments harboured the greatest number of AOB OTUs, with a total of 62 OTUs; 55 OTUs were found in the biofilm, and 61 OTUs were present in water (Fig. 3.6). During both sampling occasions, there was a high degree of specificity of AOB communities in the different seagrass meadow microenvironments, with ≤16 OTUs shared among the three microenvironments during each season (Fig. 3.6, A & B). Observed differences at the OTU level were confirmed by UniFrac analysis (Table 3.8). However, while the weighted UniFrac detected significant differences when comparing all microenvironments ($p<0.001$), the unweighted UniFrac did not detect any significant differences (Table 3.8). The Weighted UniFrac also indicated significant differences when comparing AOB communities between the two times ($p<0.001$).

Similar to AOB, seagrass sediments harboured the greatest number of AOA OTUs (88), while biofilm and water hosted 54 and 31 OTUs, respectively. These values were consistent with the values of Chao1 and Simpson indices reported in Table 3.6, with a higher diversity detected for sediment samples (varying between 0.05 and 0.14).AOA showed a high degree of "compartment specificity" among OTUs, with only 3 OTUs shared between the different seagrass meadow microenvironments during summer (Fig. 3.6 C), and none during winter (Fig. 3.6 D). The biofilm and sediment microenvironments shared a greater number of OTUs than either compartment shared with the water column, during both seasons (Fig. 3.6 C & D).

The observed differences in community composition among different microenvironments were confirmed by UniFrac analysis, comparing the AOA community between the three microenvironments (Table 3.8). The weighted UniFrac measure indicated significant differences ($p<0.001$) in AOA community structure between the three microenvironments. The unweighted UniFrac, which does not account for OTU abundances, showed a significant difference in biofilm *vs.* water and sediment *vs.* water ($p<0.05$), but no difference in biofilm *vs.* sediment. Temporal variations in AOA community structure were also evident, whereby the two sampling times shared 8 OTUs for biofilm, 23 OTUs for sediment, but only one OTU for water ($p<0.001$, Table 3.8).

Table 3.7 Bacterial and archaeal *amoA* cover percentage (%), community richness (Chao1) and diversity (Simpson) indices.

Bacterial *amoA*				Richness estimator	Diversity index
Group	Num. seqs observed	OTUs obtained	(%) Coverage	Chao1	Simpson
Biofilm M1_summer	213	7	89	16	0.22
Biofilm M2_summer	338	11	92	15	0.11
Biofilm M3_summer	143	9	90	10	0.12
Biofilm M1_winter	362	12	92	18	0.06
Biofilm M2_winter	229	6	88	11	0.11
Biofilm M3_winter	317	8	89	13	0.21
Sediment M1_summer	334	13	94	17	0.05
Sediment M2_summer	141	7	88	9	0.03
Sediment M3_summer	156	8	87	10	0.07
Sediment M1_winter	312	11	93	16	0.05
Sediment M2_winter	223	9	89	12	0.11
Sediment M3_winter	440	15	94	20	0.03
Water M1_summer	227	9	89	14	0.08
Water M2_summer	345	12	91	18	0.21
Water M3_summer	432	15	96	18	0.11
Water M1_winter	215	11	91	13	0.09
Water M2_winter	165	8	89	9	0.22
Water M3_winter	192	7	88	11	0.18
Archaeal *amoA*					
Biofilm M1_summer	213	6	90	12	0.22
Biofilm M2_summer	338	6	91	15	0.24
Biofilm M3_summer	113	4	90	7	0.42
Biofilm M1_winter	362	12	92	17	0.17
Biofilm M2_winter	334	11	94	16	0.22
Biofilm M3_winter	523	17	96	27	0.16
Sediment M1_summer	334	15	94	17	0.07
Sediment M2_summer	141	13	88	13	0.14
Sediment M3_summer	435	20	96	24	0.05
Sediment M1_winter	156	10	93	11	0.08
Sediment M2_winter	312	16	90	16	0.09
Sediment M3_winter	278	12	94	12	0.12
Water M1_summer	157	5	88	12	0.08
Water M2_summer	345	6	91	18	0.21
Water M3_summer	214	6	93	14	0.27
Water M1_winter	113	6	91	8	0.09
Water M2_winter	88	4	89	6	0.42
Water M3_winter	192	7	93	11	0.41

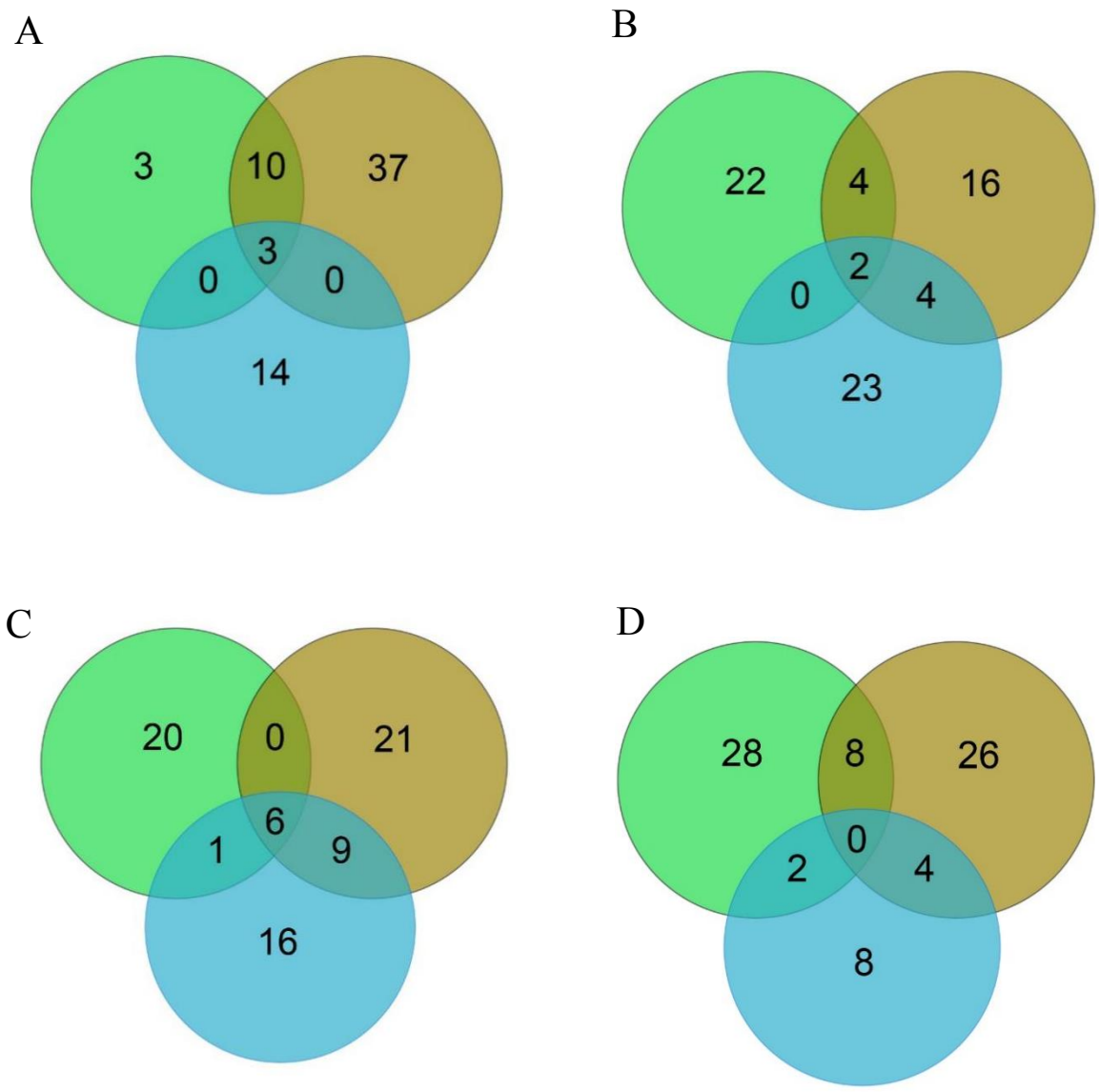


Fig. 3.6 - Venn Diagram of AOB (A, B) and AOA (C, D) OTUs shared among the three habitats in summer (A, C), and winter (B, D). The different colours refer to the diffetrent seagrass microenvironments (green for biofilm, blue for water and brown for the sediment).

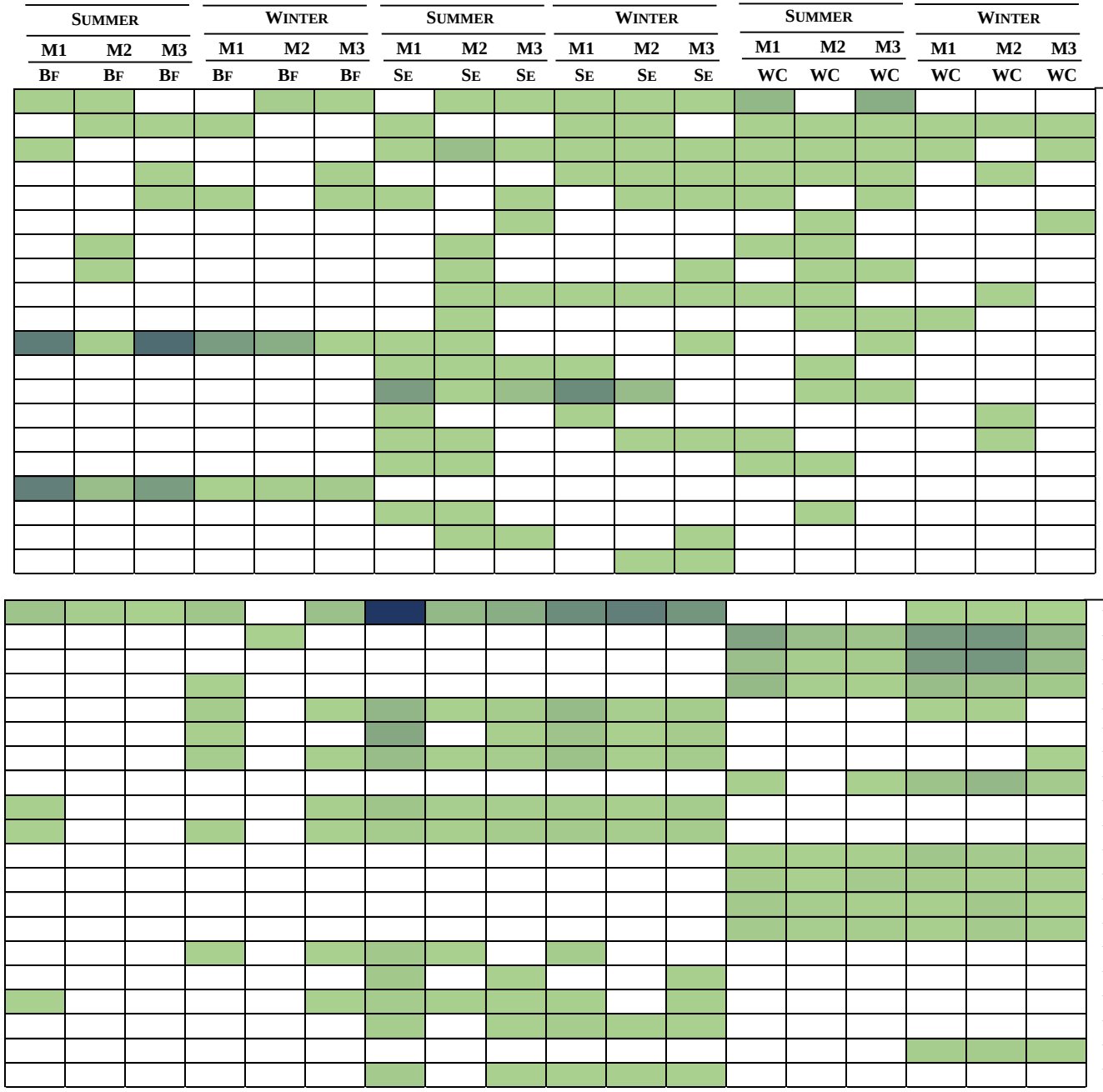
Table 3.8 Weighted and Unweighted UniFrac Test on the bacterial and archaeal community composition among different microenvironments of *P. sinuosa* meadows (biofilm, sediment and water) and the two sampling seasons (summer, December 2013, and winter, August 2014).

UniFrac Test	Groups Compared	Score	P
AOB	biofilm-sediment	0.97	0.001
Weighted	sediment-water	0.98	<0.001
	biofilm-water	0.99	<0.001
AOB	biofilm-sediment	0.99	0.44
Unweighted	sediment-water	0.99	0.88
	biofilm-water	0.99	0.74
AOB	biofilm_summer-biofilm_winter	0.99	<0.002
Weighted	sediment_summer-sediment_winter	0.96	<0.001
	water_summer-water_winter	0.93	0.001
AOA	biofilm-sediment	0.98	<0.001
Weighted	sediment-water	0.93	<0.001
	Biofilm-water	0.93	<0.001
AOA	biofilm-sediment	1	0.055
Unweighted	sediment-water	0.99	0.002
	biofilm-water	1	0.048
AOA	biofilm_summer-biofilm_winter	0.94	0.002
Weighted	sediment_summer-sediment_winter	0.97	0.001
	water_ summer-water_winter	0.93	<0.001

3.4.4 Phylogeny of bacterial and archaeal ammonia oxidisers

Bacterial phylogenetic data for the *amoA* gene showed that four of the 20 most abundant OTUs belonged to Betaproteobacteria, which corroborated my previous results on the bacterial 16S rRNA phylogenetic data that showed a high abundance of Betaproteobacteria within the sample analysed (Fig. 3.7). However, despite all the OTUs matching sequences retrieved from other environmental studies (except for OTU 16, 96% match with an uncultured ammonia-oxidising bacterium), many were

identified as ‘uncultured bacterium’ or ‘uncultured ammonia-oxidizing bacterium’ (Fig. 3.7). For archaea, the 20 most abundant *amoA* OTUs were mainly related to Thaumarchaeota or uncultured crerchaeota/archaeaon. Similar to bacteria, almost all OTUs matched sequences retrieved from other environmental studies, except OTU 9, 10 and 18 (95%, 96% and 92% match with known organisms) (Fig. 3.7).



BACTERIAL *AMOA* TAXONOMIC AFFILIATION (N SEQUENCES=4784 TOTAL)

Uncultured bacterium	99%	JN177548
Betaproteobacteria	99%	KF560825
Betaproteobacteria	99%	KF560833
Uncultured bacterium	99%	AB571284
Uncultured ammonia-oxidizing bacterium	99%	AB449332
Uncultured bacterium	99%	JX232870
Uncultured ammonia-oxidizing bacterium	100%	AB449324
Uncultured ammonia-oxidizing bacterium	98%	AB373431
Betaproteobacteria	98%	DQ289946
Uncultured ammonia-oxidizing bacterium	99%	JF715647
Uncultured ammonia oxidising	98%	HQ247936
Uncultured ammonia-oxidizing	98%	KP781060
Betaproteobacteria	99%	JX049860
Betaproteobacteria	98%	JX049860
Uncultured ammonia oxidising	98%	HQ247902
Uncultured ammonia-oxidizing bacterium	96%	JF715554
Uncultured ammonia-oxidizing beta proteobacterium	98%	KF362416
Uncultured ammonia-oxidizing beta proteobacterium	98%	AY352964
Uncultured bacterium	99%	EU244561
Uncultured ammonia-oxidizing bacterium	97%	AB373554

ARCHAEAL *AMOA* TAXONOMIC AFFILIATION (N SEQUENCES=4648 TOTAL)

Uncultured marine thaumarchaeote	99%	JX537705
Uncultured marine thaumarchaeote	98%	JX537705
Uncultured ammonia-oxidizing archaeon	99%	JF715770
Uncultured crenarchaeote	97%	EF382428
Uncultured archaeon	99%	JN183785
Uncultured Crenarchaeota	99%	JN190841
Uncultured archaeon	98%	KC758387
Uncultured archaeon	99%	KT306728
Uncultured marine thaumarchaeote	95%	JX537705
Uncultured crenarchaeote	96%	EF382428
Uncultured archaeon	99%	KF438280
Uncultured marine thaumarchaeote	99%	KM109539
Uncultured archaeon	98%	EU125516
Uncultured thaumarchaeote	98%	JF924273
Uncultured crenarchaeote	98%	GQ250776
Uncultured archaeon	98%	KJ526750
Uncultured marine thaumarchaeote	99%	KP994447
Uncultured archaeon	92%	JF924263
Uncultured crenarchaeote	98%	EU025171
Uncultured ammonia-oxidizing archaeon	99%	JF715707

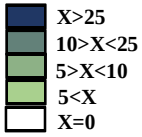


Fig. 3.7 - Heatmap of bacterial and archaeal *amoA* (97% sequence identity cutoff) obtained from biofilm (Bf), sediment (Se) and water column (WC) of three *P. sinuosa* meadows (M1, M2, M3) in two sampling occasions (Summer and Winter). The 20 most abundant OTUs are represented with their closest sequence match determined from Genebank BLAST and its corresponding accession number.

Phylogenetic tree construction showed that the all the AOB OTUs retrieved from this study belonged to the class Betaproteobacteria. Cluster 1 comprised only 4 sequences, all retrieved from sediment, and were related to the *Nitrosomonas ureae* cluster identified from previous studies (Francis et al., 2003). The remaining OTUs formed two further clusters (Cluster 2 and Cluster 3), which were *Nitrosomonas sp.* related (Fig. 3.8). Cluster 2 comprised two Subclusters, whereby OTUs from Subcluster 1 were related to an AOB OTU retrieved from marine sponges (Mohamed et al., 2009), while Subcluster 2 comprised 10 unknown OTUs, all belonging to the water column compartment. Finally, Cluster 3 OTUs represented all of the seagrass meadow microenvironments. 13 out of 28 OTUs belonging to the water column compartment were unknown, 8 out of 24 biofilm OTUs were unknown, and 9 out of 30 sediment OTUs were unknown. Most of the known sequences from Clusters 2 and 3 were closely related to *Nitrosomonas sp.*-like OTUs previously identified on macroalgae (*Osmundaria volubilis, Phyllophora crispa, and Laminaria rodriguezii*) from the Mediterranean Sea (Trias et al., 2012), sediment from South China Sea (Cao et al., 2011), and the water column from San Francisco Bay (Francis et al., 2003; Beman & Francis 2006).

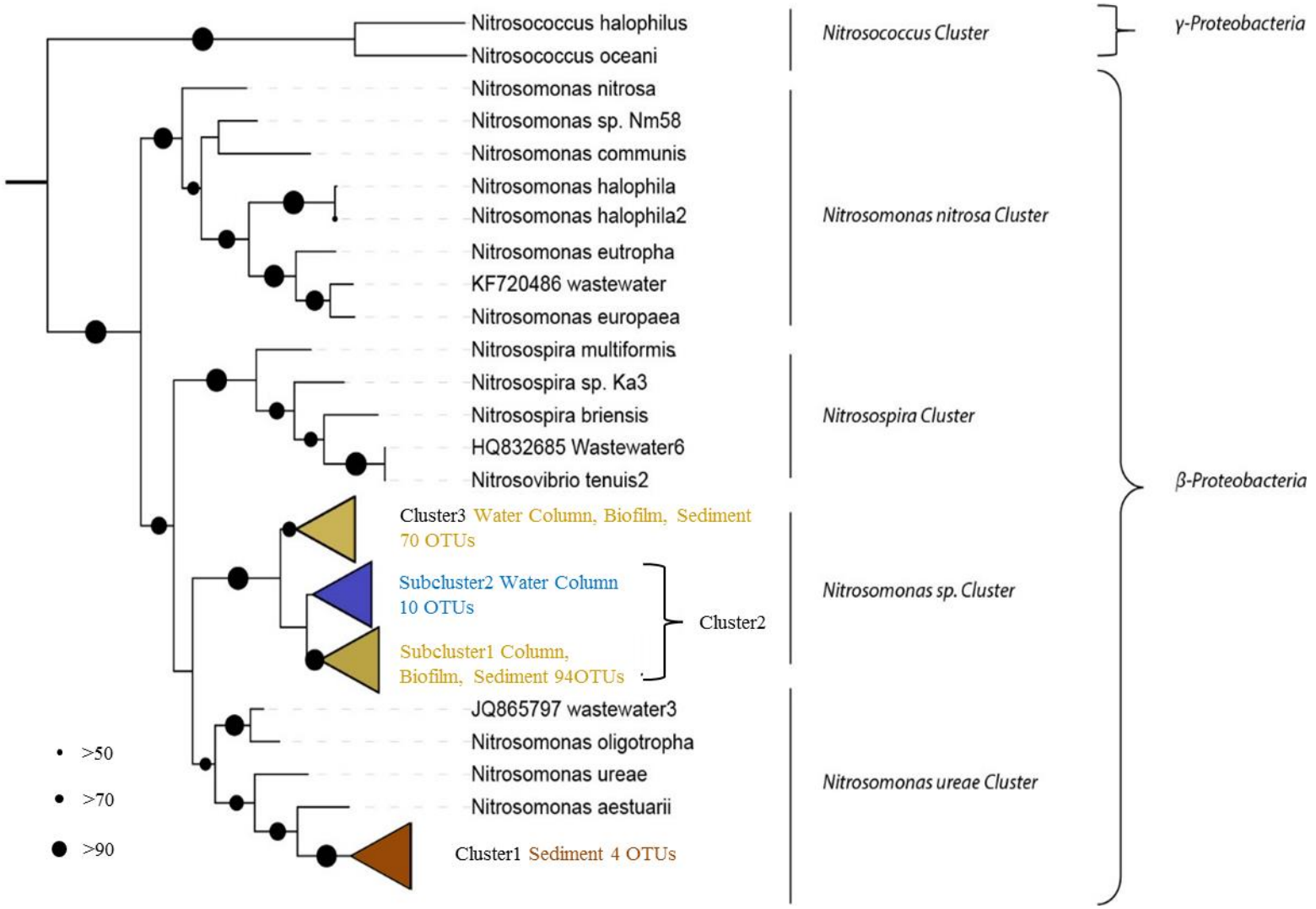


Fig. 3.8 AOB Phylogenetic Tree. The black dots represents nodes with bootstrap values >50. Smaller dots have lower bootstrap values, while bigger dots have values >70 and >90 as shown in the figure. Cluster1 is represented by sequences retrieved from sediment. Cluster2 comprises two subclusters, Subcluster1 host 10 OTUs all retrieved from water column, while the 94 OTUs of Subcluster2 are representative of all the microenvironments. OTUs from Cluster3 belonged to all the microenvironments. All the OTUs were related to the Betaproteobacteria, *Nitrosomonas* sp.-like organisms.

Compared with the AOB tree, the phylogeny of achaeal *amoA* OTUs was more complex (Fig. 3.9). Phylogenetic tree construction showed that the archaeal *amoA* OTUs were divided into 5 distinct clusters, two of which clearly separated according to seagrass meadow compartment (Fig. 3.9). Cluster 1 fell into a phylogenetic group comprising only 11 sequences, all retrieved from biofilm samples and related to *Nitrosotalea sp.* Only 4 OTUs were closely related to OTUs retrieved from other marine systems, globally, including: Indian Ocean water column (Kuma et al., 2011, unpublished), estuarine sediment (Bano et al., 2008, unpublished), freshwater aquarium biofilters (Sauder et al., 2011) and Red Sea cold seeps (Cao et al., 2014, unpublished). All the remaining biofilm OTUs were unknown. The highest number of OTUs (Cluster 2; 97 out of 173) were related to the Thaumarchaeal Group 1.a, which included the cultured species *Nitrosopumilus maritimus* and *Crenarchaeum symbiosum.* This cluster contained OTUs belonging to all seagrass meadow microenvironments, as well as OTUs retrieved from previous studies, including: coastal/estuarine (i.e. San Francisco Bay Estuary, Pacific Ocean; Mosier & Francis, 2008), as well as coastal sediment from the South China Sea, Japan (Cao et al., 2011) and seagrass (*Zostera marina*) meadow (Ando et al., 2009). Cluster 3 comprised 16 OTUs, all related to biofilm and sediment. Of these OTUs, 7 were related to OTUs previously associated with macroalgae (*O. volubilis, P. crispa, and L. rodriguezii)* and biofilm (collected near the Balearic Islands, in the Mediterranean Sea; Trias et al., 2012), sediment from the East China Sea (He et al., 2016), and aquarium biofilters (Sauder et al., 2011). The remaining 9 did not have any match with known OTUs. 41 OTUs fell into Cluster 4, representating biofilm and sediment. Of the OTUs belonging to the sediment compartment, 11 out of 26 OTUs were unknown, while 10 out of 13 OTUs belonging to the biofilm compartment were unknown. The remaining OTUs were related to OTUs identified in sediment samples from the South China Sea (Cao et al., 2011), surface sediment from the Western Pacific (Cao et al., 2011), and biofilm (Trias et al., 2012) and sponge (*Mycale laxissima*) tissue from the Western Mediterranean Sea (Zhang et al., 2014). Finally, all of the 9 OTUs comprising cluster 5 were from samples belonging to the water column compartment and hey did not match any previously identified *amoA* OTUs.

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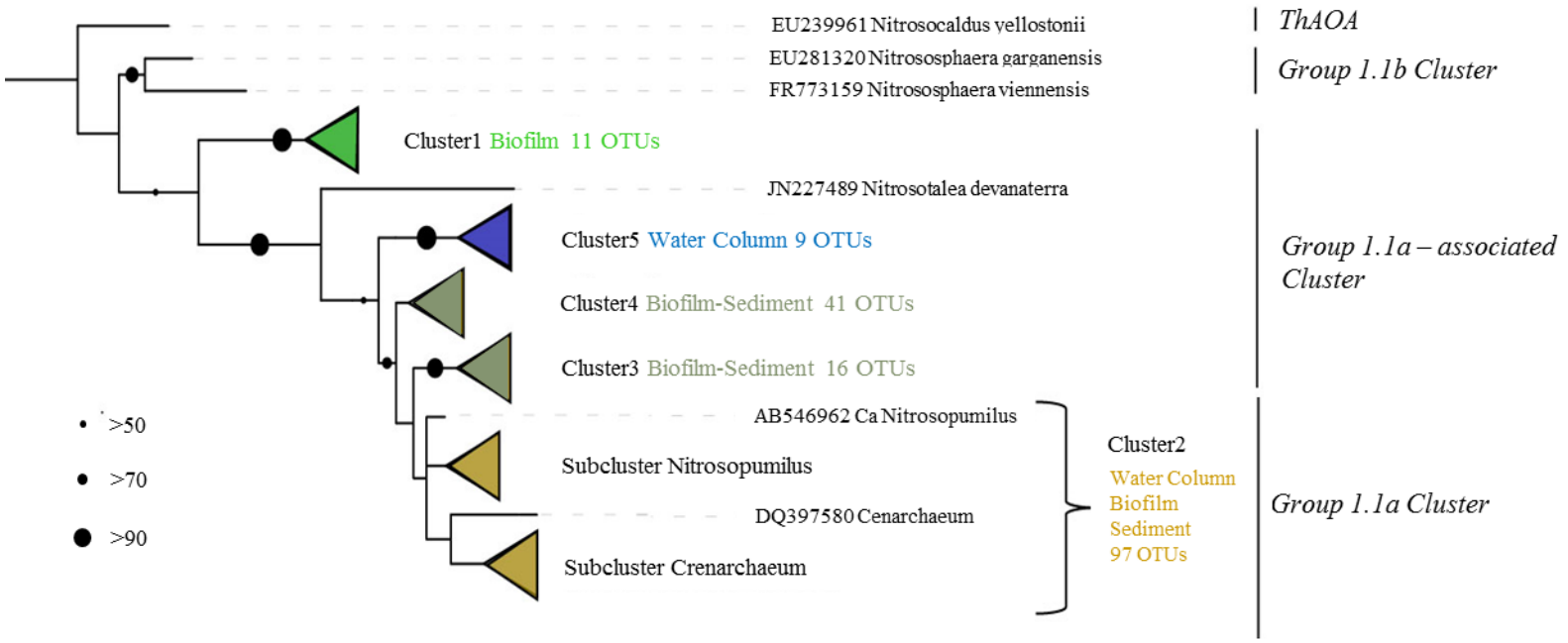


Fig. 3.9 AOA Phylogenetic Tree. The black dots represents nodes with bootstrap values >50. Smaller dots have lower bootstrap values, while bigger dots have values >70 and >90 as shown in the figure. Cluster1 is represented by sequences retrieved from biofilm and sequences from Cluster5 belonged to water. Cluster 3 and 4 hosted sequences belonging to biofilm and sediment, while OTUs from Cluster2 were representative of all the seagrass environment. All the sequences belonged to the Thaumarchaeota group I.Ia and Thaumarchaeota group I.Ia associated cluster.

3.5 Discussion

In this study, I investigated the abundance and diversity of the microbial (bacterial and archaeal) community associated with the biofilm attached to the leaves of the seagrass *P. sinuosa* and the surrounding environment (sediment and water). Specifically, I investigated the abundance and diversity of microorganisms potentially involved in N-cycling processes within *P. sinuosa* meadows: ammonia-oxidising microorganisms and ureolytic bacteria, which could link the consumption of DON to the production of DIN in the seagrass meadow ecosystem.

16S rRNA gene abundances clearly indicate that *P. sinuosa* leaf surface represents a suitable habitat for the settlement of both bacteria and archaea. The abundance of microorganisms associated with the *P. sinuosa* biofilm was always higher than abundances in the surrounding environment during both seasons (summer and winter) These results are in agreement with Wahbeh and Mahasneh (1984), who found the highest number of bacteria associated with leaves of three species of seagrass (*Halophila ovalis*, *Halophila stipulacea* and *Halodule universis*), compared to various other vegetation microenvironments (stem, root, rhizome).

Furthermore, 16S rRNA gene phylogenetic analyses suggest that the microbial community associated with the *P. sinuosa* leaf surface strongly differs from the communities present in the surrounding environment. As previously shown for several other marine plants (*Halophila stipulacea, Potamogeton perfoliatus*, V*allisneria americana, Zostera marina and Spartia. pectinata pectinata*; Weidner et al., 2000; Crump & Koch, 2008), it seems likely that marine plants harbour a "core epiphytic microbiome", probably due to the strong influence exerted by seagrasses on their leaf surfaces, with respect to the release of oxygen, nutrients and antibiotics (Kirchman et al., 1984; Engel et al., 2006; Newby et al., 2006). In fact, one of the 20 most abundant bacteria isolated form *P. sinuosa* biofilm is related to *Hydrogenophilus hirschii,* a chemolithoautotrophic bacterium that grows using hydrogen as electron donor and oxygen as electron acceptor (Stohr et al., 2001). Based on its metabolism, *H. hirschii* seems suited for living an endophytic life associated with an autotroph organism. Additionally, two abundant bacteria associated with *P. sinuosa* biofilm belong to the Bacteroidetes (Flavobacteriaceae) and Verrucomicrobiaceae (*Luteolibacter cuticulihirudinis*), which have been found

associated with the biofilm of another seagrass (*Halodule beaduettei*) from the Upper Laguna Madre, Gulf of Mexico (Chilton & Cammarata, unpublished) and the surface of the medicinal leech *Hirudo medicinalis*, respectively (Zhang et al., 2017). This is interesting, because it implies that these bacteria favour an epiphytic lifestyle, and may therefore be common members of the "seagrass microbiome". The water column population seems dominated by organisms related to *Vibrio sp*. Sediment and water also host organisms related to *Halelia sp.* and *Halelia mediterranea* (isolated from the Western Mediterranean Sea) which are capable of degrading amino acids. Regarding the community structure of the archaeal population, it is clear that there is a strong affiliation between *P. sinuosa* and *Nitrosopumilu*s-like Thaumarchaeotes in the leaf biofilm, although the majority of archaea were represented by 'unknown' uncultured microorganisms.

Since N is considered a key element for seagrass growth and productivity, and is usually limited within the marine environment, it seems likely that *P. sinuosa* would preferentially host epiphytic microorganisms that would be involved in N transformations. Using q-PCR I showed that, within three *P. sinuosa* meadows and four seagrass microenvironments (surface water, canopy water, sediment and biofilm), ureolytic bacteria and ammonia-oxidising bacteria (AOB) and archaea (AOA) were significantly (up to 6 orders of magnitude) more abundant in the leaf biofilm than the surrounding environment. However, since this is the first study to investigate the presence and abundance of ureolytic microorganisms on marine plants, I was unable to compare my results to previous studies. This highlights the significant, and important, work yet to be done in marine microbiome research.

The abundances of ureolytic bacteria were between 5 and 50 times greater than AOB abundances in all seagrass meadow microenvironments. The primer pair chosen for targeting the bacterial *ureC* in the present study should only match with the AOB community; consequently, *ureC* and AOB should have shown similar abundances. Possible explanations for discrepancies between bacterial *ureC* and *amoA* gene abundances in all meadow microenvironments may be: (i) the *ureC* gene is present in higher copy numbers than bacterial *amoA* (Koper et al., 2004), or (ii) there exists either unspecificity or bias in the primer sets used here. Additionally, several studies have shown that AOA retrieved from the Arctic Ocean and Mediterranean Sea possess the ability to hydrolyse urea (Yakimov et al., 2011), yet I

was unable to detect the *ureC* gene in my samples, using previous designed primer pairs. In the current study, I tried two different primer pairs for targeting *ureC* within the AOA community: one primer pair designed using OTUs retrieved from Arctic Ocean samples (Alonso-Saez et al., 2012), and one primer pair designed using OTUs identified in AOA populations from the Mediterranean Sea (Yakimov et al., 2011). However, neither of the Thaumarchaeal ammonia oxidisers *Nitrosopumilus maritimus* (Walker et al., 2010) or *Nitrosoarchaeum limnia* (Blainey et al., 2011; Mosier et al., 2012) contain ureases, indicating that the potential to degrade urea is not ubiquitous among marine Thaumarchaeotes. Indeed, ureases are rare in the Archaea domain (Solomon et al., 2010). According to my results I can therefore hypothesise that: (i) ammonia-oxidising archaea of Western Australian coastal environments do not possess the ability of hydrolyse urea, or (ii) a divergence exists between previously designed primer pairs and the *ureC* gene present among the Western Australian coastal AOA community. A further step to confirm this would be to design my own primer pairs; however, in the absence of any archaeal *ureC* sequences from my environment of interest, this would require significantly more sequencing effort than was possible during this current study.

In the case of ammonia oxidising microorganisms, my results suggest that *P. sinuosa* leaves could be important and selective habitats for AOB. In previously studied marine environments, such as sponges (Radax et al., 2011) and open waters (Santoro et al., 2011), the abundance of AOA is higher in comparison to AOB. These results show the opposite for AOB/AOA abundances in the *P. sinuosa* biofilm, agreeing with previous observations for three macroalgae (*O. volubilis, P. crispa, and L. rodriguezii*) from the temperate waters of Western Mediterranean Sea (Trias et al., 2012). In the current study, epiphytic AOB were 1 to 2 orders of magnitude more abundant than epiphytic AOA. Possible explanations for AOB outnumbering AOA may be: (i) AOB outcompete AOA in the presence of advantageous/stable physicochemical conditions, such as high levels of oxygen on the plant surface (Trias et al., 2012), (ii) marine plants selectively "choose" AOB rather than AOA, through a combination of epiphyte-host interactions, or (iii) a combination of both (i) and (ii). It has been suggested that the high abundance of AOA over AOB within sponges and corals may be a consequence of unstable environmental conditions, such as nitrogen and oxygen fluctuations within these

animals (Siboni et al., 2008, Radax et al., 2011, Han et al., 2013). During phases of low sponge ventilation and at night time, when zooxanthellae release CO_2 within corals as a consequence of respiration, a temporal accumulation of ammonium (NH_4^+) and fluctuations of oxygen (O_2) level can occur (Hoffmann et al., 2009; Siboni et al., 2008). The few cultivated AOA strains are adapted to survive and grow at low NH_4^+ and O_2 concentrations, while AOB are generally active only at higher concentrations (Hatzenpichler et al., 2008; Martens-Habbena et al., 2009). Assuming that the ecopyhsiology of AOA and AOB in sponges and corals is similar to that of cultivated strains, high NH_4^+ concentrations in sponge tissues and coral mucus should favour AOB growth; however, because of the concurrently low O_2 concentrations, AOA could outcompete AOB (Santoro et al., 2011). In contrast to the sponge and coral biofilm environments, on seagrass leaf surfaces O_2 levels are relatively stable, due to the exudation of photosynthetic O_2 from the leaf surface (Borum et al., 2007). Oxygen, however, can also be used by seaweeds and marine plants as a defence against bacterial attack, through the production of harmful reactive oxygen species (ROS), such as superoxide ions and hydrogen peroxides (Weinberger, 2007); to protect themselves, resident bacteria can express different oxidases that degrade ROS and reduce the oxidative cell damage (Egan et al., 2012). The importance of these seagrass/macroalgal defences can be indirectly gleaned from the genomes of several macroalgal-associated bacteria, which contain genes related to the oxidative stress response (Thomas et al., 2008; Burke et al., 2011b; Fernandes et al., 2011). In addition to O_2 release, seagrasses are able to influence the physiochemical properties of the rhizosphere and leaf surfaces by exuding small organic (carbon-containing) molecules (Larkum et al., 2007; Kirchman et al., 1984). This mechanism has been proposed to be the key process driving plant surface colonisation, through acting selectively on the epiphytic microbial community (Harlin, 1971; Kirchman., 1984).

Similar to Mediterranean macroalgae (Trias et al., 2012), I observed that the *P. sinuosa* epiphytic AOB represented approximately 1 to 4% of the total bacterial population of the biofilm. However, the total abundances were notably different, with the seagrass epiphytic AOB being ≤40 times more abundant than reported for macroalgae (Trias et al., 2012). AOA comprised approximately 1% of the entire epiphytic seagrass archaeal community. Total AOA abundance was 1 to 13 times

greater in the *P. sinuosa* biofilm than previously reported for macroalgae (Trias et al., 2012). During sample collection and biofilm sampling, which involved carefully scraping the biofilm from the leaf surface, I observed a complex 3-dimensional structure on the seagrass leaf surface, including epiphytic micro- and macroalgae. I propose that these other members of the epiphytic microbiome could facilitate high bacterial and archaeal abundances on seagrass leaf surfaces, by increasing the 3-dimensional surface available for microbial colonisation.

I also detected changes in the *amoA* gene abundances between the two sampling points, but not for either 16S rRNA or *ureC* genes. During winter (August 2014), in comparison to summer (December 2013), I observed an increase in the *amoA* community within the biofilm for both bacteria and archaea, an increase in AOB abundance in the sediment, and a decrease of AOA abundance in both microenvironments of the water column (surface and seagrass canopy). Additionally, the AOM community structure varied significantly between summer and winter, for all seagrass meadow microenvironments. While it has been demonstrated that variability in the abundances of AOB and AOA can be related to changes in environmental conditions (e.g. temperature, nutrient availability, pH and oxygen level) (Groeneweg et al., 1994; Sahrawat et al., 2008; Erguder et al., 2009), there is growing evidence that these functional gene abundances can be related to plant activity, whereby the microbial community is strongly influenced by the plant itself. For example, the ammonia-oxidising community associated with mangrove sediment in Mai Po Nature Reserve (Hong Kong) is strongly influenced by plant root activity (Li et al., 2011): in summer, when the plant is active and growing, the roots uptake most of the NH_4^+ available, thus outcompeting ammonia-oxidisers for substrate. In contrast, during winter, low levels of NH_4^+ in the sediment are instead due to strong AOA activity, and ammonia-oxidising gene abundances are higher (Li et al., 2011). *P. sinuosa* is also affected by seasonal growth, with higher biomass accumulation in summer than in winter (Collier et al., 2007). In the current study, the lower abundances of epiphytic and sediment AOB and AOA during summer may be due to competition with the plant for NH_4^+ uptake, while during winter, low seagrass activity and nutrient uptake may favour a higher abundance of ammonia-oxidisers on the plant surface.

Interestingly, weighted UniFrac analyses revealed that both AOA and AOB epiphytes were significantly different from those belonging to sediment and water samples. This constitutes further evidence for plant-epiphyte interactions, and indicates that marine plants host specific epiphytic bacterial communities, similar to sponges and corals (Bourne et al., 2005; Siboni et al., 2008; Mohamed et al, 2010). In agreement with the current study, others have shown that a core epiphytic bacterial community can be defined at the algal/animal species level, indicating a specific selection for particular microbial epiphytes (Radax et al., 2011; Trias et al., 2012; Zhang et al., 2014). However, the unweighted UniFrac algorithm, which does not account for differences in OTU abundance, but only for differences in community composition among microenvironments, indicated that epiphytic biofilms shared a similar AOA community with the sediment, while the community inhabiting the water column was separate from both biofilm and sediment microenvironments. In contrast, the unweighted UniFrac algorithm for the AOB community detected no significant differences among the AOB community from the biofilm, sediment and water column, indicating that abundances, rather than the presence of specific OTUs, are responsible for any differences among these habitats. It therefore appears that, even if AOA are less abundant than AOB, they present a greater degree of partitioning between the different seagrass meadow microenvironments, and epiphytic AOA could be recruited from the sediment, rather than the water. My results also suggest a temporal variability within the epiphytic core microbiome, as well as for AOM present in the sediment and overlying water, which may be driven by changes in environmental conditions and/or plant physiology (Li et al., 2011; Wang et al., 2013). The archaeal and bacterial *amoA* sequences I obtained here were closely related to sequences retrieved from corals and sponges, and from sediment from eelgrass and mangroves, indicating that similarities exist among microbial communities associated with macrofauna and –flora, across global spatial scales. However, for both AOA and AOB, some of the sequences belonging to the water column did not match with any known sequences, suggesting unique ammonia-oxidising communities occurring within the bacterioplankton populations of coastal southwestern Australia.

3.6 Summary and conclusions

In summary, I have shown that the abundant Australian seagrass, *Posidonia sinuosa*, harbours on its surface specific communities of bacteria and archaea that differ from the communities inhabiting the surrounding environment. My work suggests that the *P. sinuosa* leaf surface may provide a specific ecological niche for AOB in the marine environment, which is, in general, considered to be dominated by AOA. Here, I have shown that abundances of epiphytic AOB can be up to 40 times greater than AOA. However, AOA appeared to have a higher host specificity than AOB, and were potentially recruited from the surrounding sediment, rather than the overlying water. I can therefore conclude that the *P. sinuosa* leaf microbiome supports abundant and unique communities of ammonia oxidising bacteria and archaea, which are temporally variable; either in response to plant or epiphyte dynamics, or changes to the surrounding environment, or both. Within the AOB community, ureolytic genes were detected in high abundances, suggesting a possible link between the degradation of organic matter, consumption of DON, and transformation of DIN in seagrass meadow environments; and particularly within the leaf biofilm. In conclusion, *P. sinuosa* hosts a core epiphytic microbiome that includes host-specific communities of important N-cycling bacteria and archaea. I suggest that important functional plant-microorganism relationships exist, which may facilitate plant N uptake, and thus support seagrass growth and productivity.

CHAPTER FOUR: MICROORGANISMS FACILITATE THE UPTAKE OF DISSOLVED ORGANIC NITROGEN BY SEAGRASS LEAVES

4.1 Abstract

Microorganisms play a critical role in nitrogen cycling by mineralising dissolved organic nitrogen (DON) into bioavailable inorganic forms (DIN). Although DIN is crucial for seagrass growth, the hypothesis that seagrass leaf associated-microorganisms could convert DON to forms available for plant uptake has never been tested. We carried out a laboratory-based experiment in which seagrass (*Posidonia sinuosa)* leaves were incubated with ^{15}N-enriched amino acids, with and without associated microorganisms. Samples were collected after 0.5, 2, 6 and 12hours. Both bulk stable isotope and nanoscale secondary ion mass spectrometry (NanoSIMS) analysis showed high accumulation of ^{15}N within seagrass leaf tissues with an associated microbiota, but not in plants devoid of microorganisms. These results significantly change our understanding of the mechanisms of seagrass nitrogen use and provide evidence that seagrass microbiota increase nitrogen availability for uptake by seagrass leaves by mineralising DON compounds, thus enhancing growth and productivity of these important coastal ecosystems.

4.2 Introduction

Nitrogen (N) is an essential element for maintaining seagrass growth and productivity (Zimmerman et al., 1987; Williams et al., 1990), yet seagrasses are often abundant in N limited systems (Pedersen et al., 1993; Touchette & Burkholder, 2000; Gobert et al., 2002). High productivity of seagrasses, therefore, lies in their ability to maximise N sequestration from the surrounding environment (Stapel et al., 1996, Touchette & Burkholder, 2000), with leaves contributing up to 74% to their total N requirement (Alexandre et al., 2015). Canopy leaves have a preference for inorganic forms of N (dissolved inorganic N, DIN), especially ammonium (NH_4^+), whose uptake affinity can be twice that of nitrate (NO_3^-) (Touchette & Burkholder, 2000). However, the coastal marine environment is usually characterised by scarce DIN availability and high concentrations of dissolved organic nitrogen (DON), due to autochthonous organic nitrogen production and allochthonous inputs (e.g. terrestrial inputs; Berman & Bronk 2003).

Despite its long-standing recognition as a potential nutrient source, the utilisation of DON by primary producers is not well understood (Bronk et al., 2007). Although the possibility that small DON compounds could serve as a direct N source for aquatic plants was never considered (Romero et al., 2006), the uptake of DON has relatively recently been demonstrated for a variety of macroalgae (Bird et al., 1998, Tyler et al., 2003). Uptake of DON by different algae in axenic cultures has been reported (Bird et al., 1998, Tarutani et al., 2004), implying the existence of a process for the direct utilisation of DON by seaweeds. However, it is not yet known to what extent seagrasses share this capability. Recent studies suggest that seagrasses may possess the ability to take up small DON compounds (e.g. amino acids and urea; Vonk et al., 2008; Van Engeland et al., 2011; La Nafie et al., 2014; Alexandre et al., 2015) by both roots and leaves. Although these studies provide indirect evidence of utilisation of DON by seagrasses, it remains unresolved whether marine plants have the physiological capacity to take up DON intact or whether plant-associated microorganisms and/or epiphytes mediate foliar DON absorption.

Microorganisms can be highly abundant on the surface of macrophytes (Trias et al., 2012; Thompson et al., 2014; Flemming et al., 2016), forming functional partnerships with organisms belonging to different domains, which often play a

critical role in nutrient acquisition from oligotrophic waters (Wahl et al., 2012). For seagrasses, the leaf environment, or phyllosphere, provides a physical substratum for a rich epiphytic community of both heterotrophic and autotrophic microorganisms (Borowitzka et al., 1990; Weidner et al., 2000; Uku & Björk 2001; Borowitzka et al., 2007; Uku et al., 2007; Crump & Koch, 2008a; Hamsi et al., 2013). As I have shown in Chapter 3, a significant proportion of this epiphytic microbiome possesses the genetic capacity to transform organic (urea) and inorganic (NH_4^+) forms of N. Surprisingly, while the contribution of the autotrophic microbiota (e.g. N-fixing bacteria) to enhancing N availability to seagrasses has been extensively reported (Welsh, 2000; Pereg-Gerk et al., 2002; Cole & McGlathery, 2012; Agawin et al., 2016), the significance of heterotrophic microorganisms on seagrass leaves has been largely overlooked. Obvious advantages obtained by the heterotrophic microbial community inhabiting seagrass leaves include favourable microhabitat for settlement, and exudation of O_2 and organic compounds (e.g. organic carbon and vitamins) (Brylinski, 1971; Harlin, 1971; Kirchmanl 1984). Yet, much remains to be learned of the advantages that seagrasses obtain from this partnership. Since microorganisms are able to cleave DON by extracellular enzymes to produce DIN (Chrost, 1991; Romaní et al., 2012; Flemming et al., 2016), they could support seagrass N demand by mineralising DON to more bioavailable forms, such as NH_4^+ and NO_3^- (Flemming & Wingender, 2010).

The seagrass phyllosphere also represents a suitable habitat for the settlement of a variety of epiphytic algae. Filamentous and crustose rhodophytes (i.e. *ceramium, fosliella, chondria, centroceras, polysiphonia,* etc.), chlorophytes (i.e. *cladophora*), phaephyceae (such as *polycera sp.*) and diatoms cohabit and share the surface of seagrass leaves with microorganisms (Sullivan 1979; Trautman & Borowitzka, 1999). In addition to seagrasses, epiphytic algae may benefit from their close proximity to microorganisms, which could enhance N availability to the algae, as well as the plant. For example, exchange of N has been reported to occur between some species of diatoms and their epiphytic N-fixing bacteria (Foster et al., 2011), and in these studies, microorganisms provided N to the algae, while diatoms exuded organic carbon consumed by associated microorganisms (Amin et al., 2012). Many algal taxa can also use DON directly, through processing mechanisms such as urease activity and amino acid oxidation (Palenik & Morel, 1991; Mulholland et al., 2002,

Stoecker & Gustafson 2003, Solomon & Glibert, 2008). Although the relationship between seagrasses and their epiphytic algae has always been controversial, since algae are considered to compete with marine plants for light and nutrient uptake (Wetzel, 1993; Williams & Ruckelshaus, 1993; Coleman & Burkholder, 1994, 1995), extracellular mineralisation of DON by epiphytic algae may increase DIN availability at the seagrass-biofilm interface (Pohlon et al., 2009).

To test the role of microorganisms and epiphytes in competing with or facilitating the uptake of DON into seagrass leaves, I experimentally examined the uptake of ^{15}N-enriched amino acids into the leaves of the seagrass *Posidonia sinuosa* with and without epiphytic organisms. Accumulation of ^{15}N into seagrass leaves and epiphytic algae was firstly measured by isotope ratio mass spectrometry (IRMS), after which, a subset of samples was analysed by nanoscale secondary ion mass spectrometry (NanoSIMS) to measure ^{15}N accumulation in microorganisms and algae on the seagrass leaf surface, and in discrete sub-cellular seagrass components (cell wall, cytosol, vacuole and chloroplasts). NanoSIMS maps enriched stable isotope (e.g. ^{13}C, ^{15}N, etc.) tracers at the cellular scale, providing an important tool for imaging and co-quantifying the process of organic ^{15}N flow and uptake at a subcellular level. NanoSIMS technology, together with IRMS, has already been used to show the incorporation and translocation of labelled ammonium ($^{15}NH_4^+$) from microorganisms associated with plant roots into belowground plant tissue, within a few hours of ^{15}N incubations (Jones et al., 2013). Here, I applied this approach to examine the potential uptake of organic nitrogen by seagrasses and their epiphytes.

4.3 Materials and Methods

An enrichment experiment was performed by incubating seagrass shoots, with and without microorganisms and epiphytic algae, with a ^{15}N-enriched algal amino acid mixture. Samples were collected at different incubation times (0.5, 2, 6 and 12 hours) and were analysed through IRMS and NanoSIMS to examine the uptake of ^{15}N in the bulk tissue and in the microorganisms and algae on seagrass leaf surface and discrete sub-cellular seagrass components (cell wall, cytosol, vacuole and chloroplasts). In addition, samples from the different incubation times were analysed for microbial presence/absence using the LIVE/DEAD® BacLight™ . Based on the

evidence that, after 3 hours from seagrass incubation with $DO^{15}N$, the enriched substrate was already detectable within seagrass tissue (Van Engeland et al., 2011), collection times were chosen to follow potential significant changes before and after this time.

4.3.1 Field collection and $DO^{15}N$ Experiment

48 *Posidonia sinuosa* shoots (2.4 to 2.9 g) were collected from Marmion Marine Park (31°48.240' S 115°44.123' E) located offshore Perth (South-Western Australia) , and transported in aerated water at ambient seawater temperature (22 ºC) in the shade, before being placed in 30 L aquaria under natural illumination for 24 h to acclimatise. Light intensity (130 µmol m^{-2} s^{-1}) was measured using a Photosynthetically Active Radiance (PAR) sensor for underwater applications (Micro PAR, In-Situ Marine Optics, Perth) to ensure seagrasses photosynthesised. Prior to the experiment, the photosynthetic efficiency (dark yield adaptation) of six random seagrass shoots was measured with the pulse-amplitude modulater (PAM) chlorophyll fluorometer to ensure seagrasses did not show any sign of stress. After the acclimation time, 24 seagrass shoots were gently 'scraped' with a sterile razor blade to remove obvious epiphytic organisms, washed in artificial filtered (0.2 µm) seawater (Red Sea Marine Salt, ASF), and placed into individual incubation cylinders containing 1 L ASF. To further remove any residual microorganisms, scraped leaves were incubated in 5 ml L^{-1} of antibiotic mixture (comprising 10,000 units penicillin, 10 mg mL^{-1} streptomycin and 25 µg ml^{-1}amphotericin B; Sigma Aldrich; Kritzinger et al., 1997), acetazolamide (0.22 mg l^{-1}, Sigma Aldrich; Zimmerman et al., 2004; Khelaifia & Drancourt, 2012) and imidazole (1 mg L^{-1}, Sigma Aldrich; Dridi et al., 2011; Khelaifia & Drancourt, 2012) for 24 h prior to the addition of the $DO^{15}N$. The effectiveness of epiphyte removal and antibiotic incubation was monitored for each leaf at the end of the experiment using the LIVE/DEAD® BacLight™ kit with fluorescent imaging using confocal microscopy (described in detail in Appendix 4 'LIVE/DEAD® BacLight™ and antibiotic treatment'). Briefly, the LIVE/DEAD® BacLight™ kit (ThermoFisher) allowed to infer whether for the treatment 'seagrass without microorganisms' the chosen antibiotic mixture targeted epiphytic microorganisms and, whether it was possible to use the kit to reveal the efficacy of antibiotic incubation. The remaining 24 shoots

were left with epiphytes and placed into individual incubation cylinders containing 1 L ASF.

All 48 cylinders were maintained at 22 ºC under continuous aeration and natural light for 12 h during substrate incubation and sampling period. Twelve seagrass leaves with and 12 leaves without microorganisms were randomly selected to be incubated in 50 µM $DO^{15}N$ (99% ^{15}N) of algal-derived amino acid mixture (NLM-2161-0, Cambridge Isotope Laboratories, USA). The remaining 24 *P. sinuosa* leaves (12 with microorganisms and 12 without) were used as controls and incubated with 50 µM $DO^{14}N$ (ULM-2314-1, Cambridge Isotope Laboratories). Before the end of every incubation time interval and shoot collection, I assessed seagrass photosynthetic activity (as a proxy for stress) using PAM fluorometry.

4.3.2 Sample collection for IRMS and NanoSIMS

For each time point (0.5, 2, 6 and 12 hours), 3 seagrass leaves were removed from cylinders and washed briefly in ASF to remove any residual ^{15}N. Three ~1 cm^2 sections were cut 6 cm from the growing tip, using a sterile scalpel. The sections were fixed in 2.5% glutaraldehyde (Sigma-Aldich) in 0.1 M PBS and stored at 4 °C. Cutting and fixation of samples for NanoSIMS analyses took place within a few seconds, in order to minimise $DO^{15}N$ leaching from the plant tissue. Leaf tissue (~1 cm^2), adjacent to areas used for NanoSIMS analysis was collected and immediately stained and fixed with the LIVE/DEAD® BacLight™ kit in order to verify the antibiotic effect for the treatment, as described above. All remaining leaf material (for treatments and controls) was stored at -20 °C and processed for IRMS analyses.

4.3.3 Isotope ratio mass spectrometry analysis

Frozen leaves were oven-dried at 58º C for 48 h before being ground (ball-mill grinder; Retsch, Haan, Germany) to fine powder, and weighed (~1.5 mg) into 6 x 4mm tin capsules (Elemental Microanalysis, UK), which were crimped manually. Prior to drying, seagrass leaves covered with epiphytic organisms (treatment 'with microorganisms') were subjected to scraping using a razor blade in order to remove obvious epiphytes and obtain a clean isotopic signal of the seagrass material and epiphytic algae. Samples were analysed for nitrogen elemental composition (%) and

isotope ratios ($\delta^{15}N$) by continuous flow isotope ratio mass spectrometry, using an Automated Nitrogen Carbon Analyser-Mass Spectrometer system (20/20 Europa Scientific Ltd., Crewe, UK). Measures of $\delta^{15}N$ of the ^{15}N enriched plant samples were compared against reference samples incubated with $DO^{14}N$, which had been previously calibrated against standard International Atomic Energy Agency (IAEA, Vienna) reference materials with uncertainty of 0.0005 ^{15}N atom% (Skrzypek, 2013).

4.3.4 NanoSIMS sample preparation and analysis

Samples were dehydrated in a graded series of ethanol (50%, 70%, 90%, 100%, anhydrous 100%) and anhydrous acetone (100%). After dehydration, samples were gradually infiltrated in anhydrous acetone: araldite resin mixtures until total resin embedding (100% araldite concentration) and cured at 60 °C for 24 h. Resin blocks with intact samples were sectioned (200 nm) using a Leica EM UC6 Ultramicrotome (Leica Microsystems, Wetzlar, Germany) and a 45 degree diamond knife. Wet cut sections were mounted either on a Silicon wafer for NanoSIMS analysis or a glass slide for optical imaging to map sample ultrastructure. Silicon wafers with adhered (air-dried) samples were coated with 5 nm gold for subsequent analysis in a CAMECA NanoSIMS-50 ion microprobe (CAMECA, France) at the Centre for Microscopy Characterisation and Analysis (CMCA), The University of Western Australia.

4.3.5 Image Sample analysis

For the analysis of images of seagrass tissues processed with the LIVE/DEAD® BacLight™ kit, the confocal microscope was tuned to detect emission at 500 nm and 630 nm after excitation at 480 nm and 500 nm for the green-fluorescent and the red-fluorescent nucleic acid stains, respectively.

For the pilot studies, images obtained under confocal microscopy of seagrass sections with and without microorganisms were analysed using an imaging software NIS-Elements viewer (4.20 - Nikon, https://www.nikoninstruments.com/Products/Software/NIS-Elements-Advanced-Research/NIS-Elements-Viewer). For each image obtained with the NIS-Elements viewer (e.g. *E. coli* cells incubated with and without ethanol and seagrass sections

with and without microorganisms), microbial cells present within the images were highlighted using the OpenMIMS data analysis software plugin in ImageJ (http://www.nrims.hms.harvard.edu/software.php). The percent coverage of microorganisms (comprising the few remaining microbial cells for the treatment seagrass 'without microorganisms') was estimated by measuring the total area (pixel) of the seagrass sections compared to the area covered by microorganisms. Values were then used for statistical analyses, to evaluate the effectiveness of antibiotic incubation at 24 h versus 48 h.

The NanoSIMS-50 was calibrated to measure $^{12}C–$, $^{13}C^{12}C–$, $^{12}C^{14}N–$, $^{12}C^{15}N$, $^{32}S-$. This allowed determination of $^{15}N/^{14}N$ ratios along with $^{13}C/^{12}C$ ratios, ^{32}S and secondary electron imaging (for identification of cellular and sub-cellular structures).

Prior to analysis, selected areas of interest were sputtered (Cs+ implanted) by rastering a defocused primary ion beam (current density 2.5×10^{15} ions cm^{-2}) over a slightly larger area to allow samples to reach sputtering equilibrium (60 ms/pixel). Generally, analysis was performed in a chained method to allow 'stitching together' of many smaller images to create an image of a larger sample area (Fig. 4.5). This approach is useful for minimising sample spot bias and analysing co-occurring organisms. A correction factor was applied to all NanoSIMS data to make it directly comparable to IRMS data. The correction factor was based on analysis of a yeast (*Saccharomyces cerevisiae*) standard with known $^{15}N/^{14}N$ abundance. Briefly, representative samples of the yeast were analysed by IRMS with data further standardised against internationally recognised standards (see 'Isotope ratio mass spectrometry analysis' section). A portion of yeast from the same source was dried onto a silicon wafer (gold coated 10 nm) and analysed by NanoSIMS in the same manner described for seagrass samples. A correction factor was calculated daily and applied to all data collected on that day. Images were processed and analysed using the OpenMIMS data analysis software plugin in ImageJ. Enrichment of the ^{15}N isotope in ratio images is expressed as colour on a Hue Saturation and Intensity (HSI) scale, where the minimum (blue) was set to natural isotopic abundance of nitrogen (37), and the maximum (magenta) was set to an arbitrary ratio value of 100. Individual images were first processed using a pixel-by-pixel detector dead time correction (44 ns), and data were extracted from pixels within manually drawn

regions-of-interest (ROI) encircling enriched microorganisms, algae and seagrass sub-cellular structures for quantification of $^{12}C^{15}N$ /$^{12}C^{14}N$ ratios (total number of ROIs =1754 comprising controls).

ROI (Table 4.2) were selected based on ultrastructural information from secondary electron imaging, optical, charged coupled device (CCD) and individual ion images. $^{15}N/^{14}N$ data corrected using yeast standard correction factor was further transformed to delta notation ($\delta^{15}N$) for consistency with IRMS data. The use of these two element ratio images ($^{14}N/^{14}C$ and $^{15}N/^{14}N$ with increased scale) was applied to the analyses of all NanoSIMS images of seagrass cells to identify the different sub-cellular components and the epiphytic community where present (Fig. 4.1). Although it was not possible to infer the taxonomy of the epiphytic algae found in this study, analysis of the secondary electron imaging and $^{14}N/^{14}C$ images highlighted visible differences between algae and other epiphytic organisms, which were used for the identification of algal ROIs (Fig. 4.1). NanoSIMS analysis of seagrass leaves with intact microorganisms clearly distinguished the epiphytic microbial community from epiphytic algae and seagrass cells, with resolution sufficient to distinguish the sub-cellular components of the seagrass leaf (Fig. 4.1). NanoSIMS image analysis was repeated three times to obtain a reliable method for the identification of the epiphytic community and seagrass sub-cellular components.

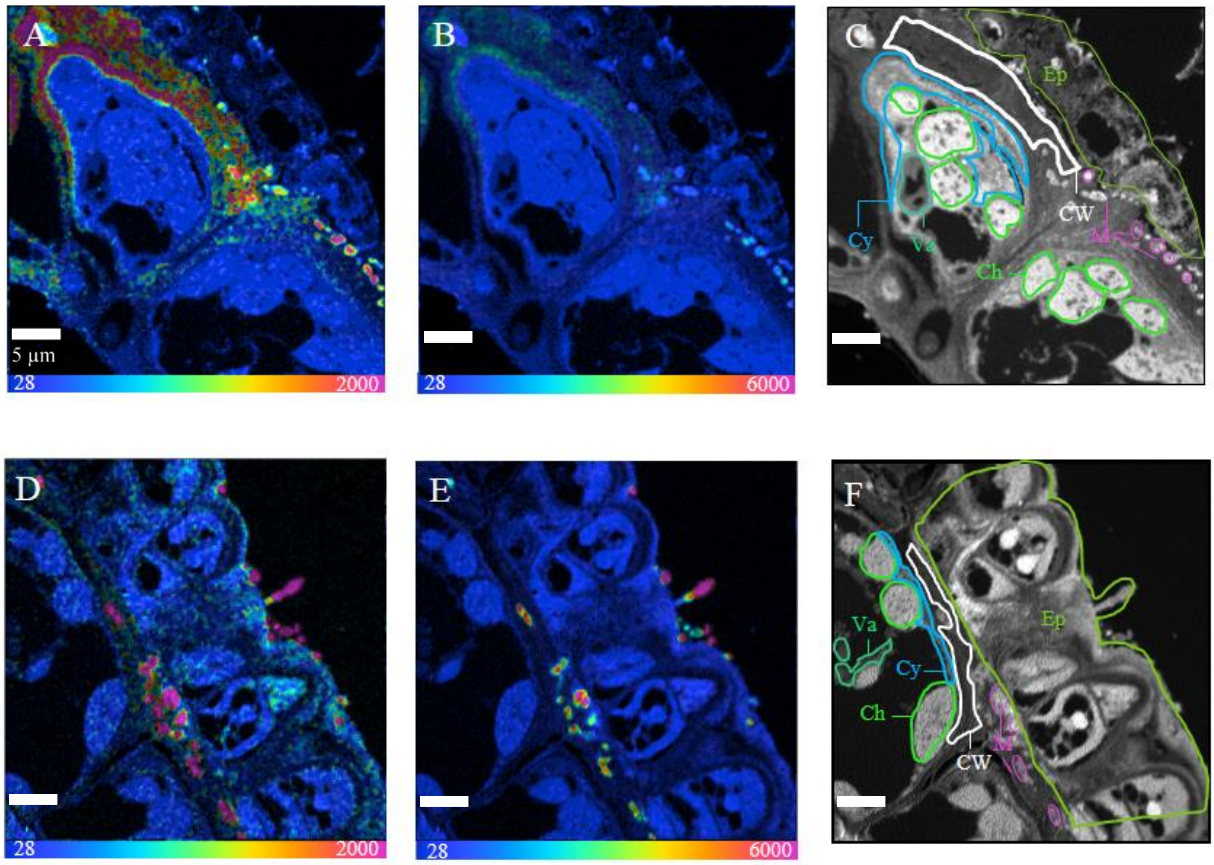


Fig. 4.1 - NanoSIMS image analysis description. The images represent two (A, B & C) or one (D, E & F) seagrass cells from shoots collected at 0.5 and 2 h from DO^{15}N spike, respectively. In figures A, B, D & E, ^{15}N-enrichment is expressed as hue saturation intensity, where blue represents the natural isotopic abundance of nitrogen (δ^{15}N‰= 28) and enrichment is shown as a shift towards magenta (colour scale label in δ^{15}N‰). In A & D, maximum ^{15}N enrichment is represented by the value of 100 % enrichment set to 2000 (δ^{15}N‰); in B & E ^{15}N/^{14}N ratio is reported with increased (3X) scale (6000 δ^{15}N‰). The increased scale together with images with natural isotopic element abundances ^{14}N/^{14}C (C & F) was used to better discriminate between different seagrass sub-cellular components and the epiphytic community, where present. C & F images reveal several clear seagrass sub-cellular components, highlighted in different colours (correlating with the colours in the box plot graphs (Fig. 4.4), except for the cell wall in white), which were used to draw our regions of interest (ROIs). Epiphytes and sub-cellular components are additionally labelled: epiphytic microbial community (M), epiphytic microalgae (Ep), cell wall (C), vacuole (Va), cytosol (Cy), and chloroplast (Ch). From the image analysis of the epiphytic community (C & F), epiphytic algae were clearly differentiated from other *P. sinuosa* epiphytes (e.g. bacteria and archaea), and these visible differences were used to drawn the algal and microbial ROIs. Scale bar represents 5µm.

Table 4.1 The number of regions of interest (ROIs) drawn for each image analysis component (microorganisms, cell wall, cytosol, vacuole and chloroplast) is reported for all time points and treatment (with and without microorganisms) and indicated with an asterisk. Only the first ten ^{15}N data (reported in $\delta^{15}N‰$) extracted from ROIs are shown; the complete dataset was used to draw box plot graphs (Fig. 4.4).

Time points	Cell wall		Cytosol		Vacuole		Chloroplast		Microorganisms
	With micro.	Without micro.	With micro.	Without micro.	With micro.	Without micro.	With micro.	Without micro.	
0.5h	24*	28*	21*	20*	55*	19*	32*	34*	74*
	1454,2	45,8	109,6	54	196,3	116,5	78,4	29,3	2284,3
	1204,7	30,5	83,9	82	156,5	152,2	77,1	28,9	1514,5
	2274,8	14,8	64,5	32,4	193,2	88,5	75,4	61	1410,5
	1067,4	48,4	113,6	55,7	205,7	77,6	61,4	40,1	2200,9
	1359,2	34,6	114,7	34,8	190,3	25,4	67,5	30,1	2235,6
	2232,6	36	196,7	60,8	207	26,5	61,9	42,5	2257,2
	1217,9	35	209	42,8	142,1	57	64,7	48,5	1565,4
	1391,8	29,5	256,8	32,4	112,5	47,3	71,1	38,4	2301,7
	999,4	31	236,3	26,8	232	64,9	73,8	20,6	2745,5
	1278,9	60,1	80,6	46	85,1	60	73,2	64,6	3365,4
2h	41*	26*	18*	47*	59*	53*	83*	44*	104*
	112,7	68,3	191,6	157,5	348	139,4	128,2	135,2	2198,2
	96,9	73,3	197,7	185,9	244,2	297,3	156,6	212,2	2648,4
	197,6	75,2	115,3	139,2	236,3	161,6	112,3	148,6	6291,4
	229,2	79,6	265,8	195,8	251,1	95,6	113,3	93,6	3513,7
	88,4	89,2	134,3	295,8	183,5	54,1	136,2	111,5	3745,6
	96,2	97,6	172,8	285,9	168,6	86,4	138,2	117,5	2888,5
	100,7	66,9	224,4	162,7	194,1	60,1	141,5	110,1	3630,2
	116,2	69,4	306,3	165,8	110,4	142,3	161,6	91,9	2550,9
	92,9	62,6	289	160,8	172,8	139,6	119,3	150,9	5651,2
	93,3	67,5	253,9	89,8	182,2	83,8	132,5	92,3	5191,5
6h	26*	25*	48*	24*	67*	48*	28	21*	104*
	143,2	247,5	471,9	394,7	624,4	178,3	128,2	190,5	4999,7
	113,3	344,5	500,1	363,9	632,8	204,6	183,9	98,1	5729,6
	116,9	84,1	508,5	153,6	404,3	130,2	198,6	80,1	6391,5
	113,1	94,7	551,2	167	427,9	231,1	188,5	80	7896,4
	140,7	186	571,8	222,3	452,2	178,1	189,4	98,1	4452,7
	117,1	214,9	577,5	66,9	535,0	219,6	184,8	80,1	6657,2
	109,1	43,6	585,2	146,4	596,5	104,1	214	138,1	5440,7
	64,6	78,8	596	104,8	630,1	123,7	247,8	80	6015
	108,3	74,3	613,9	74,3	705,9	90,1	232,2	98,1	5181,7
	149,4	87,6	642,8	19,9	488,8	46,3	189,3	80,1	8345,3
12h	29*	53*	80*	19*	40*	63*	58*	19*	105*
	400,1	84	1500,5	25,6	485,3	170,6	1167,3	21,5	14976,1
	284,2	48,8	1658,6	118,9	510,6	32,3	1130,2	27,9	10050,1
	298,1	70,3	1162,6	30,4	577	63,6	1243,3	30,2	10020,6
	346,5	40,9	1138,8	13,9	625,7	60,5	1069,1	98,4	9397,9
	284	100,8	1781,5	27,8	700,4	52,9	1293,9	78,1	7073,9
	273,7	87,3	2090,5	82,9	627,3	47,3	1169,6	29,9	11072,5
	223,8	94,6	1480,4	26,7	499	55,4	1079,2	21,5	13354,7
	378,2	75,3	1426	29,6	636,2	104,1	1400,2	90,2	12312,4
	341,5	49,6	1496,6	26,6	477,9	123,7	1087	58,4	12856,6
	424,7	74	1476,6	34,6	486,1	90,1	1118,8	48,1	18204,7

Statistical analyses.

For image analysis of microbial cell percentage cover for seagrass leaves incubated in antibiotics at 24 and 48 h, and seagrass leaves with and without microorganisms at the end of the DO^{15}N incubation experiment, One-Way ANOVA was performed in SPSS (Appendix 4).

For isotope ratio mass spectrometry analyses, differences in $^{15}N/^{14}N$ abundances between samples collected at different times were tested using a 2-WAY ANOVA in SPSS. The factors were represented by plant type (i.e. seagrass with and without microorganisms and epiphytic algae) and collection time (0.5, 2, 6 and 12 h). Prior to analysis, data were natural log-transformed to meet the homogeneity requirement using Levene's test.

4.3 Results and Discussion

Evidence that microorganisms associated with *P. sinuosa* leaves facilitate seagrass uptake of DON (in this case, ^{15}N derived from amino acids) was provided by isotope mass spectometry analyses of bulk tissue (IRMS) of seagrass leaves with and without intact microorganisms (Fig. 4.2, Table 4.3). After 12 h, ^{15}N accumulation was 4.5 times greater in leaves with intact microorganisms, compared to those where microorganisms had been removed ($p=0.001$). Samples from preceding collection times also had greater ^{15}N accumulation in leaves with intact microbial communities, compared to those without (Fig. 4.2). Considering that in different studies where seagrass leaves incubated with DO^{15}N showed a lower enrichment in the presence of epiphytic algae (Cunningham et al., 2002; Hyndes et al., 2012), the higher seagrass enrichment I detected in the 'seagrass with microorganisms' treatment compared to the 'seagrass without microorganisms' ($p=0.001$) highlights the pivotal role that microorganisms play in enhancing N availability for seagrass uptake regardless of the presence of epiphytic algae. IRMS results also showed that for the treatment 'seagrass with microorganisms', epiphytic algae presented a higher ^{15}N enrichment compared to seagrass tissue with associated microorganisms at all collection times ($p=0.001$; Fig. 4.2). These results suggest that epiphytic algae possibly compete with seagrass leaves for uptake of N.

Differences in N uptake between seagrass leaves and the epiphytic community members were expected, since microorganisms, and some species of algae/phytoplankton, possess different paths for the internalisation of N from the environment. Traditionally, DON uptake by phytoplankton, and algae in general, has not been considered a significant process in the marine environment (Mulholland & Lee 2009; Moschonas et al., 2017). Rather, bacteria were thought to be the primary consumers of DON (Zehr & Ward 2002; Bronk et al., 2007). However, genomes of marine diatoms, such as *Thalassiosira pseudonana*, has revealed the presence of amino acid transporters in the plasma membrane (Armbrust et al., 2004). In fact, small organic N molecules, such as some amino acids and urea, can be taken up intact through active transport driven by a sodium ion pump, or through facilitated diffusion if the concentrations outside the cell are high enough to create a concentration gradient (Mulholland & Lomas, 2008). For larger DON compounds (>1kD, e.g. proteins and polypeptides), DON mineralisation can occur within or outside the algal cell, through the activity of intracellular and extracellular enzymes, such as amino-peptidases, hydrolases, oxidases and deaminases, that break down DON to produce DIN (Mulholland et al., 2002; Bronk et al., 2007).

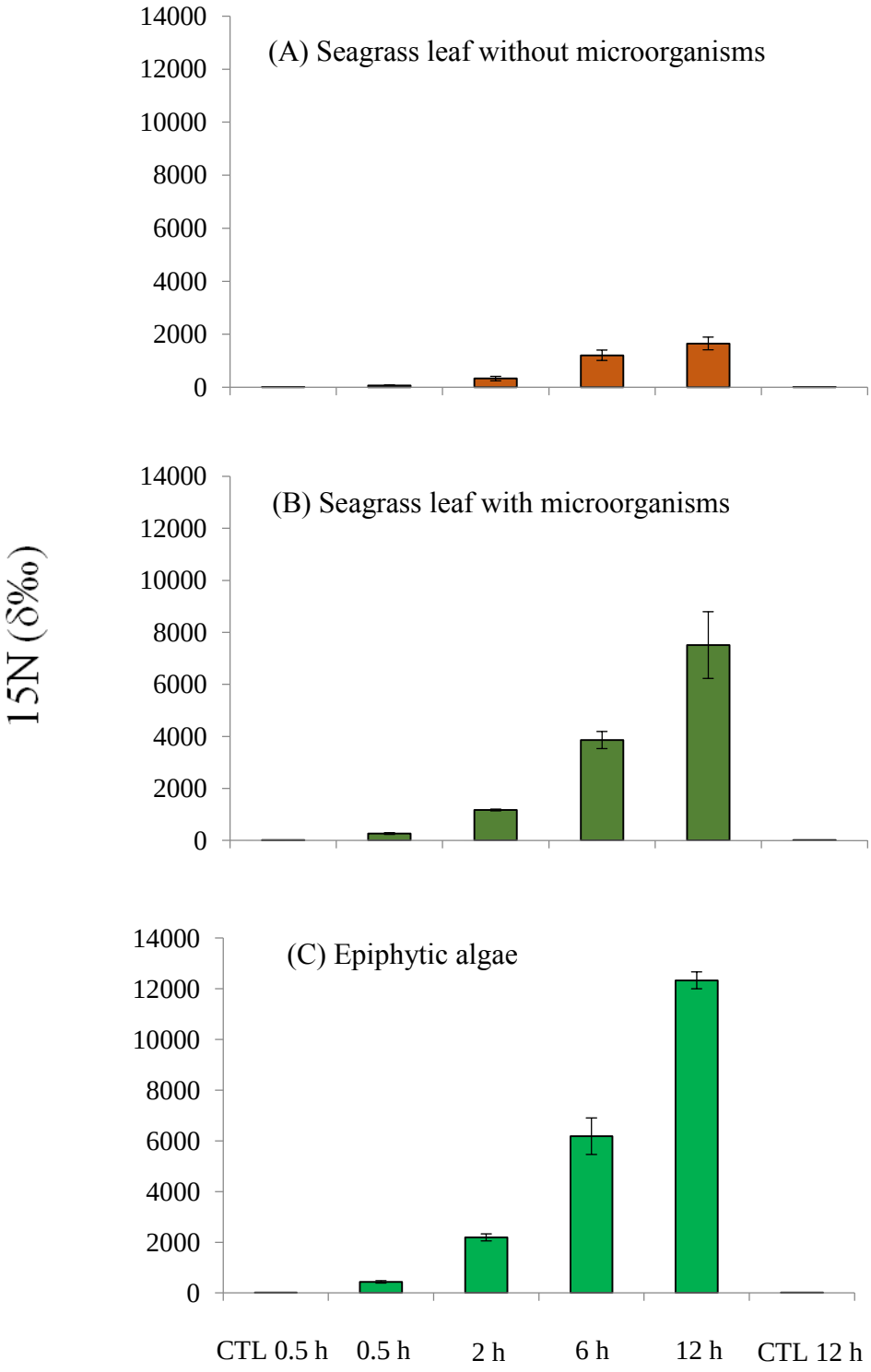


Fig. 4.2 - Mean (±SE) $\delta^{15}N$ values for (A,B) bulk tissue of seagrass leaves (n=3 per treatment and collection time), after $DO^{15}N$ enrichment (A) without, and (B) with epiphytic microorganisms, and (C) $\delta^{15}N$ values for epiphytic algae scraped from seagrass leaves, at different time points of incubation (0.5 h, 2 h, 6 h and 12 h). Control material (Ctl) bars represent values obtained incubation with $DO^{14}N$ for seagrass leaves (n=3) (A) and (B), and for the epiphytic community scraped from seagrass leaves (C), taken at the first (Ctl 0.5 h) and last time point (Ctl 12 h).

Table 4.2 2-WAY ANOVA test results of seagrass leaves incubated with and without microorganisms collected at different times. **Pl** refers to plant type (seagrass with microorganisms, seagrass without microorganisms and epiphytic algae) and, **Ti** refers to the collection time (0.5, 2, 6 and 12 h). Data were natural log-transformed prior to analysis to meet requirements of homogeneity using Levene's test (F=2.764, p= 0.131).

Source	*df*	*MS*	*Pseudo-F*	*P(perm)*
Plant type (Pl)	2	3283	112.59	0.001
Time (Ti)	3	7224	119.78	0.001
Pl x Ti	6	854	12.235	0.001
Res	24	13		
Total	35			

Although *P. sinuosa* leaves 'without microorganisms' had significantly lower ^{15}N enrichment compared to the tissue where microorganisms were present (p=0.001), they were still characterised by a slight elevation of ^{15}N enrichment during the experiment (Fig. 4.2 A). Bulk isotope measures alone are unable to discern specific isotope tracer accumulation points or the source of slight ^{15}N enrichment detected in leaves removed of microorganisms. Subsequently, nanoscale secondary ion mass spectrometry (NanoSIMS) was performed on representative samples to resolve ^{15}N accumulation in the finer structures of the seagrass leaves and the flow of ^{15}N through the epiphytic community.

In general, NanoSIMS image analysis showed that seagrass leaves 'without microorganisms' were characterised by the absence of ^{15}N-enrichment within their tissue (Fig. 4.3). Differences between ^{15}N-enriched sub-cellular microenvironments versus non-enriched samples, obtained from the 'without microorganisms' treatment, appear even more evident when observing seagrass cell images after 12 h following $DO^{15}N$ enrichment (Fig. 4.3 D & I). However, for a few samples of the seagrass leaves 'without microorganisms', I detected a slight enrichment of seagrass sub-cellular microenvironments (Fig. 4.4), and the possible explanations regarding these samples are discussed later. NanoSIMS images showed that, after 0.5 h incubation in $DO^{15}N$, microorganisms (bacteria/archaea) on the surface of the seagrass leaf were highly enriched compared to the surrounding seagrass cells (Figs 4.3 and 4.4). The microorganisms remained more ^{15}N-enriched than adjacent sub-cellular components of the seagrass leaf at each time point of the experiment, and their enrichment was

characterised by an exponential ^{15}N accumulation over time (Fig. 4.4). By 12h, microorganisms were ~200 times more enriched (~10000 ^{15}N per mil) than unlabelled reference (control) samples. These results, combined with the rapid ability of microbes to mineralise DON (Chrost, 1991; Flemming et al., 2016), indicate that epiphytic microorganisms strongly influence the accumulation of ^{15}N into different components of the seagrass tissue over the timescale of the experiment (Figs. 4.3 and 4.4). Within 0.5 h of incubation, ^{15}N was 26 times higher in the outer cell wall of epidermal cells of seagrass leaves with microorganisms present than those with microorganisms removed (Fig. 4.4). While the mechanisms of absorption of solutes through the cuticle are not fully characterised (Riederer & Schreiber, 1995; Khayet & Fernández, 2012), the enhanced enrichment of ^{15}N in the cell wall, followed by a decrease in enrichment (Figs. 4.3 – A & C and 4.4) suggests that: (i) the movement of N across the seagrass cuticle is a rapid process (Riederer & Schreiber, 1995; Khayet & Fernández, 2012), and (ii) the plant is able to limit N uptake once it has reached substrate saturation (Touchette & Burkholder, 2000). Slight ^{15}N accumulation was first observed in cytosol and vacuole after 0.5 h, followed by an exponential accumulation over proceeding sampling times (Fig. 4.4). The very large and obvious chloroplasts that proliferate at the outer edge of the seagrass epidermal cells accumulated ^{15}N more slowly than other sub-components of seagrass leaves and values were still rising at the final 12 h sampling time.

Based on the ^{15}N accumulation dynamics of the different seagrass sub-cellular structures observed, my results suggest that DIN, in the form of nitrate (NO_3^-), is the primary form of N available for seagrass uptake, for the following reasons. Seagrass leaves can acquire DIN as either NO_3^- and/or ammonium (NH_4^+), though they tend to have a greater affinity for the former (Touchette & Burkholder, 2000). Assimilated NO_3^- is stored in the vacuole or reduced to nitrite (NO_2^-) within the cytosol and then transported into the chloroplasts for further reduction to NH_4^+ (Touchette & Burkholder, 2000), a pattern consistent with my findings. The slight elevation of ^{15}N within leaf chloroplasts at 2 h and 6 h, appearing not to have reached a maximum after 12 h (Fig. 4.4), indicates the deliberate transport of ^{15}N through the seagrass sub-cellular components towards the chloroplasts; thus implicating the role of the chloroplast in the reduction of NO_3^- to NH_4^+. The preferential uptake of NO_3^- by seagrass tissues, even in the presence of excess concentrations of DON, highlights

the potentially vital functional role of ureolytic and ammonia-oxidising microorganisms that I identified in Chapter 3. Since these microbial groups connect the degradation of organic matter (DON production) with the transformation of DON to DIN, they could be key components of the processes shown in the current study. Further work, aimed at identifying these microbial groups using additional microscopic techniques, would confirm this hypothesis.

A lack of knowledge on the interactions that regulate seagrass–epiphyte relationships has thus far limited our ability to understand the N dynamics between marine plants and members of the epiphytic community. For example, several studies have documented a detrimental effect of excessive epiphytic algal growth on seagrass viability, particularly in areas with increasing anthropogenic nutrient inputs to coastal waters (Silberstein et al., 1986, Hauxwell et al., 2001, McGlathery, 2001). However, it has also been shown that, in oligotrophic waters (such as those in the current study region), epiphytic algae can play an important role in ecosystem functioning by contributing to the capture of scarce N resources from the water column, and thus promoting nutrient cycling (McGlathery, 2001). Indeed, seagrass leaves are able to modify the microenvironment of their phyllosphere to create a habitat suitable for specific epiphytic organisms, and it has been shown that the dominant microalgal groups in the epiphytes on the leaves of the seagrasses *Zostera marina* and *Halodule wrightii* were cyanobacteria and diatoms (Pinckney & Micheli; 1998), which may directly benefit seagrasses. In fact, the biofilm is not as inert as previously assumed. The extracellular polymeric substances (termed matrix) excreted by microbial cells has a characteristic sponge-like nature, which plays a pivotal role in the absorption of nutrients from the environment and increase of the retention time of molecules within the matrix (Flemming et al., 2016). A variety of extracellular enzymes (e.g. microbial- or algal-derived) that degrade organic matter have been described within biofilms (Brukmeier et al., 2005; Romani et al., 2008; Pohlon et al., 2009), and it seems likely that the secreted enzymes retained within the matrix provide nutrients (N in this case) for all the members of both the epiphytic and the host community.

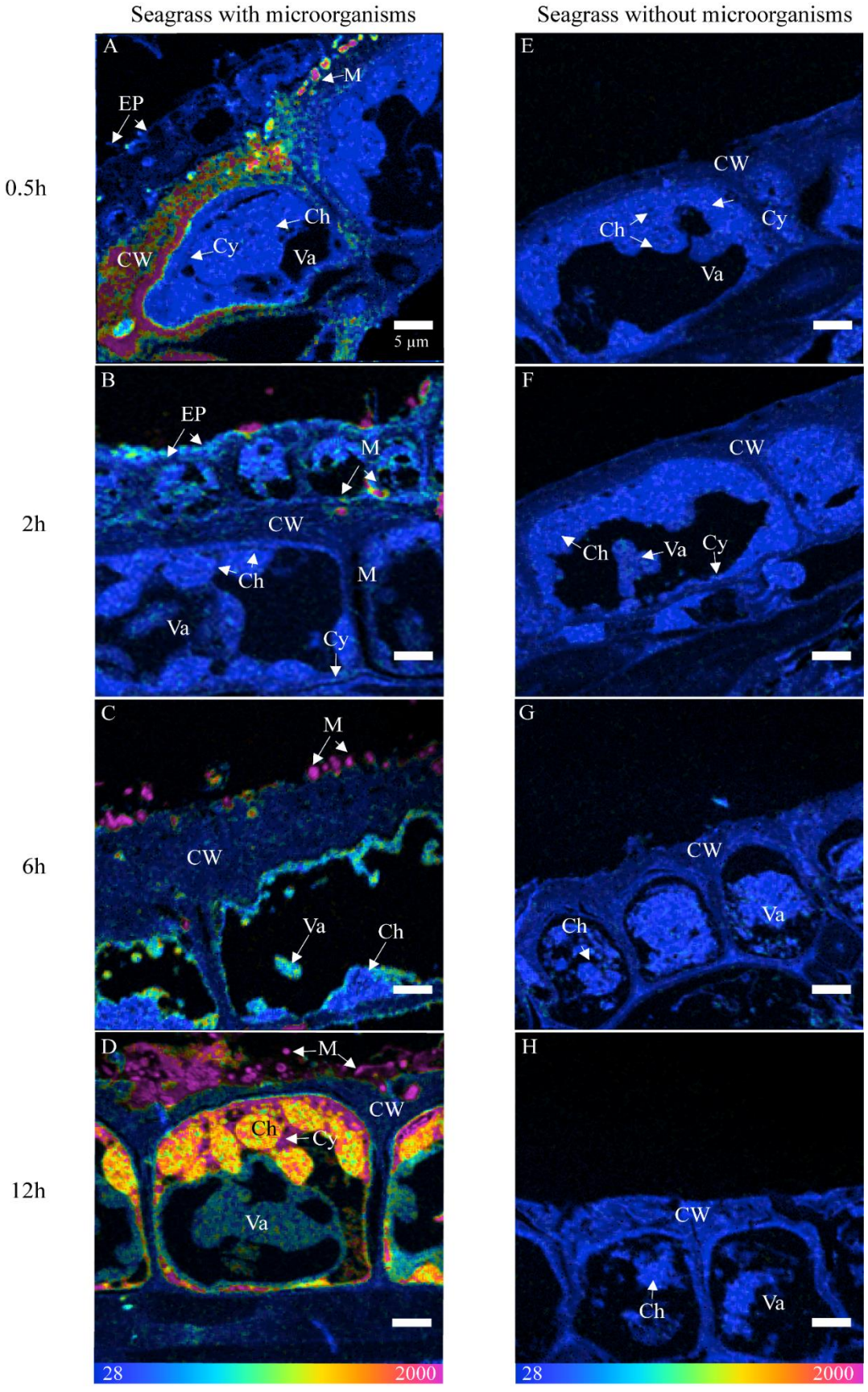

Seagrass with microorganisms
Seagrass without microorganisms
0.5h
2h
6h
12h
A
B
C
D
E
F
G
H
EP
M
Ch
Cy
CW
Va
5 µm
28
2000

Fig. 4.3 – ^{15}N concentration images of seagrass (*P. sinuosa*) leaf cells after incubation in enriched $DO^{15}N$ (amino acid mix) (A-D) with or (E-H) without microorganisms. Seagrass leaves were incubated for (A,E) 0.5 h, (B,F) 2 h, (C,G) 6 h, and (D,H) 12 h. Seagrass sub-cellular components are indicated (CW=Cell wall, Ch=Chloroplast, Va=Vacuole, Cy=Cytosol), as well as microorganisms (M) and epiphytic algae (EP). Enrichment is expressed as hue saturation intensity, where blue represents the natural isotopic abundance of nitrogen ($\delta^{15}N‰ = 28$) and enrichment is shown as a shift towards magenta (color scale label in $\delta^{15}N‰$). The images show a ^{15}N enrichment of seagrass cell wall after (A) 0.5h, and the appearance of enriched substrate within the (B) cytosol, (C) vacuole, and (D) chloroplasts over the incubation time. Where microorganisms are absent (E-H), a clear enrichment is absent. In every image the scale bar represents 5μm.

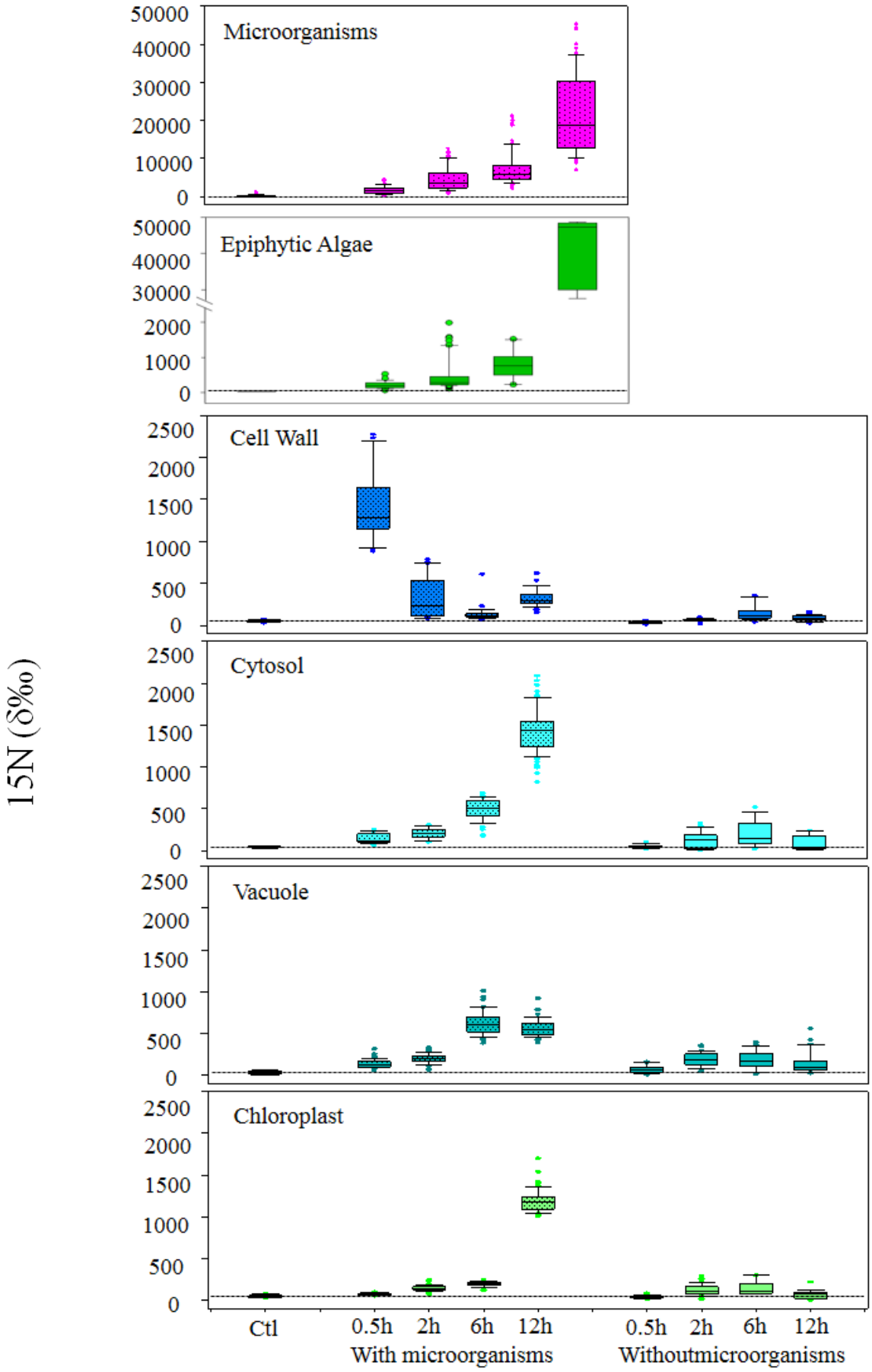

15N (δ‰)
Microorganisms
Epiphytic Algae
Cell Wall
Cytosol
Vacuole
Chloroplast
50000
40000
30000
20000
10000
0
2000
1000
2500
1500
500
Ctl
0.5h
2h
6h
12h
With microorganisms
Withoutmicroorganisms

Fig. 4.4 – Box-Whisker plots (median, interquartile range = box, extremes = whiskers) of ^{15}N enrichment of identifiable microorganisms, epiphytic algae and sub-cellular seagrass components: cell wall, cytosol, vacuole, and chloroplast, from NanoSIMS analysis of seagrass leaves incubated (left) with, or (right) or without leaf-associated microorganisms, for each incubation time (0.5, 2, 6 and 12 h). Control values (Ctl), representing $\delta^{15}N$ natural abundance for microorganisms, epiphytic algae and seagrass sub-cellular microenvironments obtained from incubation with non-enriched $DO^{14}N$, are displayed for each graph by the horizontal line. Note the difference in y-axis scales between microorganisms, epiphytic algae and plant sub-cellular components.

For seagrass leaves where microorganisms were removed, I observed only the slightest ^{15}N accumulation in the various cellular structures (Fig. 4.5). I postulate that this elevation in ^{15}N may be due to: (i) persistence of some epiphytic microorganisms that presented antibiotic resistance and converted $DO^{15}N$ to inorganic forms over time, (ii) antibiotics altering the bacterial cell wall and causing leaching of enzymes from dead bacterial cells, with subsequent $DO^{15}N$ degradation (Hałemejko & Chrost, 1986; Kiersztyn et al., 2011), or (iii) seagrass leaves taking up small amounts of ^{15}N-labelled amino acids over time. However, I argue that the first of these options is the likely cause, since NanoSIMS analysis and corresponding LIVE/DEAD® BacLight™ kit cell counts revealed very few examples where microorganisms were not entirely removed (<1% leaf surface; Fig. 4.5). In these specific areas, microorganisms appeared to be protected from antibiotic penetration by the remaining epiphytic algae. The few remaining microorganisms were 20 times more ^{15}N-enriched than those of unlabelled reference samples, and only seagrass cells directly adjacent to the remaining microbiome exhibited significant ^{15}N enrichment (Fig. 4.5). Overall, these results cast doubts over the ability of seagrasses to directly utilise amino acids directly as a source of essential N (Vonk et al., 2008; Van Engeland et al., 2013; La Nefie et al., 2014; Alexander et al., 2015). In other studies that have investigated seagrass ability to utilise DON (Vonk et al., 2008; Van Engeland et al., 2013; La Nefie et al., 2014; Alexander et al., 2015), microbial epiphytes were never completely removed and, based on our findings, even small numbers of microorganisms on seagrass leaves do influence the uptake of N derived from DON.

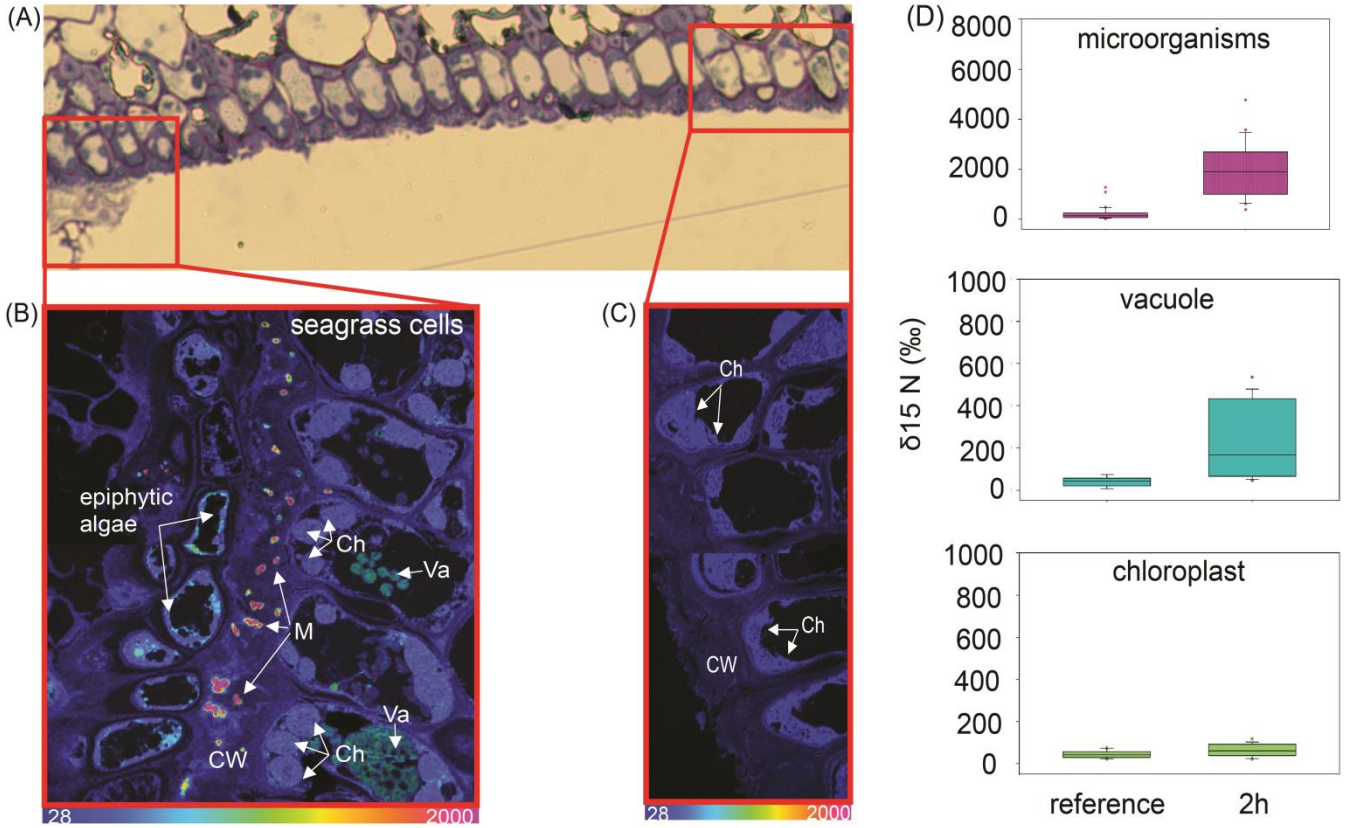


Fig. 4.5 - Images of seagrass (*P. sinuosa*) leaf cells after incubation in enriched $DO^{15}N$ (amino acid mix) for the 'without microorganisms' treatment, where there was evidence of the presence of residual epiphytic microorganisms. (A) Optical image of the seagrass leaf (transverse section) for treatment 'without microorganisms' after 2 h of incubation in $DO^{15}N$, (B) NanoSIMS image of the section void of microbes, (C) NanoSIMS image of a different section of the same leaf, where algae have not been entirely removed, thereby protecting the microorganisms beneath from antibiotic penetration and effect. Enrichment is expressed as a hue saturation intensity image, where blue represents the natural isotopic abundance of nitrogen ($\delta^{15}N‰ = 28$) and enrichment is shown as a shift towards magenta (color scale label in $\delta^{15}N‰$). (D) Box-Whisker plots of ^{15}N enrichment of identifiable epiphytic microorganisms and sub-cellular (vacuole and chloroplast) seagrass components against reference values. $\delta^{15}N$ values are reported on the y axes.

4.5 Summary and conclusions

The results presented in this study, show for the first time, that epiphytic microorganisms inhabiting the seagrass leaf biofilm facilitate the uptake of nitrogen (derived from DON) in seagrasses. By mineralising DON generated within seagrass meadows (Wahl et al., 2012), or DON imported from other coastal ecosystems (Säwström et al., 2016), epiphytic microorganisms on *P. sinuosa* leaves link the organic and inorganic components of the elemental N cycle in seagrass meadows, and are likely to contribute significantly to the high productivity of these important coastal ecosystems. Additionally, seagrass algal epiphytes include a variety of micro- and macroalgae, which represent a substantial component of the seagrass ecosystem (Sullivan 1979; Trautman & Borowitzka, 1999; Smit et al., 2006). Like microorganisms, some algae are capable of utilising DON directly, while others may benefit from microbial mineralisation of organic-N, as I have concluded is the case for the seagrass *P. sinuosa*. In Chapter 3, I identified important N-cycling members of the epiphytic microbiome of *P. sinuosa*: ureolytic bacteria, and ammonia-oxidising bacteria and archaea. The results presented in this current chapter furthermore suggest that these specific functional groups could play a key role in transforming DON into DIN for uptake by seagrass leaves.

Seagrasses and seagrass epiphytes are responsible for the high primary productivity of many benthic coastal systems (Borrowitzka & Lethbridge, 1989; Moncreiff et al., 1992; Larkum et al., 2007). From this current study, I conclude that microbial mineralisation of DON within the seagrass leaf biofilm could support this high productivity, by processing DON into DIN, which is more readily bioavailable for uptake by seagrasses and their epiphytes. This is likely to be the case for other macrophytes and their epiphytic algae in aquatic systems, which also have an association with heterotrophic epiphytic microorganisms. The present work therefore represents a significant advance in our understanding of the functional relationship between macrophytes (*P. sinuosa*) and their associated epiphytic microbiome and

encourage the exploration of different seagrass species and their associated microbiomes.

CHAPTER FIVE: COMPLEX CYCLING OF NITROGEN ASSOCIATED WITH SEAGRASS (*POSIDONIA SINUOSA*) LEAF MICROBIAL COMMUNITY

5.1 Abstract

Within the marine ecosystem, boundaries between different habitats are permeable and allow for passive and active movement of organisms and nutrients. Traditionally, seagrass ecology has focused on seagrass habitats independent of their surroundings, however, some studies have shown that a relative high biomass of allochthonous material (i.e. seston and kelp) is trapped within seagrass beds contributing to seagrass nitrogen (N) demand. Seagrasses can host a high abundance of ammonia-oxidising microorganisms (AOM) on leaf surface, providing a pathway for recycling N at biofilm-leaf interface. The aims of this study were: i) to evaluate the potential role of *P. sinuosa* epiphytic microorganisms in enhancing inorganic N (DIN) availability for uptake by seagrasses by processing allochthonous sources of organic N (DON); and ii) clarify the relationship between the seagrass *P. sinuosa* and AOM in terms of competing for or enhancing N availability. Hence, I performed a series of experiments using enriched (^{15}N) algal and kelp-amino acids in order to mimic the two potential N allochthonous sources for seagrass meadows of Western Australia. I used allylthiourea (ATU), a common inhibitor for nitrification, to discriminate AOM competition/contribution to the plant N demand to the other microorganisms present on seagrass surface.,

Epiphytic microorganisms were able to efficiently mineralise, not only simple amino acids, but more complex DON molecules (such as kelp leachate), increasing DIN availability for seagrass uptake. ATU addition resulted in greater ^{15}N enrichment of both seagrass leaves and their epiphytic algae, showing that a proportion of DON is consumed by AOM. These results suggest the possibility that competition between seagrass leaves, seagrass epiphytes, and their associated AOM could occur on the seagrass leaf surface. However, several studies have documented a detrimental effect of excessive epiphytic algal growth on seagrass viability, particularly in areas with increasing nutrient inputs to coastal waters. Competition for NH_4^+ uptake between epiphytes and their seagrass host may be used by seagrass leaves as a form of 'biocontrol' over the growth of excess algae.

5.2 Introduction

Within the marine ecosystem, boundaries between different habitats are permeable and allow for movement of resources, including the active movement of organisms and the passive flow of nutrients (Polis et al., 1997). These cross-ecosystem transfers of energy and nutrients are called 'spatial subsidies' and play a key role in linking habitats, and can dramatically affect productivity and trophic and community dynamics in 'recipient' systems (Polis et al., 1997; Suchaneket et al.,1985; Polis & Hurd, 1996; Duarte, 2002; Heck et al., 2008). Understanding trophic interactions among habitats and the direction and magnitude of material flow through food webs are fundamental to our basic comprehension of ecosystem function and management.

In terms of nutrient transfer, seagrass ecology has mainly focused on the export of nutrients from the seagrass meadow canopy to 'recipient ecosystems' (e.g. offshore reefs), and this has mainly focused on particulate material (Mateo et al., 2003; Mateo et al., 2006; Romero et al., 2006; Heck al., 2008). Seagrass meadow productivity in Western Australia has been estimated at 500 g C m^{-2} y^{-1} (Hyndes et al., 2013), of which approximately 74% is exported to adjacent recipient habitats (Wolff, 1980; Suchanek et al.,1985; Duarte, 2002; Heck et al., 2008), where seagrass nutrients can enhance food web structure and productivity (Vetter, 1994; Vetter, 1998; Heck et al., 2008; Hyndes et al., 2014). However, the large export (up to 70%) of leaves and sources from seagrass systems (Mateo et al., 2006, Heck et al., 2008) suggests that external nutrient subsidies into seagrass systems are necessary to balance the loss of nitrogen (N) through organic matter export (Cambridge & Hocking, 1997; Heck et al., 2008). Thus, the import of nutrients into seagrass meadows from 'donor' habitats could be particularly important for seagrass survival.

Seagrass meadows can receive nutrients in the form of particulate and dissolved organic matter (POM/DOM) or dissolved inorganic matter (DIM) from a range of donor sources, including macrophytes and seston (non-living particulate matter). For example, in South Western Australia, rocky reef communities are

dominated by kelps – large brown algae in the order *Laminariales* (Kirkman & Kendrick, 1997; Steneck et al., 2002; Wernberg et al., 2003) – which often occur close to seagrass meadows (Sanderson, 1997). Kelp biomass can be lost through detachment or erosion of the thalli (Wernberg et al., 2006, de Bettignies et al., 2013), and detached thalli from kelp reefs, where primary production can exceed 1 kg C m^{-2} y^{-1}, can accumulate in seagrass meadows (Hyndes et al., 2014). Kelp reefs therefore act as 'donor habitats', providing a source of nutrients, including N, to seagrass meadows (Hyndes et al., 2012). There is growing evidence that seston represents another important contributor to the POM/DOM pool in seagrass sediments, with inputs estimated between 0.1 and 0.3 Kg C m^{-2} yr^{-1} (Mann, 1979; Kenworthy & Thayer, 1984) providing approximately 7 to 45 g N m^{-2} yr^{-1} for seagrass uptake (Hemminga et al., 1991). Import of allochthonous material could provide seagrasses with a supplemental source of N that could be especially important in South Western Australia due to the constantly low environmental N concentration (Thompson 1984; Holloway & Nye, 1985) that results from the low-rainfall climate and the influence of the Leeuwin Current (Thompson 1984; Holloway & Nye, 1985).

Once allochthonous material has been trapped within a seagrass meadow, the regeneration of nutrients through the activities of heterotrophic microorganisms enhances the availability of N for seagrass uptake (Evrard et al., 2005). The degradation of POM (e.g. kelp thalli) releases DOM through leaching, providing a substrate for residential microorganisms of recipient habitats (Blum, & Mills, 1991; Shilla et al., 2006 Lavery et al., 2013). It appears that DOM lost as leachate from macroalgae represents a readily available nutrient source, subject to subsequent microbial mineralisation (Fankboner & De Burgh, 1977). Yet, despite the emphasis placed on transported organic material as a vector for spatial subsidies across a range of coastal habitats (Hyndes et al., 2014), relatively little is known about the role of microbial mineralisation of organic material in facilitating coastal connectivity (Säwström et al., 2016) and even less for microbial mineralisation of organic N.

Under oxic conditions, the mineralisation of organic N is linked to ammonia oxidation, the first and rate-limiting step of nitrification, whereby ammonium (NH_4^+) is oxidised to nitrite (NO_2^-) (Kowalchuk & Stephen, 2001; Francis et al., 2005; Francis et al., 2007). The second step of the nitrification pathway is the oxidation of NO_2^- to nitrate (NO_3^-), with both NO_2^- and NO_3^- being released by microbial cells during nitrification (Rotthauwe et al., 1997; Kowalchuk & Stephen, 2001). In Chapter 3, I showed that the surface of *P. sinuosa* leaves hosts an abundant and diverse community of epiphytic ammonia-oxidising microorganisms (AOMs). Following the mineralisation of DON by heterotrophic microorganisms, DIN released into the immediate seagrass environment (e.g. the leaf, or phyllosphere) could be taken up by the plant itself (Chapeter 4) and/or remineralised further by different microbial functional groups. Surprisingly, very little is known of the relationship between AOM and seagrass leaves in terms of competition for or provision of N, including the N 'donated' from adjacent habitats. In other words, while studies of the seagrass microbiome are in their infancy, we know little of the contribution of seagrass epiphytes to spatial subsidies and N transport in seagrass systems.

Based on the evidence that seagrass leaves are incapable of uptake of DON directly (Chapter 4), the aim of the present study was to: (i) determine the role of leaf-associated microorganisms in providing N to seagrasses via mineralisation of the DON made available from allochthonous material, and (ii) define the seagrass-AOM relationship in terms of DIN uptake. Hence, I performed a series of experiments using a mixture of simple ^{15}N-algal amino acids and more complex organic ^{15}N-compounds derived from kelp exudates, in order to mimic two common, indirect, allochthonous N sources for seagrass meadows of southwestern Australia. To help determine the significance of N cycled by microorganisms hosted by *P. sinuosa* meadows, I have measured nitrification rates associated with AOMs inhabiting the *P. sinuosa* leaf biofilm and tested whether these AOMs may enhance, or compete with, seagrass DIN uptake.

5.3 Materials and Methods

In this study, I performed two linked experiments. Firstly, I determined the ammonia oxidation rates of microorganisms associated with *P. sinuosa* leaves. This experiment also provided the concentration of allylthiourea (ATU) that was capable of inhibiting ammonia oxidation by those microorganisms to be used in the second experiment. Secondly, I used ATU as an ammonia oxidation inhibitor, to measure the uptake of enriched ^{15}N derived from a mixture of simple ^{15}N-algal amino acids and more complex organic ^{15}N-compounds derived from kelp, in order to determine the relationship between seagrasses and their epiphytic ammonia-oxidising microorganisms.

5.3.1 Ammonia oxidation experimental design

Allylthiourea (ATU) is a common inhibitor of ammonia oxidation, which represents the first and limiting step of nitrification, and it has been extensively used in experiments to exclude the ammonia oxidation process (Bedard & Knowles, 1989). However, recent studies have shown that some ammonia-oxidising archaea (AOA) remain uninhibited by ATU concentrations up to 1 µM (Santoro & Casciotti, 2011). In this experiment, I examined: (i) the ammonia oxidation rates of AOM associated with *P. sinuosa* shoots in southwestern Australia, and (ii) the ATU (Sigma Aldrich, USA) concentration that was capable of inhibiting ammonia oxdation of epiphytic AOM, and which could therefore be used for the second series of experiments. To achieve these aims, I used microorganisms collected from the *P. sinuosa* leaf biofilm, which were incubated without ATU (CTL; to obtain the ammonia oxidation rates) and with two ATU concentrations (100 mg L^{-1} and 200 mg L^{-1} equal to 0.9 and 1.75 mM). Every control and treatment experiment was measured in triplicate.

5.3.2 Nitrification experiment

Twenty *P. sinuosa* shoots were collected from a meadow (31°48'12.8" S 115°43'07.2" E) in Marmion Marine Park, offshore from Perth, Western Australia. Seagrass shoots were collected with rhizomes and roots attached to minimise plant stress, and transported in aerated seawater to aquarium facilities at Edith Cowan University (ECU). At ECU, seagrasses were placed in 30 L aquaria under ambient seawater temperature (18 $^{\circ}$C) with natural light cycle. After 24 h acclimatisation, a total of 18 healthy-looking seagrass shoots were chosen for the experiment and placed in artificial filtered (0.2 µm) seawater (ASF).

Epiphytic organisms were detached from leaves by placing seagrass shoots in ASF and gently scraping the leaves with a razor blade. The epiphytic microbes needed to be incubated in isolation from seagrass due to the inability to avoid seagrass uptake of inorganic nitrogen if it was present, thus affecting the nitrogen concentration in the medium and consequent nitrification measurements. The resulting 'solution' (1.8 L final volume) of microorganisms and epiphytic algae was then placed into a sterile glass bottle. The glass bottle was placed in the dark to minimise algal growth, and placed on a shaker (MaxQ 3000 Benchtop Orbital Shaker, Thermofisher) at low ramp speed (100 rpm) overnight (approximately 12 h) to detach microorganisms from epiphytic algae present within the solution. The slurry was filtered through 25 µm, 7 µm and 5 µm mesh nets, in order to separate the epiphytic algae from microorganisms, and then mixed using a magnet stirrer to create a homogeneous solution. For the treatments (TRT) and control (CTL), an inoculum of 25 ml was removed from the slurry and placed into 9 autoclaved glass bottles, containing 225 ml of ASF (250 mL final volume). 3 replicates per treatment and control were set up with: (i) no ATU addition (CTL); (ii) incubation with 100 mg ATU L^{-1} (0.9 mM final concentration) (TRT1); and (iii) incubation with 200 mg ATU L^{-1} (1.75 mM final concentration) (TRT2) (Santoro & Casciotti, 2011). Shortly after the introduction of ATU, NH_4^+ (10 µM final concentration) was added to each bottle of treatments and controls (obtained by dissolving NH_4Cl in 2 ml sterile,

ultrapure water). Bottles were incubated with air bubblers and magnetic stirrers at 18 °C in a temperature-controlled room in the dark. 20 mL of water samples were taken from each bottle at each time point (time 0, 8 and 24 h), and immediately frozen at -80 °C until analysis. 20 ml of water samples were also collected from the 1.8 L slurry, filtered through a 0.2 μm filter (Millipore) and frozen at -80 °C, to detect DIN background values. DIN analyses were performed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Perth (Floreat). Concentrations of NO_2^- and NO_3^- were detected using a Lachat FIA QickChem 8000 series, whereas NH_4^+ concentrations were determined with gas diffusion into orthophthaldiadehyde (OPA) with fluorescent detection (Aminot et al., 2001). Biofilm wet weight scraped from the older and/or medium *P. sinuosa* leaf can vary from ca 0.8g to 2.5 g (wet wt) of an entire shoot. For ammonia oxidation measurements, 25 ml of microbial inoculum (corresponding to ca 3 g. of biofilm, equal to the amount of biofilm we would expect to find on an entire shoot) were used to estimate ammonia oxidation rates associated with a *P. sinuosa* shoot. For both CTL and treatments, net nitrification rates were calculated by measuring NO_2^- and NO_3^- concentration over the time course of the laboratory incubation experiments, (with background N values subtracted) following the method of Radax et al.,2012. Average NO_2^- and NO_3^- (NO_x) concentrations were plotted for each time point and the slopes of the linear trend in NO_x concentration over time were used to calculate net nitrification rates. The R^2 values for the linear relationships used to calculate net nitrification were 0.88 for CTL, 0.36 for TRT1 and 0.28 for TRT2.

An upscaling calculation was performed based on the daily ammonia oxidation rate associated with a single *P. sinuosa* shoot and the average density of *P. sinuosa* shoots related to the surface covered (information obtained from the Australian Department of Parks and Wildlife, DPAW, for Marmion Marine Park) to calculate nitrification rates that could occur within seagrass meadows in the Perth region.

At the beginning of the ammonia oxidation experiment, prior to the incubation with NH_4^+, 1.8 ml of water was collected from treatment and control flasks to

evaluate differences in microbial abundances between the treatments. Water samples were fixed in glutaraldheyde (Sigma-Aldrich, USA) 2.5% final concentration and used for bacterial count.

5.3.3 Microbial counts

For the enumeration of bacteria, I followed the method of Chen and colleagues (2001): 1.8 ml of sampled was fixed using 25% 0.2 μm-filtered glutaraldheyde (final concentration 2.5%), before being vacuum filtered through 0.2 μm black polycarbonate filters (Isopore Membrane Filters, Millipore), using vacuum pressure no greater than 20 kPa. Immediately after this, filters were mounted on a microscope slide (Lom Scientific Pty Ltd) and stained with 10μl SYBR Gold (X10000 original dilution, Invitrogen, USA) diluted 1:1000 in ultrapure, 0.2 μm-filtered water. Filters were incubated with SYBR Gold for 10 minutes in the dark. A drop of anti-fade mounting reagent (Slow Fade Light, Invitrogen, USA) was placed on the stained filters after they were completely dry, and coverslips were applied. One drop of immersion oil was added to the top of the cover slip, and analysis for microbial abundances was undertaken using epifluorescence microscopy under blue-green light excitation (Fitch filter, excitation at 480-495nm). Two replicate preparations were counted for each sample and for each replicate, 5 fields of view were counted.

5.3.4 ^{15}N experimental design

I designed this experiment to determine the relationship between seagrasses and their associated ammonia-oxidising microorganisms (AOM), with respect to the competition for or provision of N under natural conditions. Since the majority of the N pool in coastal waters is usually in the form of DON (Sharp, 1983; Hasegawa et al., 2000), this was tested through two experiments using different donor sources of enriched N: (i) a ^{15}N-enriched algal amino acid mixture, and (ii) leachate from kelp that had been previously enriched with ^{15}N. Each experiment comprised two

treatment categories: plant type (3 levels, fixed), and ammonia oxidation (2 levels, fixed). Five replicates were established for each level of each treatment. The 'plant type' category consisted of 3 treatments: (i) seagrass leaves 'without epiphytes', to test for the uptake of N by seagrasses in the absence of competition with epiphytic algae, but with attached bacteria left undisturbed, and (ii) seagrass shoots 'with epiphytes' to test for uptake by seagrasses in presence of epiphytic algae. From the treatment seagrass 'with epiphytes' I obtained the third 'plant type' treatment: (iii) 'epiphytic algal ^{15}N-enrichment'. The experiment was performed twice: in the presence and absence of ATU, to test for the uptake of N by seagrass leaves with and without microbial ammonia oxidation ('ammonia oxidation' treatment). Five seagrass leaves were kept within the glass cylinders without DO^{15}N spike for the duration of experiment to be used as reference samples.

5.3.5 Environmental parameters and nitrogen concentration

To determine the concentration of algal and *Ecklonia radiata*-DO^{15}N to be added for seagrass shoot incubation experiments, I determined physical parameters and collected water samples for inorganic and organic nitrogen concentrations in 3 *P. sinuosa* (31°48'12.8" S 115°43'07.2" E) meadows located in the Marmion Marine Park, on two different sampling occasions (December 2013 and August 2014). Dissolved oxygen, temperature, salinity and pH were measured using a YEO-KAL water quality analyser. For N analyses, triplicate 1 L water samples from surface (SW; 0.5 m) and canopy water (CW; 4 m) were collected in acid-washed PVPP bottles. All samples were kept on ice in a shaded enclosure until arrival at ECU facilities, where they were immediately placed in a -80 °C freezer. Total nitrogen (TN), total dissolved nitrogen (TDN), ammonium (NH_4^+), and nitrite and nitrate (NO_2^- and NO_3^- as NOx) concentrations were analysed using a Lachat QuikCem flow injection analyser at ECU and used as reference for DO^{15}N concentration during seagrass incubation experiments.

5.3.6 Preparation of enriched nitrogen sources

Two sporophytes of the kelp *Ecklonia radiata* (about 200 g wet weight), including the stipe and holdfast to minimise stress, were detached from the reef immediately adjacent to the 3 seagrass meadows, and transported in aerated seawater to ECU facilities. At ECU, the kelp sporophytes were placed in 45 L of artificial seawater (Red Sea)under natural light conditions and ambient temperature for 24 h. Light intensity (130 µmol m^{-2} s^{-1}) was measured by using a Photosynthetically Active Radiance (PAR) sensor for underwater applications (Micro PAR, In-Situ Marine Optics, Perth) to ensure the kelp photosynthesised. The thallus from one sporophyte was used as a reference, while the other was enriched with ^{15}N.

Reference thallus. After acclimatisation, about 2.5 g of fresh blades (wet wt) were taken from the non-enriched kelp to be used as reference (natural $^{15}N/^{14}N$ abundance composition), and processed for stable isotope analysis. 100 g (wet wt) of blades were placed in 1 L of ASF (previously sterilised under UV light for 30 minutes) and left for 48 h to allow organic matter to leach from the thallus, this was then compared against the enriched leachate.

Enriched thallus. For the enriched kelp, prior to and during the enrichment period, the kelp photosynthetic efficiency (dark yield adaptation) was measured with the pulse-amplitude modulater (PAM) chlorophyll fluorometer to ensure the kelp did not show any sign of stress. The ^{15}N enrichment of the thallus was carried out using ammonium nitrate ($^{15}NH_4{}^{15}NO_3$, enriched at 98%, Sigma Aldrich), following methods adapted from Boschker et al., (2000). I added 2 g of $^{15}NH_4{}^{15}NO_3$ dissolved in ultrapure, sterile water to the aquarium over a time period of 10 days (200 mg $^{15}NH_4{}^{15}NO_3$ dissolved in 10 ml water d^{-1}). At the end of the incubation, ^{15}N-enriched *Ecklonia radiata* was transferred to a different aquarium filled with 45 L ASF for about 30 minutes, in order to remove excess ^{15}N from the plant surface. The kelp surface was cleaned from algal epiphytes by gentle scraping using a razor blade, and sterilised by wiping it with 70% ethylic alcohol, in order to preserve kelp leachate

from microbial degradation (Mazure et al., 1980; Corre et al., al., 1990; Vollmers et al., 2017). As for the reference thallus, 2.5 g (wet wt) ^{15}N-enriched thallus was processed for stable isotope analysis and 100 g of ^{15}N-enriched thallus were placed in 1 L ASF for 48 h, to collect DO^{15}N leachate.

Both the enriched and non-enriched leachate were filtered, firstly through 0.45 μm filter membrane (Whatman), followed by 0.2 μm filtration (Millipore), to eliminate all particulate organic nitrogen present within the solution. 20 ml of leachate samples were taken from the 0.20 μm solutions to measure total dissolved nitrogen (TDN), and both inorganic (ammonium, nitrate and nitrite) and organic N (DON). N was measured using a Lachat QuikCem flow injection analyser at ECU. In order to eliminate any DIN potentially present within *E. radiata* 0.2 μm leachate, the solution was treated following the method of Van Engeland et al., (2011): 50 mg of Devadarda alloy and 25 mg of MgCl dissolved in 100 mL ultrapure water were added to 1 L of kelp leachate, shakenfor 48 h at room temperature in order to convert NH_4^+ to NO_3^-, and then NO_3- to the gas form, allowing it to evaporate off the sample.

A further step was performed to separate and concentrate more complex kelp leachate molecules from peptides and simple amino acids using tangential flow filtration (TFF) with a Pellicon tangential flow device (Millipore) equipped with a fluorocarbon membrane 0.11 m^2 Ultracell 30 kDa (Merck, Germany). The cassette size used for the present study concentrated N compounds characterised by a molecular weight higher than 30 kDa, and eliminated smaller compounds such as remaining DIN and small peptides. About 10 ml of the concentrated kelp leachate was firstly frozen at -80 °C for 48 h and then freeze-dried to be analysed for stable isotope analyses at The University of Western Australia. The remaining leachate was frozen at -80 °C and kept for the seagrass enrichment experiment. The concentrations of DIN and DON within the concentrated leachate were measured using a Lachat QuikCem flow injection analyser at ECU. The final concentration of N added to the seagrass cylinders was 50 μM for both substrates (amino acids and

more complex kelp DON). By knowing the DON concentration of the kelp leachate and its level of enrichment through stable isotope analysis, I calculated the ^{15}N concentration within the added leachate to be 7.2 µM final concentration.

5.3.7 Experimental procedures

80 seagrass shoots were collected from the selected *P. sinuosa* meadow in Marmion Marine Park and treated in the same way as described for the ammonia oxidation experiment, above. For the 'seagrass without' treatments, seagrass shoots containing minimal epiphytic growth were selected and gently scraped with a razor blade to remove obvious epiphytes, whereas seagrass shoots with relatively high levels of epiphytic cover were selected for the other treatments and references. Approximately 3 g (wet wt) of seagrass shoot with epiphytes and approximately 2.2 g (wet wt) of seagrass shoot without epiphytes were placed in 500 ml glass cylinders in aerated artificial seawater. All the glass cylinders had been sterilised previously by acid-washing and autoclaving, and ASF had been previously sterilised under UV light for 30 minutes

From the ammonia oxidation experiment described above, inhibition of ammonia oxidation was detected from 8 to 24 h at a concentration of 200 mg ATU L^{-1}. Thus, 8 h before the introduction of any labelled substrate into the experiments, 200 mg ATU L^{-1} were placed within the cylinders designed for ammonia oxidation inhibition (+ATU). For each experiment, after 8 h incubation with ATU, either 50 µM of algal ^{15}N-amino acids or 7.2 µM of ^{15}N-kelp leachate were added to the cylinders containing seagrass for the two ammonia oxidation treatments (+ATU, -ATU). Photosynthetic yield of seagrass shoots used for the experiments were measured at the beginning and at the end of the experiment using PAM fluorometry as a proxy for plant stress. Seagrass shoots with and without epiphytes were removed after 6 h incubation with $DO^{15}N$ (Vonk et al., 2008). Samples were rinsed in filtered

seawater to remove excess ^{15}N and frozen at -20 °C until they were processed for $\delta^{15}N$ determination (see 'Stable isotope analyses' below).

5.3.8 Stable isotope analyses

Prior to the experiments, ^{15}N enrichment of the kelp blade and leachate was determined. Freeze-dried kelp ^{15}N leachate was mixed with non-enriched ^{14}N-leachate powder in 1:10 ratio, due to the high ^{15}N atom% enrichment we were expecting. Kelp blades were oven-dried for 48 h at 58 °C and ground (ball-mill grinder; Retsch, Haan, Germany) to fine powder. Similar to the leachate procedure, the enriched thallus powder was mixed with the non-enriched powder in the same ratio. The mixed leachate and thallus powder were weighed (approximately 1.5 mg) into 6 x 4 mm tin capsules (Elemental Microanalysis, UK) and crimped manually.

For both seagrass samples incubated in $DO^{15}N$ and reference material, frozen leaves covered with epiphytic organisms were gently scraped using a razor blade in order to detach and collect epiphytic algal material and obtain a clean isotopic signal for both the seagrass material and epiphytes. Seagrass and algal samples were oven-dried for 48 h at 58 °C, ground (ball-mill grinder; Retsch, Haan, Germany) to fine powder and weighed (approximately 1.5 mg) into 6 x 4 mm tin capsules (Elemental Microanalysis, UK) that were crimped manually. Samples were analysed for nitrogen elemental composition (%) and isotope ratios ($\delta^{15}N$) by continuous flow isotope ratio mass spectrometry using an Automated Nitrogen Carbon Analyser system, consisting of a Sercon 20-22 mass spectrometer and an elemental analyser (SERCON, UK), and were compared against reference samples that had been previously calibrated against standard International Atomic Energy Agency (IAEA, Vienna) reference materials with a precision and accuracy of <0.0005‰ (Skrzypek et al., 2013). Kelp enrichment level and the concentration of $DO^{15}N$ in the leachate were used to calculate the DON concentration that was added to the seagrass shoots during the incubation experiment. While for the algal ammino acid mixture I simply

added 50µM of "amino acid mixture" (NLM-2161-0, Cambridge Isotope Laboratories), I calculcated the kelp leachate that nedded to be added for seagrass incubation experiment. By knowing the final kelp DON concentration L^{-1} within the kelp $DO^{15}N$ leachate, I calculated the concentration related to ml^{-1}, then I simply calculated the amount (in ml) to be added to the seagrass cylinders (final volume of 0.5 L) to have a final N concentration of 50 µM (350 µg N 0.5 L^{-1}, equal to 0.7 µg N ml^{-1}). By knowing the isotopic enrichment ($^{15}N‰$) of the kelp $DO^{15}N$ I retrieved the molarity of ^{15}N added into the cylinders as part of the 50 µM N pool (350 µg N 0.5 L^{-1}):

14% ^{15}N of 350 µg N (0.5 L^{-1}) is equal to 49 µg ^{15}N 0.5 L^{-1}.

49 µg ^{15}N is equal to 7 µM of ^{15}N

5.3.9 Statistical analyses

A one-way ANOVA was performed on the data for bacterial abundance for the two ATU treatments at the beginning of the ammonia oxidation experiment, to test for similar microbial abundances among samples. For the seagrass ^{15}N incubation experiment, a 2-way ANOVA was used to test for differences in $\delta^{15}N$ among plant type categories (seagrass *P. sinuosa* leaf and epiphytic algae) and with and without ammonia oxidation. Data were natural log-transformed prior to analysis, to meet the requirements of homogeneity of variance and normality according to the Levene's and Kolmogorov-Smirnov tests (SPSS).

5.3 Results

5.3.1 Environmental parameters

Environmental concentrations of inorganic and organic nitrogen were detected from the selected *P. sinuosa* meadow in Marmion Marine Park. Concentration of total dissolved nitrogen (TDN), ammonia (NH_4^+), nitrite and nitrate (NOx) were

directly measured while values of dissolved organic nitrogen (DON) were obtained by subtracting the total inorganic nitrogen (NH_4^+ + NOx) content to the total nitrogen. In Table 5.1 nitrogen values are reported for water surface (WS; 50cm depth) and canopy water (CW; 4m depth) in two sampling occasions (summer and winter). Temperature, salinity, DO and pH were also registered and used for controlling the water characteristics during the seagrass incubations.

Table 5.1 Environmental concentrations of organic and inorganic nitrogen from above and within the canopy meadow reported as µg N L^{-1} (±SE)

	Water	NH_4^+	NOx	DON	TDN
Summer	WS	6.9 ± 4.8	2.8 ± 2.1	38.9 ± 18.5	48.8 ± 11.5
	CW	4.3 ± 1.1	2.7 ± 1.4	64.6 ± 18.8	72.3 ± 14.3
Winter	WS	5.2 ± 3.3	2.6 ± 1.7	49.0 ± 14.6	57.8 ± 5.4
	CW	3.8 ± 1.6	3.1 ± 1.3	41.5 ± 5.6	48.8 ± 3.8

5.3.2 Ammonia oxidation rates in seagrass meadows

Ammonia oxidation rates associated with *P. sinuosa* shoots were estimated during the ammonia oxidation experiment, by following the production of NO_2^- and NO_3^- detected in the control 'No ATU' over a time course of 12 hours, in comparison to the two treatments with ATU (Fig. 5.1). The amount of ATU chosen for the seagrass shoot incubation experiment was, conversely, determined by following the decrease of NO_2^- during the time course of the experiment in the samples incubated with 100 and 200 mg ATU L^{-1}, respectively. That is, the treatment resulting in no NO_2^- accumulation over time was deemed to have successfully inhibited ammonia oxidation activity. The DIN values presented in Fig. 5.1 are reported with the initial nitrogen background values subtracted to the final values.

For the control (No ATU) and both the treatments (TRT1, 100 mg ATU L^{-1}, and TRT2, 200 mg ATU L^{-1}) NH_4^+ concentration increased over the time course of the experiment (Fig. 5.1). For the control 'No ATU', NH_4^+ varied between 12 ± 1.01 µmol L^{-1} at T0 and 25.91 ± 0.63 µmol L^{-1} at T24 (Fig. 5.1), NO_2^- concentration increased from 0.27 ± 0.11 µmol L^{-1} (T0) to 4.43 ± 0.41 µmol L^{-1} (T24) (Fig. 5.1), and NO_3^- concentration increased from 0.84 ± 0.27 µmol L^{-1} at T0 to 16.71 ± 3.37 µmol L^{-1} after 24 h incubation (Fig. 5.1).

For TRT1 and TRT2, NH_4^+ concentration increased from 13.01 ± 0.86 µmol L^{-1} up to 24.87 ± 0.56 µmol L^{-1} and from 13.30 ± 3.6 µmol L^{-1} to 24.53 ± 0.77 µmol L^{-1}, respectively (Fig. 5.1). For both ATU treatments, NO_3^- concentrations increased within the first 8 h, followed by a decrease at 24 h. NO_3^- decreased from 7.70 ± 0.14 µmol L^{-1} (T8) to 7.1 ± 0.05 µmol L^{-1} (T24) and from 6.94 ± 0.12 µmol L^{-1} (T8) to 5.83 ± 0.07 µmol L^{-1} (T24), for TRT1 and TRT2, respectively. For TRT1, NO_2^- remained constant between T8 and T24, at 1.5 ± 0.7 µmole L^{-1}, while for TRT2, NO_2^- decreased from 1.14 ± 0.7 µmol L^{-1} at T8 to 0.43 ± 0.03 µmol L^{-1} at T24. After 24 h, the drop in NO_2^- concentration was significantly greater in samples incubated with 200 mg L^{-1} ATU. This experiment therefore suggested that 200 mg L^{-1} incubation was needed to inhibit ammonia oxidation.

Net nitrification rates are reported for the nitrification experiment for both the control and treatments expressed as nmol L^{-1} day^{-1} *P. sinuosa* $shoot^{-1}$.

Table 5.2 NH_4^+, NO_3^-, and NO_2^- (µM) production and net nitrification rates (± SE) (nmol L^{-1} d^{-1} $shoot^{-1}$) associated with microorganisms collected from *P. sinuosa* shoots without nitrification inhibitor (-ATU) and the two treatments (100 mg and 200 mg ATU L^{-1}).

Samples	NH_4^+ (µM)	NO_2^- (µM)	NO_3^-(µM)	Net nitrification Rates (nmol L^{-1} d^{-1}
0 mg L^{-1}ATU	25.91 ± 0.98	4.43 ± 0.41	16.71 ± 3.37	13291
100mg L^{-1}ATU	24.87 ± 0.56	1.47 ± 0.70	7.1 ± 0.05	5741
200mg L^{-1} ATU	24.53 ± 0.77	0.43 ± 0.03	5.83 ± 0.07	3556

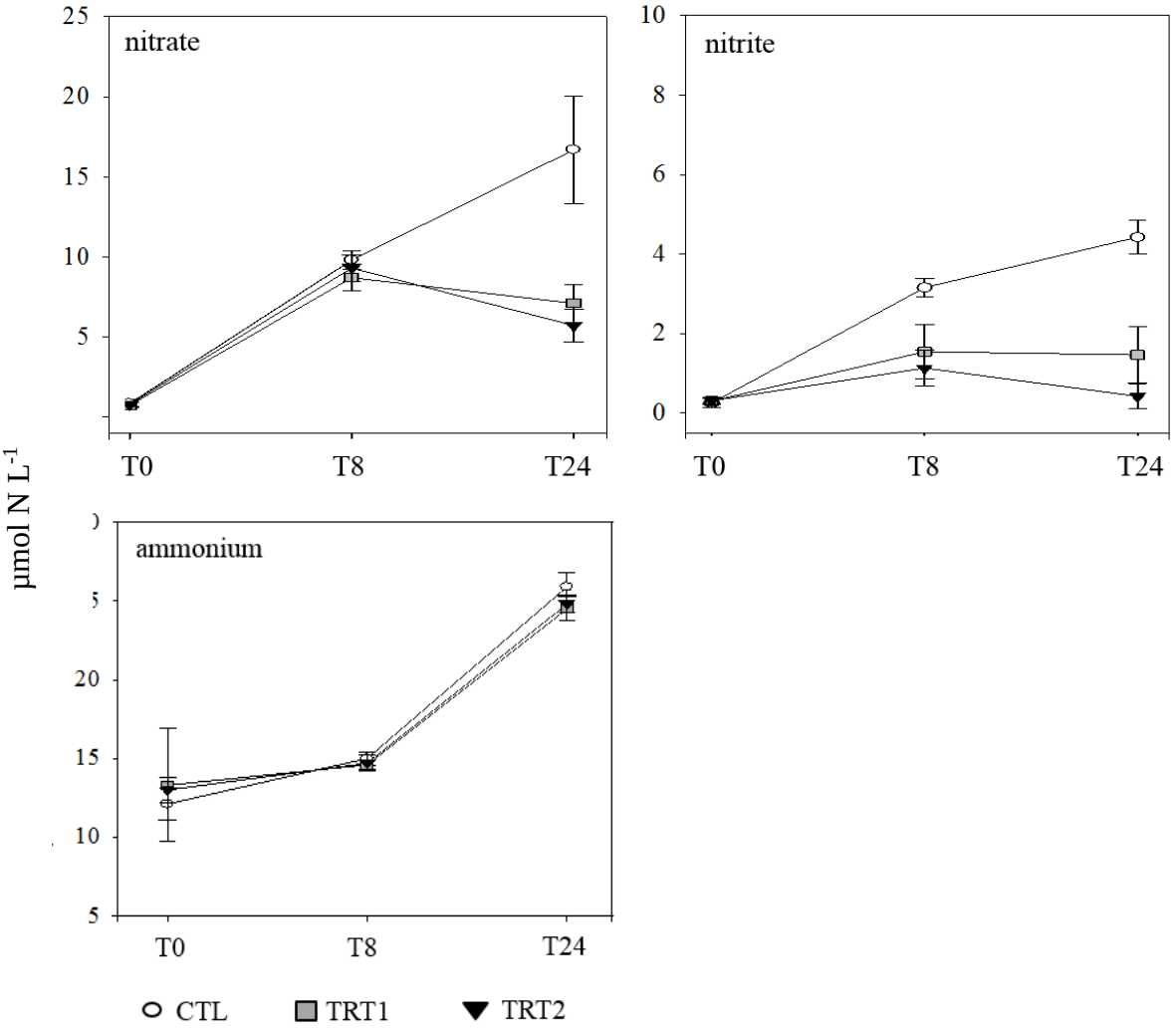


Fig. 5.1 - Change in the nutrient concentration (NH_4^+, $NO_3^=$ and $NO_2^=$) associated with *P. sinuosa* epiphytic microorganisms, in the absence of an ammonia oxidation inhibitor (CTL, open circles), and with 100 mg L^{-1} or 200 mg L^{-1} added to inhibit ammonia oxidation activity (TRT1, grey squares, and TRT2, black triangles, respectively). DIN concentrations are reported as µmole N L^{-1} $shoot^{-1}$ reported at time zero (T0), after 8 h (T8) and 12 h (T12), obtained as an average from triplicate samples, each with the background DIN values subtracted.

Subtracting the net nitrification values for TRT2 (with ATU) from the control values (without ATU) (Table 5.2), I calculated that the average ammonia oxidation rate for a single *P. sinuosa* shoot ***($N_{(shoot)}$)*** in Marmion Lagoon, was 13291 nmol L^{-1} d^{-1}. Based on an average density ***(D)*** of 55 shoots m^{-2} and the area covered by *P. sinuosa* meadows in the Marmion Marine Park (366 ha, Department of Parks and Wildlife, Australia), I calculated the total daily nitrification rate ***($TotN_{(nitr)}$)***

performed by epiphytic AOM associated with the shoots of *P. sinuosa* in Marmion Marine Park:

$$TotN_{(nitr)} = N_{(shoot)} \times D = 0.3 \text{ mol N } d^{-1}$$

5.3.3 Kelp leachate concentration and enrichment

48 h following kelp blade immersion into 1 L ASF, an average (± SE) concentration of 1674.7 ± 337.6 μg N 100 g^{-1} kelp was present in the water in the form of DON, compared to 16.8 ± 2.7 μgN 100 g^{-1} kelp as NH_4^+, and 5.33 ± 1.4 μg N 100 g^{-1} $kelp^{-1}$ as NOx. Through tangential flow filtration we were able to concentrate high molecular weight DON compounds up to 10286 ± 872.3 μgL^{-1} (about 6.2 times more concentrated) while NH_4^+ and NOx-N were below the detection limit. Kelp stable isotopic composition was evaluated both within blades and leachate for natural control and enrichment treatments. Additionally, a high level of ^{15}N-enrichment was detected for both the kelp blade and leachate, varying from 29.2 to 30.2 atm% (on average 112291.4 ± 2704.5 ^{15}N‰) and between 12 and 15 atm% (on average 37624.9 ± 652.9 ^{15}N‰) for blade and leachate, respectively (Fig 5.2; $p<0.05$, Table 5.3)

Table 5.3. *Ecklonia radiata*. Results of 2-way ANOVA testing for differences in the nitrogen stable isotope (δ^{15}N) values of different kelp type (blade and leachate) analysed in different treatments (unenriched or ^{15}N-enriched blade and ^{15}N-enriched leachate) in aquaria experiments. Data were natural log transformed prior to analysis to meet requirements of homogeneity using the Levene's test.

Factor	*df*	*MS*	*P*
Kelp Type	2	53341	<0.001
Treatment	1	37328	<0.001
Kelp Type × Treatment	1	37181	<0.001
Error	8	128	

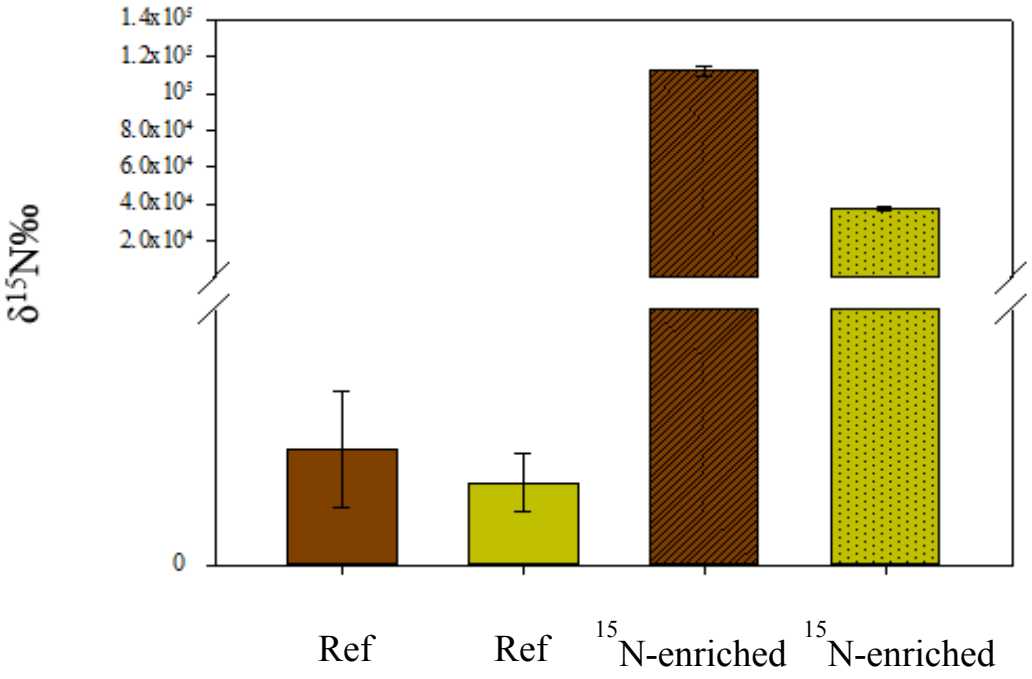


Fig. 5.2 - Kelp DO^{15}N enrichment level of *Ecklonia radiata* thallus obtained by analysing blades (dark brown) and leachate (green) by using IRMS. Enriched samples (dots fill) were compared to natural $^{15}N/^{14}N$ concentration in kelp tissue (solid fill) and values are reported as mean of three measurements with standard errors. Variations of $^{15}N/^{14}N$ ratio on the y axes are reported in delta N ($\delta^{15}N$ ‰).

5.3.3 Laboratory experiment

In the laboratory experiment, seagrass shoots and epiphytes incubated with both DO^{15}N sources (simple amino acids and complex kelp-derived DO^{15}N) showed a high ^{15}N-enrichment within their tissue compared to references (varying between 1193 and 2230 ^{15}N‰ and 124 and 318 ^{15}N‰, for seagrass enriched with algal and kelp DO^{15}N, respectively and, between 3214 and 3736^{15}N‰ and 412 and 568^{15}N‰ for epiphytic algae enriched with algal and kelp DO^{15}N, respectively). Uptake of DON derived from the two DO^{15}N sources were different, and seagrass leaves with and without ^{15}N-enriched epiphytes showed a higher ^{15}N concentration compared to the kelp ^{15}N-leachate, whether ATU was present or not. I took into consideration that

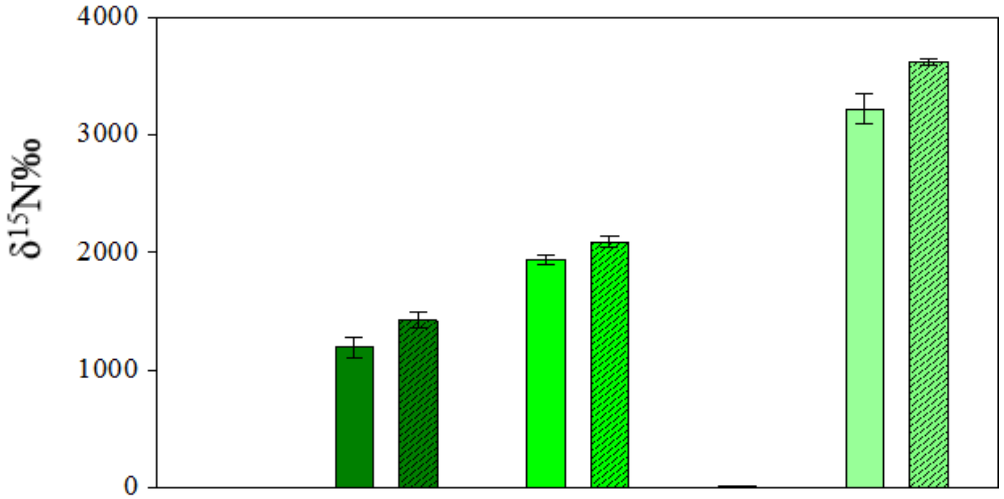
the final $DO^{15}N$ concentration of the kelp leachate added to the seagrass shoots was 7 µM (approximately 6.9 times lower than the concentration of algal amino acids); however, at the end of the incubation, uptake of algal amino acids by seagrass shoots resulted on average 8 times higher than kelp leachate. A difference related to substrate $DO^{15}N$ source was also detected between epiphytic algae, which were 8-fold more ^{15}N-enriched when incubated with algal-derived $DO^{15}N$-amino acids, compared to the kelp $DO^{15}N$ enrichment. In general, the presence of epiphytic algae negatively affected seagrass N uptake: for both treatments with and without the addition of ATU, and for both types of $DO^{15}N$ source, seagrass shoots showed a significantly higher enrichment in the treatment 'without epiphytes', compared to leaves where epiphytic algae were present ($p>0.001$). Additionally, ^{15}N enrichment into epiphytic algae was significantly higher than enrichment in seagrass shoots after every incubation, resulting in a 'plant type' effect ($p<0.001$), whereby epiphytic algae appear to be more efficient at taking up DON over the timeframe of this experiment.

For both seagrass treatments 'with epiphytes' and 'without epiphytes', differences in ^{15}N-enrichment of seagrass leaves were significant between samples incubated with and without ATU ($p<0.05$), with a greater ^{15}N-enrichment detected within seagrass tissue that had been incubated with ATU, for both DON sources (+ATU, Fig. 5.2)(1.3 and 1.4 times more enriched when incubated with amino acid and kelp $DO^{15}N$, with and without epiphytic algae, respectively). Similarly, for both of the enriched DON substrates used, epiphytic algae incubated with ATU had higher ^{15}N-enrichment compared to algae incubated without ATU. That is, or the '-ATU' treatment, epiphytic algal ^{15}N enrichment was 1.2 and 1.3 times higher when incubated with ^{15}N-amino acids and kelp ^{15}N –leachate, respectively (3214 and 3736 $^{15}N‰$ for amino acid incubation and 412 and 568 $^{15}N‰$ for kelp incubation).

Table 5.4 - Results of 2-WAY ANOVA testing for differences in the nitrogen stable isotope ($\delta^{15}N$) values of different combinations of primary producers (plant types: *Posidonia sinuosa* leaves with and without epiphytes and epiphytic algae) with and without ATU, in aquaria experiments. Data were natural log-transformed prior to analysis.

Factor	*df*	*MS*	*P*
Plant Type	2	5137.2	<0.0003
ATU	1	265.9	<0.022
Plant Type x ATU	2	19.9	<0.99
Error	12	258	

A Amino acid DO^{15}N



B Kelp leachate DO^{15}N

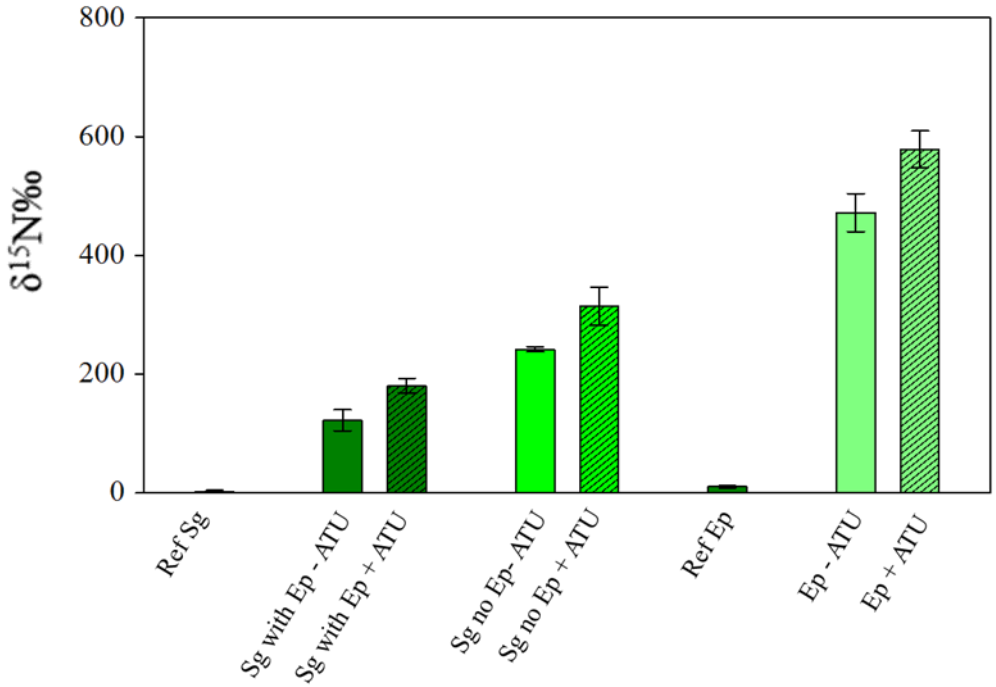


Fig. 5.3 – Seagrass shoot ^{15}N enrichment after incubation with ^{15}N-enriched (A) amino acids, and (B) kelp leachate. Reference (no enrichment) values are reported for seagrass (Ref Sg) and epiphytic algae (Ref Ep). ^{15}N enrichment is shown for seagrass shoots ('Sg') with and without epiphytes ('with Ep' and 'no Ep'), as well as with and without ATU addition ('+ATU' and '-ATU'). ^{15}N enrichment is also shown for epiphytic algae ('Ep') with and without ATU addition ('+ATU' and '-ATU'). On the y axes, ^{15}N enrichment is expressed as ^{15}N per mil. Note the differences in the scale between the graphs for the two types of DO^{15}N.

5.4 Discussion

Nitrogen is an important constituent of amino acids and proteins, including DNA (Young & Pellet, 1994). Within coastal systems, the majority of the N pool is represented by DON rather than DIN, due to both authocthnous production and allochthonous import of material from 'donor' habitats (Heck et al., 2008). In fact, marine environments are characterised by a continuous flow of organisms, nutrients, and non-living seston, which can subsidise primary production in recipient habitats (Polis et al., 1997; Suchaneket et al., 1985; Polis & Hurd, 1996; Duarte, 2002; Heck et al., 2008). The coastline of southerwestern Australia is characterised by high biomass of benthic habitats, and the common brown kelp, *Ecklonia radiata*, participates in forming the highest benthic biomass for the rocky reef communities. It has been estimated that a considerable proportion of the detached biomass produced in the region per year (1270 to 7800 t dry wt km^{-2}) would subsequently pass through seagrass meadows (Kirkman & Kendrick's 1997), resulting in a significant increase of nutrients being released into the recipient seagrass meadows. Levels of DIN can be low in this area (McGlathery et al., 2001), and the DON and DIN concentrations measured during different sampling occasions for this current study (Table 5.1) highlight the oligotrophic nature of these waters (on average 5.05 ± 1.3 NH_4^+ and 2.7 ± 0.1 NOx µg L^{-1}). However, while kelp thalli and seston may constitute a considerable amount of donor material, measurements of the DIN leached from kelp in my experiment suggests that it contributes little to the DIN pool available for uptake by *P. sinuosa*. The predominant form of N lost through leaching by the kelp in my experiment was DON (on average 98.7% of the N leachate pool). My results are therefore in accordance with the study by Hyndes et al., (2012), which showed very low NH_4^+ and undetectable NOx concentrations being released from kelp thalli during leaching. Other previous experiments have shown that a similar situation may occur, with DON being the predominant form of N released from seston (Becker et al., 2014).

Recently, short-term (≤3 h) DON uptake via both aboveground and belowground tissues has been demonstrated for seagrass species (*Zostera noltii, Cymodocea nodosa*, and *Caulerpa prolifera*) in an oligotrophic system (Van Engeland et al., 2011). In these experiments, the relative short time of incubation with enriched substrates led the authors to conclude that seagrasses could have taken up DON-derived nutrients immediately, while microbial mineralisation would have required more time to take place. However, while seagrass incubation was performed with dual-labelled (^{13}C and ^{15}N) compounds, no ^{13}C enrichment was detected within seagrass tissue. Van Engeland et al., (2011) hypothesised that the absence of clear ^{13}C enrichment was due to the sole uptake of N or that the C was lost after breakdown of the substrates in the plant. In my previous study (Chapter 4), I have shown that labile DOM can be efficiently recycled by microorganisms within 30 minutes. Since algae and seston are generally more nutritious and more easily assimilated than seagrass detritus (Klumpp & Polunin, 1989), bacterial nutrient demand during mineralisation is reduced by the presence of these allocthonous DOM sources, and external materials are quickly degraded within seagrass ecosystems (Holmer et al., 2004; Williams et al., 2009; Williams et al., 2009). Hyndes et al., (2012) hypopthesised that microorganisms associated with seagrass meadows may provide an important link between the leaching of kelp/algal-derived organic matter and seagrass N uptake; however, the ecological significance of the nutrients provided by kelp remained unknown.

Since seagrass leaves do not share with other macrophytes the capability to uptake DON directly (Chapter 4), DON mineralisation mediated by epiphytic microorganisms is essential for enhancing allochthonous N availability within seagrass meadows. In this experiment, simple algal-derived ^{15}N-labelled amino acids were 1.1 times more readily taken up by seagrass shoots (both with and without epiphytes), compared to complex kelp leachate-derived DON, while epiphytic algae were 1.2 times more enriched when incubated with algal amino acids as their source of $DO^{15}N$. However, even small amounts of complex kelp leachate could still

represent a substantial input of N for seagrass uptake, as indicated by seagrass tissue ^{15}N-enrichment in the kelp leachate treatments, compared to reference material (on average 8 times more enriched). The higher ^{15}N enrichment in epiphytic algae, compared to that observed in seagrass shoots, suggests that epiphytic algae are able to take up N more rapidly than their seagrass hosts. These results are expected, since some microalgae (e.g. diatoms) can directly access the DON pool, in contrast to seagrass leaves (see Chapter 4).

Seagrass shoots with no algal epiphytic cover, and with the addition of the ammonia oxidation inhibitor, allylthiourea (ATU), showed the highest ^{15}N enrichment compared to shoots with epiphytes, suggesting competition between seagrasses and their algal epiphytes for the uptake of DIN derived from ammonia oxidation. Thus, seagrass ^{15}N-enrichment indicated that when *P. sinuosa* shoots (with and without epiphytes) were incubated with ATU, they had a greater concentration of ^{15}N in their tissue compared to the treatments without ATU, irrespective of the DO^{15}N substrate type. Similarly, epiphytic algae were significantly, positively affected by the addition of ATU. Thus, I am able to speculate that: (i) ammonia-oxidising microorganisms compete with both the epiphytic algae and seagrass for NH_4^+ uptake, and (ii) when ammonia-oxidising microorganisms are inhibited, the competitive relationship is restricted to seagrasses and epiphytes. However, this data is presented with the caveat that, even in the absence of an algal epiphytic community, and when nitrification rates were inhibited by the addition of ATU, ^{15}N enrichment was still observed within seagrass tissues. The reason for this discrepancy could be: (i) other epiphytic microorganisms were present, that were able to transform DON to DIN sources, available for uptake by the seagrass, (ii) the concentrations of ATU used here did not entirely inhibit ammonia oxidation, so the seagrass tissue could still be enriched through DIN uptake, or (iii) a combination of both. Future work should therefore explore these mechanisms more closely.

With respect to nitrification rates, Trias et al., (2012) already hypothesised that macroalgae may represent a "hotspot" for nitrification, fueled by the continuous release of photosyntetically produced oxygen, and the epiphytic ammonia-oxidising microbial community of *P. sinuosa* may similarly benefit from the release of O_2 from seagrass leaves (Oremland & Taylor, 1976; Roberts and Moriarty, 1987; Larkum et al., 1989; Borum et., 2007). During the ammonia oxidation experiment performed here, NH_4^+ increased in all treatments, suggesting that NH_4^+ concentrations were not limiting ammonia oxidation in this study. By following the increase of nitrite in the control treatment, I was able to detect ammonia oxidation activity associated with *P. sinuosa* shoots. The net nitrification rates obtained in this study (13291 nmol N L^{-1} d^{-1} $shoot^{-1}$) are lower than those found for sponges, such as *Geodia barrette*, which support nitrification rates of 560 nmol N d^{-1} cm^{-2} sponge tissue (Radax et al., 2011). However, nitrification rates associated with seagrass shoots are much higher than the rates associated with bare sediment (18.3 nmol L^{-1} day^{-1}; Beman et al., 2012). Moreover, while the main benefit of ammonia oxidation for sponges is related to the efficient removal of the eukaryotic host's waste products, such as NH_3, the ecological significance of ammonia-oxidisers on *P. sinuosa* leaves is less clear, and AOM may even compete with the seagrass for DIN uptake under certain conditions. Indeed, seagrasses may still benefit from the presence of AOMs on their leaf surface as they may still increase DIN availability (in the form of NO_3^-) at leaf – biofilm interface.

5.5 Summary and conclusions

From the results obtained in this study, it appears clear that the epiphytic microbial transformation of allocthonous DON could play an important role in seagrass health and productivity, by increasing DIN availability to the plant. Moreover, not only simple amino acids but more complex organic N pools, such as kelp leachate, can be efficiently mineralised by seagrass leaf microorganisms, resulting in enhanced N uptake by *P. sinuosa* in the presence of the lead microbiome.

More generally, microorganisms present within 'recipient' habitats, in this case seagrass meadows, play a key role in linking the DON leached from allochthonous substrates to the regeneration of nitrogen *in situ*. The seagrass microbiome could therefore enhance total ecosystem productivity, by acting as a 'spatial subsidy', and providing regenerated nutrients for their eukaryotic host and other members of the meadow community.

Additionally, this study indicates that *P. sinuosa* meadows in South Western Australia could represent important sites for ammonia oxidation (nitrification) activity. When I performed upscaling calculations to understand the implications for epiphytic ammonia oxidation rates the entire seagrass ecosystem, I found that a high amount of the total NH_4^+ (13291 mM N d^{-1} $shoot^{-1}$) could be recycled by AOM within the *P. sinuosa* microbiome. The specific ecological significance of epiphytic AOM for the wider function of the seagrass meadow ecosystem is yet to be determined. However, my experiment suggests that the leaf microbiome of *P. sinuosa* could have important implications for the regeneration of N in the coastal waters of Western Australia.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Summary

The present thesis represents a significant advance in our understanding of the seagrass leaf-microbiome relationship and transformations of N within seagrass meadows (summarised in Fig. 6.1). Seagrass ecosystems are biologically diverse and valuable marine systems, providing a range of services including primary production, nutrient cycling, carbon burial, sediment stabilisation, and food and shelter for economically valuable biota (Thresher et al., 1992; De Lestang et al., 2006; Valentine & Duffy 2006; Barbier et al., 2011; Lavery et al., 2013; Heck et al., 2015). Nitrogen (N) is one of the most important elements required for the success of all living organisms, including seagrasses, as it is a constituent of amino acids, proteins and a variety of other organic compounds (Young & Pellet, 1994). However, despite their importance, very little is known about the N transformations within seagrass meadows and, in particular, N cycled by seagrass leaf-associated (epiphytic) microorganisms. It is widely acknowledged that the microorganisms associated with seagrass roots and leaves are a vital component of the seagrass 'holobiont': a term that incorporates all members of the microbiome, as well as the seagrass host.It has been demonstrated that microorganisms are responsible for a variety of processes within marine holobionts, including recycling of nutrients, which increase their eukaryotic host 'fitness' (health and productivity) (Steger et al., 2008; Foster et al., 2011; Amin et al., 2012; Rädecker et al., 2015). While the majority of studies have investigated the diversity and role of rhizosphere microorganisms (Jørgensen, 1982; Shieh & Yang 1997; Krutz et al., 2003; Jensen et al., 2007), little is known about the ecological significance of microorganisms in leaf biofilms and interactions between seagrass leaf microorganisms and their host plants.

My research significantly supports the seagrass holobiont concept, by showing a marked difference between the *P. sinuosa* microbial assemblage and the environmental one (Chapter 3), which agrees with the hypothesis that specific plant-microbial interactions occur during the establishment of the epiphytic community (Loucks et al., 2013; Costa et al., 2015; Zidorn, 2016). Microbial communities

associated with *P. sinuosa* meadows include microorganisms involved in different steps of N cycling (e.g. mineralisation of DON and nitrification; Fig. 6.1) that may influence the rate and efficiency of N transformations within seagrass meadows and have profound effects on plant fitness (Chapter 3, Chapter 4, Chapter 5). In particular, my research shows that seagrass epiphytic microorganisms are intimately involved in the functioning of marine plants, by enhancing canopy inorganic N uptake through DON mineralisation (Chapters 4 and 5), thus supporting seagrass growth and productivity. Moreover, my work clearly points to the importance of considering the epiphytic algal community as part of the microbiome (Chapters 4 and 5), to interpret some of the interactions that occur on the canopy leaf surface. Indeed, I have demonstrated that, while some microorganisms mediate seagrass N uptake by mineralising DON, seagrass and ammonia-oxidising microorganisms could compete for ammonium uptake (Chapter 5). However, I speculated that the strong competition that AOMs exert on epiphytic algal N uptake may, in turn, control algal growth and favour seagrasses in times of nutrient surplus, similar to the coral holobiont (Yellowlees, et al., 2008; Rädecker et al., 2015; Fig. 6.1).

Chapter 4 provides new insights into the use of imagining techniques, such the NanoSIMS, for the study of N flow among different members of the microbiome community, and may be used to resolve questions regarding seagrass organic against inorganic nutrient uptake. My results represent an important baseline for further research aimed at clarifying the interaction between seagrass leaves, microorganisms involved in N cycling, and other members of the seagrass microbiome, such as epiphytic algae. Moreover, my results are not only relevant for seagrasses as hosts, but other macrophytes in aquatic systems that may benefit from the presence of specific N-cycling microorganisms, providing an important starting point for future work in this important research area.

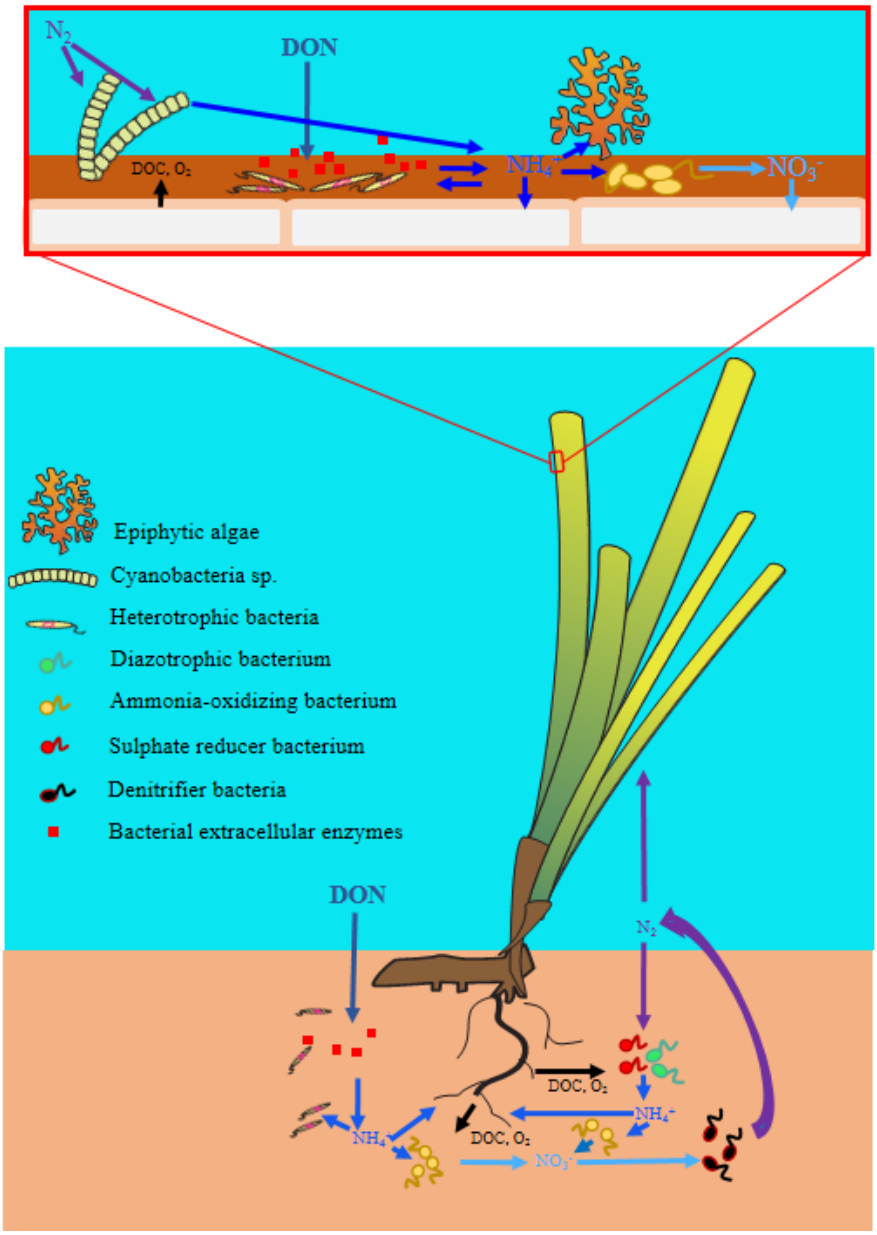


Fig. 6.1 - Key N transformations within the seagrass microbiome. On the leaf: epiphytic growth of algae, N-fixing and heterotrophic microorganisms, resulting in increased concentrations of dissolved inorganic nitrogen (DIN) at the leaf-biofilm interface; N-fixing cyanobacteria facilitating N uptake through the leaves from atmospheric N; heterotrophic microorganisms enhancing DIN availability from dissolved organic nitrogen (DON) through extracellular enzymes; heterotrophic and ammonia-oxidising microorganisms (AOM), epiphytic algae and even seagrass may compete for NH_4^+ uptake, however, the production of nitrate (NO_3^-) by AOM enables DIN uptake by seagrass leaves. Within the sediment: heterotrophic microorganisms recycle DON by producing NH_4^+, which can be utilised by seagrass, AOM or other members of the rhizosphere microbiome. Since nitrification is an oxygenic-dependent process, seagrass regulation of O_2 exuded from the roots could represent a mechanism of biocontrol over AOM activity. Nitrification can be coupled with denitrification across the oxic-anoxic interface surrounding the root-rhizosphere, and at the sediment surface. O_2 and dissolved organic carbon (DOC) released from the above- and belowground plant tissue drive microbial processes, including sulphur cycling and N fixation in the rhizosphere; thus providing a further mechanism for the recycle of N in the rhizosphere in the rhizosphere.

6.2 Discussion

Very few studies have described the abundance and diversity of microorganisms attached to the surface of aquatic macrophytes, however, those studies indicate that seagrasses host a high number of microorganisms on both above- and belowground tissue (Peduzzi & Herndl, 1994; Moriarty et al., 1985; Kirchman et al., 1984), and that microorganisms associated with the surface of macrophytes are different from the microbes present in the surrounding environments (e.g. sediments and water column) (Weidner et al., 2000; Krutz et al., 2003; Egan et al., 2012). Table 6.1 provides published data on the abundances of bacteria and archaea associated with seagrasses and macrophytes, including *P. sinuosa* leaves from this study.

Table 6.1 Microbial 16S rRNA or total cell abundances associated with seagrasses and macroalgae. Current study in bold; 1 Novak, 1984; 2 Peduzzi & Herndl, 1994; 3 Moriarty et al., 1985; 4 Kirchman et al., 1984; 5 Trias et al., 2012; 6 Mazure & Field, 1980; - no data available.

Host organism	Species	Bacteria	Archaea	Technique
Seagrass	***Posidonia sinuosa***	**10**	**10**	**16S rRNA**
	Posidonia oceanica[1]	6	-	Cell counts
	Cymodocea nodosa[2]	7	-	Cell counts
	Zostera capricrorni[3]	8	-	Cell counts
	Zostera marina[4]	7-8	-	Cell counts
Macroalga	*Phyllophora crispa*[5]	9-10	6	16S rRNA
	Osmundaria volubilis[5]	8-9.5	5-7	16S rRNA
	Laminaria rodriguezii[5]	9-10	5-7	16S rRNA
	Laminaria pallida[6]	3-7	-	Plate counts
	Ecklonia maximata[6]	3-7	-	Plate counts

The inconsistency in techniques used in previous studies (Table 6.1) does not allow for a direct comparison among all different habitats or host species; however, important information can be deduced. Firstly, seagrass leaves represent a hotspot

for microbial abundance (Chapter 3), and can support greater abundances than those found on macroalgal surfaces (Trias et al., 2012). Secondly, there is a paucity of information regarding archaeal abundance and diversity. Archaea are microorganisms with peculiar features, including the ability to grow at extremely high temperatures and to produce methane as a metabolic product; a trait that is exclusive to this domain). Archaea have been largely overlooked in previous decades and only relatively recently their potential involvement in nutrient transformations and marine biogeochemical cycles has been recognised. Evidence that archaea, as well as bacteria, are important players in the N cycle are provided by a number of studies, and seems that archaea are involved in every step of N cycling, except for nitrite oxidation (Offre et al., 2013). Archaeal diazotrophs (class Methanobacteria, Methanococci and Methanomicrobia) have been isolated from a variety of environments (Leight, 2000; Cabello et al., 2004; Offre et al., 2013), and cultivation experiments have shown that methanotrophic diazotrophs not only fix N but share it with bacterial partners in anaerobic consortia (Dekas et al., 2009). Also, archaea capable of nitrification (belonging to the Thaumarchaeota) (Könneke et al., 2005; Treusch et al., 2005; Stahl et al, 2012) and denitrification (Völkl et al., 1993) have been described, and appear to be a key component of the N-cycling epiphyte microbiome (Chapter 3).

6.2.1 Diversity of microorganisms associated with Posidonia sinuosa and functional genes

Microbial diversity associated with seagrasses, and macrophytes in general, seems to be influenced by the release of nutrients, oxygen and antibiotics from the plant surface (Zapata & McMillan, 1979; Engel et al., 2006; Kannan et al., 2010). Crump and Koch (2008) and Weidner et al., (2000) showed that different seagrass species host different bacterial communities, and associations with Gamma-, Beta- and Alphaproteobacteria are common on temperate seagrass leaves. A similar association between epiphytic bacteria and the seagrass host was found and

presented in this study for the *P. sinuosa* microbiome, characterised by a high abundance of Proteobacteria, with the only exception being Deltaproteobacteria, which was found exclusively in the sediment, and the presence of Verrucomobia and Firmicutes, which were bothabsent from the water column (Chapter 3). Futhermore, Chapter 3 showed that Euryarchaeota and Thaumarchaeota dominated the archaeal community of the *P. sinuosa* microbiome, and that the leaf-associated archaeal community differed from that in the surrounding environment; highlighting the close association between the seagrass and the archaeal component of its microbiome. Given the absence of information regarding the archaeal community associated with seagrasses, it is difficult to compare the present results with a similar habitat, but my results provide a reference point for future studies.

In addition to the information on the total microbial community structure obtained through 16S rRNA sequencing, the study of functional genes assists in providing a better understanding of the functional role of microbiomes in marine systems. By studying functional genes (e.g. ammonia-oxidising genes, *amoA*), we can obtain information on the diversity and environmental drivers of microorganisms, which could be missed when studying at bacterial species diversity or abundance alone.

Within the marine environment, the main processes of N regeneration are N fixation and mineralisation, the latter being linked to ammonia oxidation (Carpenter & Capone, 2016). Ammonia-oxidising microorganisms (AOM) have been found associated with a variety of marine habitats, including sediment (Ando et al., 2009; Matsutani et al., 2011), water column (Santoro & Casciotti., 2011; Qin et al., 2014), the surface of different substrates (e.g. macroalgae; Trias et al., 2012), and the tissues of different animals (i.e. sponge and corals; Mohamed et al., 2010; Siboni et al., 2012; Yang et al., 2013; Feng et al., 2006). Data on the bacterial (AOB) and archaeal (AOA) *amoA* abundances, collected from a variety of representative marine habitats (e.g. unvegetated and vegetated sediments, and macroalgal and animal surfaces) (Table 6.2), show that the AOM abundances observed in the current study, within the

sediment and water column of seagrass meadows, fall within the range of other aquatic habitats, including mangrove and salt marsh sediments. However, the seagrass biofilm from my study represents a hotspot for microorganisms capable of nitrification, with AOM abundance on *P. sinuosa* leaf surface being orders of magnitude higher in comparison to surrounding environments, and even greater than those found on, for example, macroalgal surfaces (Trias et al., 2012) (Table 6.2). Moreover, seagrasses, and marine autotrophs in general, present an opposite pattern of AOM abundances than those observed for marine animals (Radax et al., 2011), with AOB outnumbering AOA on their surface (Chapter 3). Indeed, abundances of AOA on the seagrass leaf surface are between 2 and 4 orders of magnitude less abundant than within marine sponges, whereas abundances of AOB on seagrass leaves are between 4 and 8 orders of magnitude higher than AOB abundances in marine sponges (Table 6.2; Radax et al., 2011). The reasons for this inverted trend could be explained by the differences of physico-chemical parameters encountered within animal tissue (fluctuating O_2 levels, temperature and NH_4^+ concentration; Hoffmann et al., 2009) in comparison to the surface of marine macrophytes, which are characterised by the release of photosynthetic O_2 (Larkum et al., 2006; Trias et al., 2012).

Table 6.2 Ammonia-oxidising gene abundances in microbiomes from different habitats and organisms. Current study in bold; 1. Mosier et al., 2008; 2. Beman et al., 2012; 3. Cao et al., 2011; 4. Li et al., 2010; 5. Moin et al., 2009; 6. Radax et al., 2011; 7. Trias et al., 2012.

Habitat		AOA Log (g^{-1}/ml^{-1})	AOB Log (g^{-1}/ml^{-1})	Location
Water column	**Seagrass *P. sinuosa***	**2**	**4**	**Temperate, Western Australia**
Sediment	Coastal[1]	6.5	5.5	Gulf of Mexico
	Coastal[2]	5-7	4-8	California
	Offshore[1]	6	3	Gulf of Mexico
	Continental shelf[3]	5-6	4-6	China Sea
	Mangrove[4]	6-7	6-8	Hong Kong
	Salt marsh[5]	6-9	3-8	Barn Island, Connecticut
	Seagrass *P. sinuosa*	**7**	**7**	**Temperate, Western Australia**
Sponge	*Geodia barretta*[6]	11	<0	Cold water
	Phakelia ventilabrum[6]	10	5	Cold water
Macroalgal thallus surface	*Phyllophora crispa*[7]	5.5	7.5	Mediterranean Sea
	Osmundaria vulibis[7]	7	8	Mediterranean Sea
	Laminaria rodriguezii[7]	6	8	Mediterranean Sea
Leaf surface	**Seagrass *P. sinuosa***	**7.5**	**8.5**	**Temperate, Western Australia**

Despite differences in the relative abundance of the AOM members, there is some similarity between AOA and AOB community composition found on *P. sinuosa* leaves in this current study (Chapter 3), and the OTUs identified in sponges (Mohamed et al., 2009) and macroalgae (Trias et al., 2012), pointing to the possibility that specific AOM taxa may be adapted to live in association with eukaryotic hosts. AOM sequences retrieved from the sediment and water column in my study (Chapter 3) were also related to OTUs identified in previous studies

performed in the Western Pacific (Cao et al., 2011), California (Francis et al., 2003; Beman & Francis, 2006), South and East China (Cao et al., 2011; He et al., 2016), and sediment from an eelgrass meadow along the coast of Japan (Zhang et al., 2014). However, the high proportion of unknown AOM sequences in my study highlights the fact that seagrass meadows may represent a habitat for uncharacterised populations of ammonia-oxidising microorganisms.

Interestingly, part of the AOM community may also be able of using urea as a source of N through the urease enzyme, thus directly linking N mineralisation to ammonia-oxidation (Alonso-Sáez et al., 2012; Lu et al., 2012). Urea is an important component of the marine organic N pool, and is rapidly turned over within coastal waters (e.g. for Chesapeake Bay, McCarthy et al., 1977; Lomas et al., 2002). Until relatively recently, the pathway for urea hydrolysis was thought to be mediated by two microorganisms: one organism capable of hydrolysing urea (identified using the *ureC* gene) through the production of CO_2 and NH_4^+, whereby the resulting NH_4^+ becomes available for a different chemolithotrophic bacterium or archaeum to perform ammonia oxidation (identified by the *amoA* gene). The possibility that some microorganisms are able to perform both reactions represents a short cut in the recycling of N, and a link between organic matter breakdown, DON consumption and DIN transofmrations (Alonso-Sáez et al., 2012). NH_4^+ released from urea hydrolysis can be incorporated into microbial biomass (Koper et al., 2004), and/or used for the reaction of ammonia-oxidation, with a subsequent release of NO_2^-; thus increasing DIN concentrations on the seagrass leaf surface (Fig. 6.2).

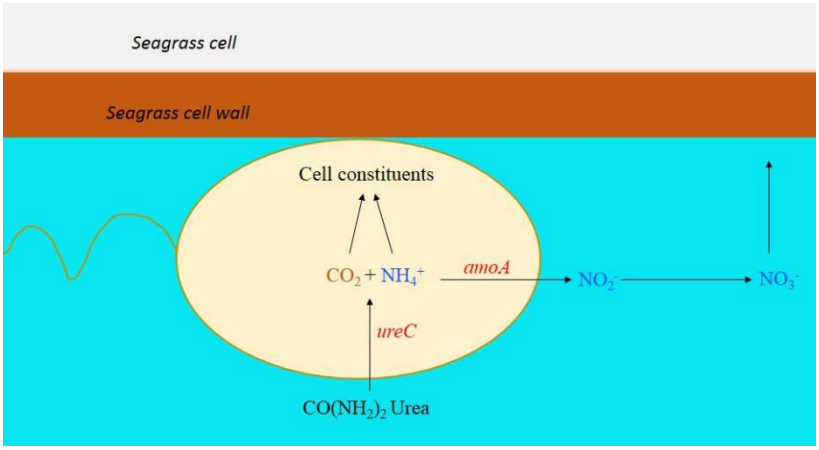


Fig. 6.2 - Hydrolysis of urea mediated by a seagrass epiphytic ammonia-oxidising microorganism. Urea is hydrolysed by the urease enzyme (gene *ureC*) into CO_2, which is fixed by the microbial cell, and NH_4^+, which can be further incorporated into cell constituents, or oxidised into NO_2^- by the ammonia monoxygenase enzyme (gene *amoA*). NH_4^+ and/or NO_2^- released from the microbial cell enhances DIN concentration at the seagrass leaf-biofilm interface.

Alonso-Sáez et al., (2012) hypothesised that AOA were able to shortener the recycle of N by hydrolysing urea and performing nitrification directly within their own cells. They found both of the relevant genes (*ureC* and *amoA*) associated with archaeal populations in the Arctic Sea. Interestingly, while I found a high abundance of ureolytic AOB in all the seagrass meadow microenvironments (Chapter 3), I was unable to detect archaeal *ureC*. However, since most of the AOA sequences in my study, especially the ones retrieved from the water column, did not match with known organisms, there is a strong possibility that a divergent or novel population of AOA taxa associated with seagrasses of Western Australia exists. Thus, different primer pairs for the detection of the *ureC* gene of Western Australian AOA population should be designed for future studies.

6.2.2 The importance of seagrass leaf microbiome

Seagrasses are extraordinarily productive, yet they occur in nutrient poor and nitrogen-limited environments (Kenworthy & Fonseca, 1992; Burkholder et al., 1994; Agawin et al., 1996; Gobert et al., 2002). The results presented in my thesis

show, for the first time, that microorganisms in the biofilm of seagrasses directly facilitate the uptake of organic nitrogen – a key nutrient for these highly valuable, and yet highly threatened, marine plants (Chapters 4 and 5). Here, I showed that leaf-associated microorganisms fulfil a nutrient-supply role for seagrasses, by processing DON into inorganic forms (NH_4^+, NO_2^-, NO_3^-) more readily available for uptake by the plant (Fig. 6.3). My research significantly changes our understanding of the mechanisms of seagrass N use, and makes a significant contribution to ourknowledge of the functional role of the seagrass epiphytic microbiome. Seagrasses preferentially take up DIN (Izumi & Hattori, 1982; Short & McRoy, 1984; Pedersen & Borum, 1992, 1993; Lee & Dunton, 1999), which accounts for less than half of the N pool in coastal systems (Voss et al., 2013). Recent work has suggested that seagrasses are also able to use the DON pool, primarily via uptake through their roots (Vonk et al., 2008; Van Engeland et al., 2011; Alexandre et al., 2015). However, my findings indicate that it is the leaf-associated microorganisms that provide the mechanism for this uptake through seagrass leaves, thus highlighting the important role of the seagrass epiphytic microbiome in supporting plant health and productivity.

The prevailing paradigm of seagrass-microbiome interactions focuses on the role of epiphytic and rhizome-associated N-fixing bacteria (Welsh, 2000; Pereg-Gerk et al., 2002; Cole & McGlathery, 2012; Agawin et al., 2016), which contribute to the exchange of N between the bacteria and the plant. Further to this, work clearly shows that seagrass leaves also host microbial consortia that transform DON into DIN, thereby increasing the pool of N readily available for uptake by the plant. Indeed, even the presence of a small number of microorganisms capable of DON mineralisation is able to significantly increase seagrass N uptake (Chapter 4).

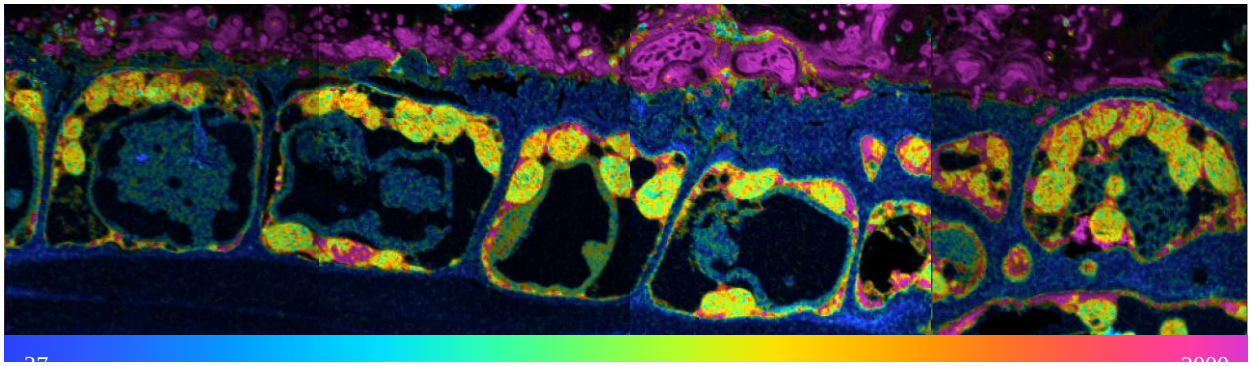


Fig. 6.3 – Chain image of seagrass cells after 12h form the DO^{15}N spike for the treatment 'with microorganisms '(Chapter 4). The epiphytic community (purple) is highly enriched; however, seagrass chloroplasts and cytosol also show a high level of ^{15}N-enrichment.

NanoSIMS image analysis of the seagrass cell components suggested that seagrass leaves were taking up NO_3^-, rather than NH_4^+, following microbial DON mineralisation on the leaf surface (Chapter 4). In fact, by following the path of ^{15}N-enrichment within different seagrass sub-cellular microenvironments (cell wall, cytosol, vacuole and chloroplasts) over 12 hours (Fig. 6.3), I was able to propose that N in the form of NH_4^+ is directly transported into seagrass chloroplasts, where it is used to produce amino acids, while NO_3^- is either stored within the vacuole or reduced to NO_2^- within the cytosol, prior to entering the chloroplast. Thus, microbial transformations represent a vital pathway by which the seagrass can increase the efficiency of its N uptake, by preferentially taking up the NO_3^- produced by nitrification (Fig. 6.4).

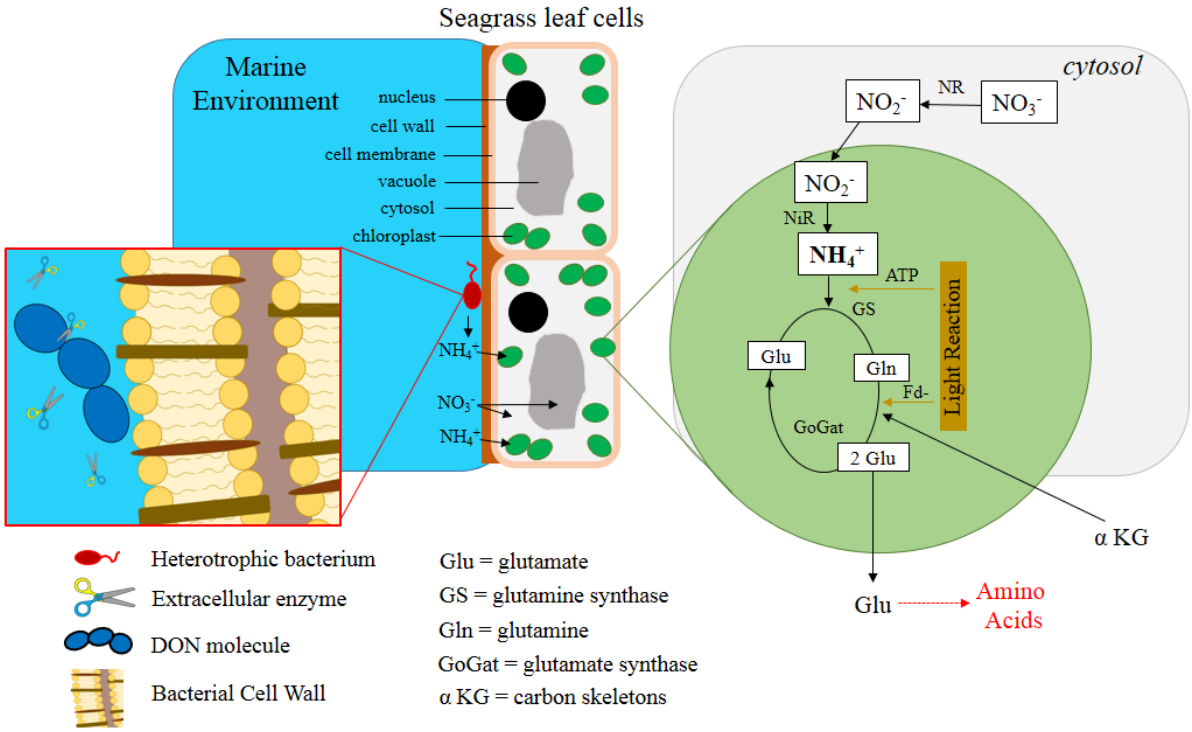


Fig. 6.4 - Dynamics of N cycling and seagrass N uptake. On the left, a heterotrophic bacterium on the seagrass leaf surface is mineralising DON into DIN through the use of extracellular enzymes. Additionally, AOM also transform NH_4^+ into DIN forms that are readily taken up by the plant (NO_2^- and NO_3^-). On the right, the path of N uptake within seagrass cells. NH_4^+ is directly transported to the chloroplasts, where it enters the GS/GOGAT cycle whereby glutamate is aminated by the enzyme glutamine synthetase (GS) to synthesise glutamine. The addition of carbon skeletons (a-ketoglutarate) allows transamination by glutamate synthase (GOGAT) to produce two glutamate molecules. One glutamate can be used to start the cycle, the other one is used to build amino acids. Once NO_3^- enters the cell, it can be stored within the vacuole or reduced to NO_2^- within the cytosol by the enzyme nitrate reductase (NR). NO_2^- can enter the chloroplast, where is further reduced to NH_4^+ by the enzyme nitrite reductase (Nir) (Touchette & Burkholder, 2000).

6.2.3 Complex relationship between seagrass leaves and epiphtyc microbiome

Further indirect evidence for the uptake of NO_3^-, rather than NH_4^+, by seagrass leaves is provided by my research using an ammonia oxidation inhibitor, allylthiourea (ATU), to investigate how AOM facilitate or compete with the plant's N uptake (Chapter 5). ATU addition resulted in greater ^{15}N enrichment of both

seagrass leaves and their epiphytic algae, showing that a proportion of DON (here provided as either algae-derived or kelp-derived DON) is consumed by ammonia-oxidation on the leaf surface. Since AOM use NH_4^+ as a substrate to fuel the first step reaction in the nitrification pathway, my results suggest the possibility that competition between seagrass leaves, seagrass epiphytes, and their associated AOM could occur on the seagrass leaf surface. However, if we consider the high AOM abundance found associated with the *P. sinuosa* biofilm (Chapter 3), and the capability of seagrasses to influence the physico-chemical environment on their leaf surface to influence the epiphytic microbial community, this hypothesis appears controversial. Nonetheless, my work has also shown that also epiphytic algae can also compete with its seagrass host for DON uptake (Chapter 5). Although some species of epiphytic algae may increase DIN availability at the seagrass-biofilm interface by mineralising DON (Tuchman et al., 2006; Cochlan et al., 2008; Wawrik et al., 2009), excessive algal growth may also become detrimental to the plant (Cambridge et al., 1986). For example, the dominant group of epiphytic algae on the leaves of the seagrasses *Zostera marina* and *Halodule wrightii* was diatoms (Pinckney & Micheli; 1998), which may benefit seagrasses since molecular evidence suggests that diatoms can mineralise DON into DIN (Armbrust et al., 2004). It has also been shown that, in oligotrophic waters, such as those of Western Australia, epiphytic algae can play an important role in and nutrient cycling and ecosystem functioning by contributing to the capture of scarce N resources from the water column (McGlathery, 2001). However, several studies have documented a detrimental effect of excessive epiphytic algal growth on seagrass viability, particularly in areas with increasing nutrient inputs to coastal waters (Silberstein et al., 1986, Hauxwell et al., 2001, McGlathery, 2001). Related to this, I postulated that competition for NH_4^+ uptake between epiphytes and their seagrass host may be used by seagrass leaves as a form of‘biocontrol’ over the growth of excess algae. A similar mechanism has been proven to exist for the coral holobiont, where control of N availability by coral-associated AOM may stabilise the functioning of the holobiont under eutrophic conditions and control the proliferation of fast-growing

Symbiodinium (Yellowlees, et al., 2008; Rädecker et al., 2015). Clearly, further studies are needed to fully characterise the relationship between seagrasses, AOM and epiphytic algae, and therefore to confirm this intriguing hypothesis.

My research has shown that seagrasses in N-limited areas are strongly supported by their associated microbiome. Even a small amount of complex DON molecules (such as the kelp-leachate DON used in Chapter 5) may be taken up by seagrasses follow microbial DON mineralisation (Fig. 6.5). However, regardless of the DON source, I have shown that the role of the seagrass leaf microbiome in sustaining host fitness may be even more vital than previously thought, through the provision of a mechanism for DIN uptake following DON mineralisation. Thus leached DON of either allochthonous or autochthonous origin is transformed to DIN by both heterotrophic and chemotrophic (ammonia-oxidising) epiphytic microorganisms, allowing increased plant N uptake (Fig. 6.5).

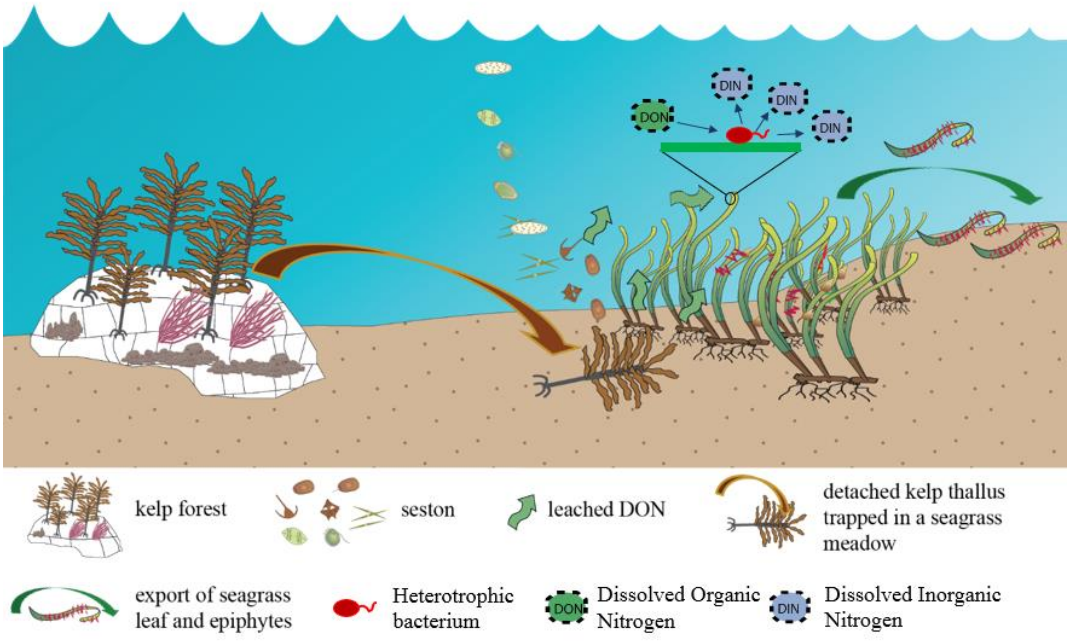


Fig. 6.5 - Conceptual diagram showing the import of autochthonous nutrient sources from 'donor' habitats, to seagrass meadows in southwestern Australia, and the role that the seagrass epiphytic microbiome plays in transforming DON and DIN, leading to enhanced DIN uptake by the host plant.

6.3 Future research directions and methodological developments in the study of the seagrass microbiome

While this work adds to the growing evidence for the importance of marine eukaryotic microbiomes, especially in supporting vital ecosystem services, much remains to be learned regarding the seagrass microbiome and its interactions with the plant host. Specifically, most studies of N transformations carried out by the seagrass epiphytic microbiome have focused on N-fixation performed by cyanobacteria (Welsh, 2000). However, my thesis highlights the presence of complex N cycling in seagrass meadows, performed by leaf-associated microorganisms that transform DON to forms of DIN that are more readily available for uptake by the seagrass leaf.

An important outcome of my research is the demonstration that the use of specific, innovative techniques, such as NanoSIMS, could help to elucidate specific plant-microbe interactions and the flow of nutrients between different members of the holobiont. NanoSIMS has been used previously to show bacterial N-fixation and NH_4^+ uptake by *Symbiodinium* (Pernice et al., 2012; Ceh et al., 2013; Pernice et al., 2014). Other recent studies (e.g. Lema et al., 2016) indicate that the combination of two imagining techniques, combining a taxon-specific probe (e.g. using fluorescence in-situ hybridisation (FISH) with an elemental tracing technique (e.g. NanoSIMS), would represent a more powerful tool for the identification of the functional role of specific microorganisms, their 'position' within the biofilm, and their interaction with other members of the seagrass holobiont.

The phylogenetic identification of microbial functional groups (Chapter 3) does not always imply their activity within the environment, and this activity may be best targeted in future using metatranscriptomic studies. In the last few years, metatranscriptomics has become a valuable tool for identifying active members of a microbial community and studying their functional response of microorganisms under changing environmental conditions (Poretsky et al., 2005; Bailly et al., 2007;

Frias- Lopez et al., 2008; Gilbert et al., 2008; Shi et al., 2009; Stewart et al., 2010). Combining ‘omics’ techniques, such as metagenomics and metatranscriptomics, with the more targeted functional approaches discussed above, will enable the rapid advancement of the new and exciting field of marine microbiome research.

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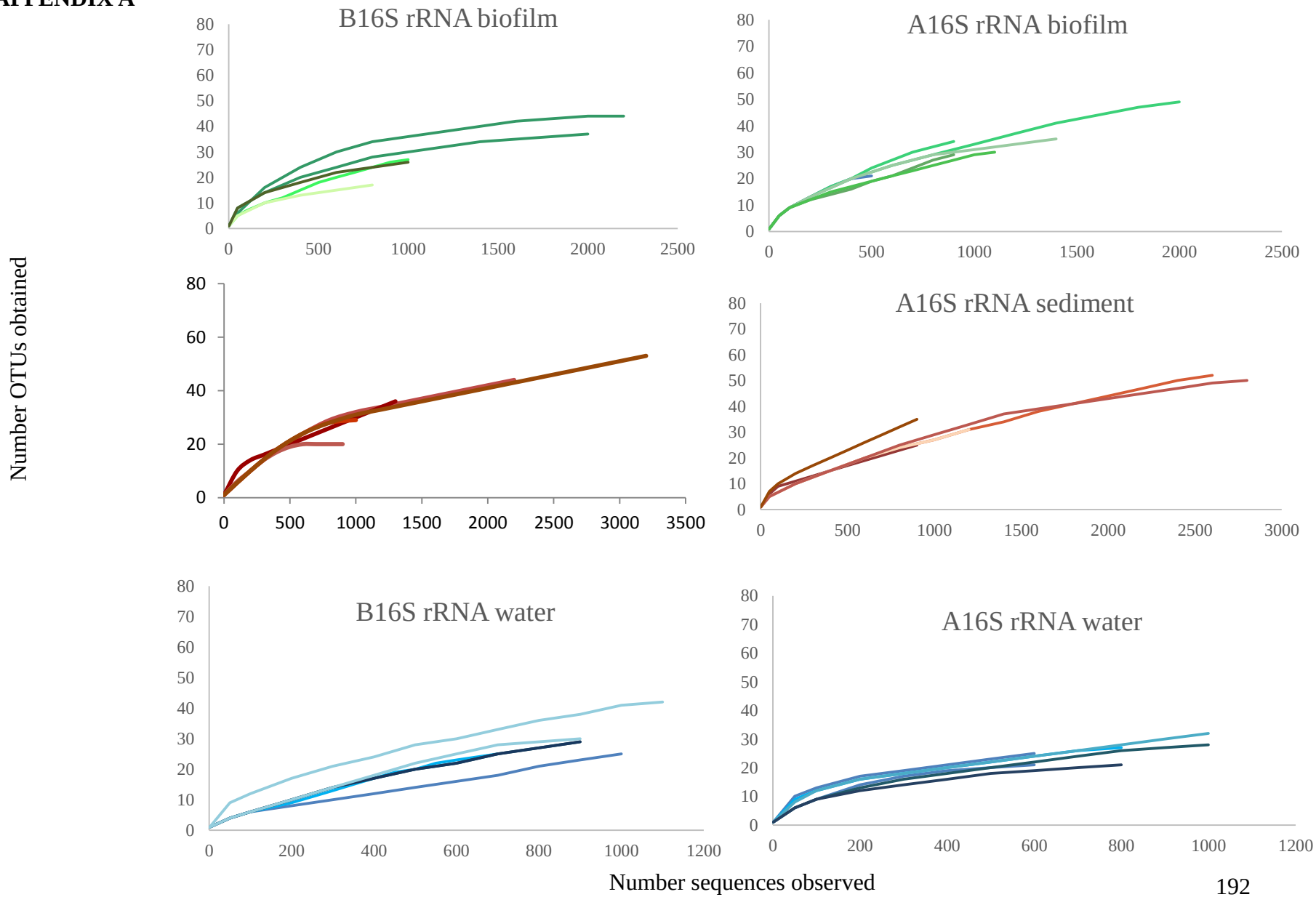
8 APPENDIX A


B16S rRNA biofilm
A16S rRNA biofilm
A16S rRNA sediment
B16S rRNA water
A16S rRNA water
Number OTUs obtained
Number sequences observed

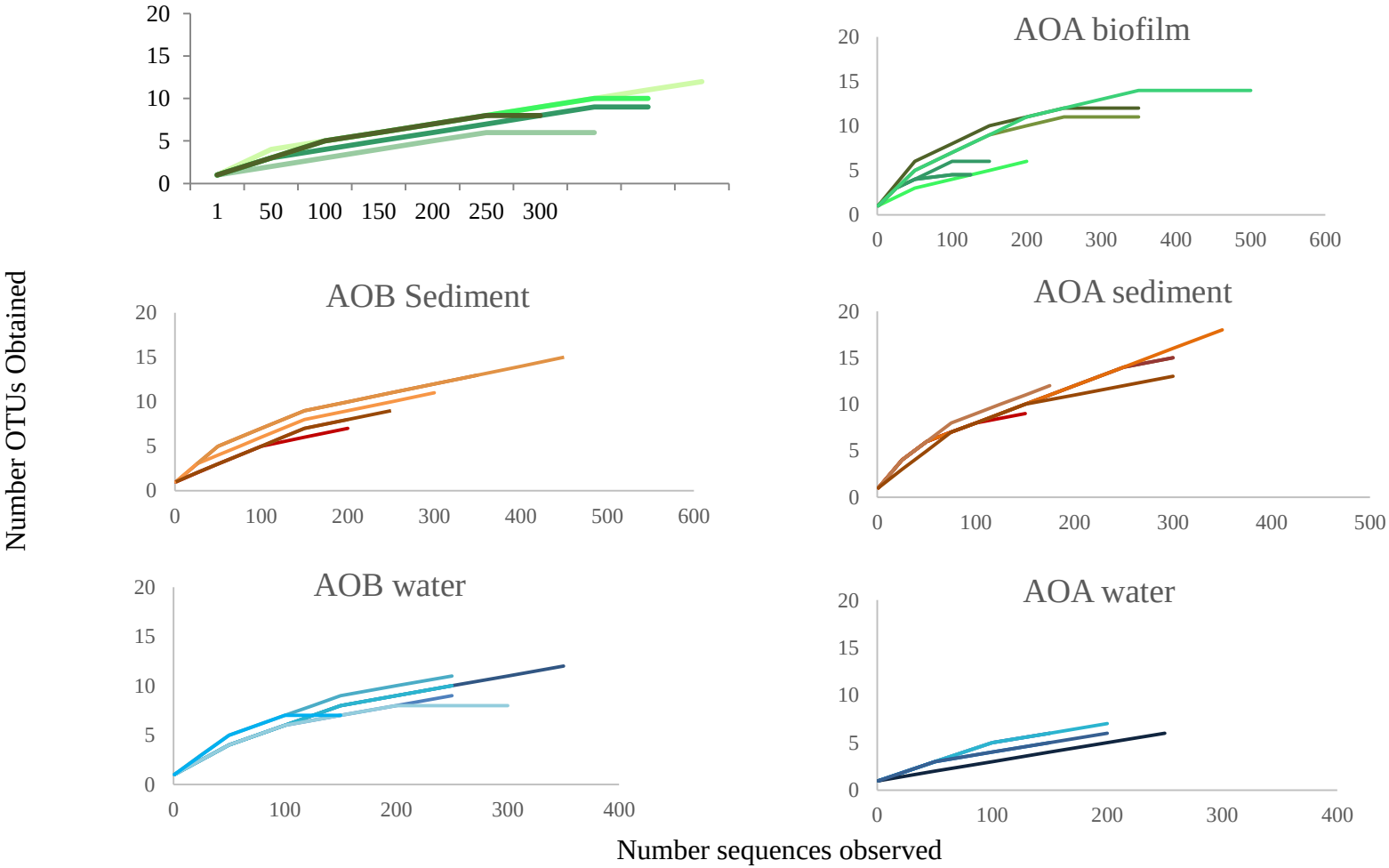


Fig. A1 - Rarefaction curves obtained from Illumina paired end sequencing of bacterial and archaeal 16S rRNA and *amoA* genes. Curves are reported for each gene (B16S rRNA, A16S rRNA, AOB and AOA), microenvironment, meadow and time point analysed.

9 Appendix B

LIVE/DEAD® BacLight™ and antibiotic treatment

The LIVE/DEAD® BacLight™ kit (ThermoFisher) is used to infer cell viability of microorganisms cultured in laboratories. The LIVE/DEAD® BacLight™ viability kit utilises mixtures of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. In order to discriminate (i) whether for the treatment 'seagrass without microorganisms' the chosen antibiotic mixture targeted epiphytic microorganisms, and (ii) whether it was possible to use the LIVE/DEAD® BacLight™ kit to reveal the efficacy of antibiotic incubation, I tested for: (i) LIVE/DEAD® BacLight™ kit reliability on *Escherichia coli* colonies, (ii) reliability of the kit on environmental seagrass biofilm, and (iii) efficacy of the scraping, together with antibiotic incubation, to eliminate epiphytic algae and microorganisms. Thus, during the ^{15}N-experiment, I applied this technique (iv) on samples of seagrass leaves for the treatment 'without microorganisms' collected at every incubation time.

(i) In order to test the reliability of the LIVE/DEAD® BacLight™, 100 µl of *E. coli* cells (JM109; Promega), $>10^8$ cfu/µg, were incubated for 2 hours in 1.9 ml of pre-warmed (37 ºC) SOC medium [2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$, and 20mM glucose] (ThermoFisher) with shaking at 250 rpm (Hanahan, 1983). At the end of the incubation, 200 µl of the medium containing cells was plated on agar plates containing Luria Bertani broth [15g agar, 10g tryptone, 5g yeast extract, and 5g $NaClL^{-1}$] (Sigma Aldrich) and left overnight at 37 ^oC. Following incubation, small samples (approximately 1 cm^2) of the medium presenting *E. coli* colonies on the surface were cut using a sterile scalpel, and placed within 8-well chambers (Nunc Lab-Tek USA Scientific, USA). 200 µL of 70% ethylic alcohol were added within 4 of the 8 chambers in order to target *E. coli* cells, and the remaining 4 samples were left with live microbial cells. 2

hours after the incubation, a series of washing steps and kit incubation were performed. Ethylic alcohol was removed by gentle pipetting, and all the wells were washed twice with 0.1 M phosphate-buffered saline solution (PBS). 200 µL of LIVE/DEAD® BacLight™ reagents, mixed according to the manufacturer's instructions, was added to each well, and kept in the dark at room temperature for 15 minutes. After incubation, sections were rinsed twice with PBS to remove the excess reagents, and fixed in 200 µL of 10% natural buffered formalin for 45 minutes at room temperature in the dark. Sections were then rinsed twice with 0.1 M PBS, walls were removed from the slide and a coverslip was applied (Jurcisek et al, 2011). Bacterial cells were then observed using confocal scanning laser microscopic imaging (Nikon Ti-E inverted motorised microscope with Nikon A1Si spectral detector confocal system running NIS-C Elements software) to discriminate whether the SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain were reliable (confocal microscope settings are described in the 'Image Sample analysis' paragraph).

(ii) Fluorescence imaging in plants has unique challenges and methodologies. Plant staining is complicated by endogenous autofluorescence of plant tissues (Fricker & White, 1992), therefore the main problem for the applicability of the LIVE/DEAD® BacLight™ kit was the interference of plant background fluorescence during the analysis of stained biofilm. In order to prove the reliability of the kit on environmental biofilm, I collected two *P. sinuosa* leaves and kept them in oxygenated seawater until analysis. I then cut 8 samples of about 1 cm^2 from each leaf; 2 sections were randomly retrieved from the tip (older brown part) and 2 from the younger green part of the leaf. I removed the biofilm from two of the older and younger sections of *P. sinuosa* leaves by gentle scarping with a razor blade, and the remaining 4 samples were kept with biofilm intact. Leaf samples were placed within 8-well chambers and treated with the LIVE/DEAD® BacLight™ kit, as described above. *P. sinuosa* sections with and without epiphytic organisms were observed using confocal microscopy.

(iii) Biofilms are organised communities of mixed microorganisms attached to a surface (Davey et al., 2000). Biofilms are not only bacteria-specific; there is growing evidence that bacteria co-occur with archaea, and that both may possess some resistance to oxidative stress/antibiotics produced by the plant (Dridi et al., 2011). Different antibiotics are classified based on their mechanism of action, which can be: (i) inhibition of bacterial cell wall synthesis (e.g. penicillins and cephalosporins), (ii) inhibition of protein synthesis (e.g. tetracyclines, macrolides and clindamycin), (iii) blocking of important metabolic steps (e.g. sulfonamides and trimethoprim), or (iv) interference with nucleic acid synthesis (e.g. metronidazole and quinolones). Both bacteria and archaea commonly present forms of resistance to antibiotics, which can be intrinsic or acquired through horizontal transfer of antibiotic resistance genes from other microorganisms (Martínez, 2008; Khelaifia & Drancourt, 2012). Also, many of the agents commonly used against bacteria appear to be ineffective in targeting archaea. From the study performed in Chapter 3, I know that archaea (including ammonia-oxidising archaea) can be highly abundant on *P. sinuosa* leaf surface. In the study of Tourna and colleagues (2011), the ammonia-oxidising archaea *Nitrososphaera viennensis* was not affected by the antibiotics streptomycin, kanamycin, ampicillin, and carbenicillin. The half-maximal effective concentration of the antibiotic sulfathiazole to the ammonia-oxidising bacterium *Nitrosospira multiformis* was 7.70 μM, while the same measure for ammonia-oxidising archaea (*N. viennensis* EN76) was over 1500 μM (Shen et al., 2013).

Cell-walls ynthesis inhibitors	DNA-interfering antimicrobials	Protein synthesis inhibitors	Cell-wall alterating antimicrobials

(−) β-Lactams – **Penicillin**	(−) Ansamycins	(−) Tetracyclines	(−) Polymyxins
(−) Glycopeptide and lipoglycopeptide	(+) Quinolones	(−) Macrolides	(−) **Amphotericin B**
(−) Fosfomycin	(+) Novobiocin	(−) Lincosamides	(+) Squalamine
	(+) **Imidazole**	(−) Erythromycin	
	(+) Nitrofurans	(−) Phenicols	
	(+) Sulphonamides - **Acetazolamide**	(+) Aminoglycosides - **Streptomycin**	
	(+) Benzylpyrimidines	(+) Fusidic acid	

Table B1 Classification of antimicrobial agents according to their mode of action from the study of Khelaifia & Drancourt, 2012. (−), no anti-archaeal activity observed; (+), anti-archaeal activity observed. In bold are reported the antimicrobial and antifungal agents used for the present study.

In order to maximise the inhibition of bacterial and archaeal cells present in the biofilm of *P. sinuosa l*eaves, I chose for the present study a mixture of antibiotics with different mechanisms of action. For bacteria, I chose an antibiotics mixture comprising 10,000 units penicillin, 10 mg ml^{-1} streptomycin and 25 μg m^{-1} amphotericin B. Penicillin (family beta-lactams) (Sigma Aldrich), which acts by inhibiting bacterial cell-wall synthesis. Streptomycin (aminoglycosides) inhibits prokaryotic protein synthesis by preventing the transition from initiation complex to chain-elongating ribosome, thus causing miscoding. Amphotericin B interferes also with fungal membrane permeability by forming channels in the membranes and causing small molecules to leak out which lead to dead. However, as shown in Table 4.1, there is evidence for the ineffectiveness of these antibiotics on archaeal cells. β-Lactams are so called because they interfere with β-lactamase activity, which lead to the formation of peptidoglycans: essential proteins of the bacterial cell wall. Archaea possess a different cell wall devoid of peptidoglycan (Albers & Meyer, 2011), so that β-Lactams are ineffective for archaea, due to a lack of β-lactamase activity (Martin & König, 1996; Khelaifia & Drancourt, 2012). Susceptibility of some archaea has been reported for aminoglycosides, however, resistance for gentamicin and streptomycin, with minimal inhibitory concentrations of >100 mgL^{-1} has been

reported (Dridi et al., 2011). Thus, in order to target archaea, two different antibiotics were chosen: 0.22 mg L^{-1} acetazolamide (Sigma Aldrich; Khelaifia & Drancourt, 2012; Zimmerman et al., 2004) and 1mg L^{-1} imidazole (Sigma Aldrich; Dridi et al., 2011; Khelaifia & Drancourt, 2012). Acetazolamide belongs to the sulphonamides family and inhibits the synthesis of folic acid, a key cofactor in the synthesis of purine and pyrimidine bases in prokaryotes. Imidazole binds to DNA regions rich in adenine and thymine and cause oxidative cleavage of DNA stretches. Such DNA lesions are followed by the death of the microorganism.

In order to discriminate whether the chosen antibiotic mixture was effective against the seagrass epiphytic microbial community, I performed a study using 9 seagrass shoots. Three seagrass shoots were used as control and the remaining 6 as treatment. The 9 seagrass shoots were collected from a meadow in Marmion Marine Park (31°48.240' S 115°44.123' E) and kept in oxygenated seawater until analysis. They were then placed in aquarium filled with oxygenated seawater (10 L), kept at ambient seawater temperature and overnight. Nine glass cylinders (previously acid washed and autoclaved) were filled with 1 L ASF and placed for 30 minutes under UV light to ensure sterility. Both the control and treatment leaves were carefully scraped using a razor blade to remove bigger epiphytes, rinsed in ASF and placed within the cylinders. For the controls, I did not add the antibiotic mixture, and for the treatments, antibiotics were added to the cylinders just before the seagrass leaves were placed into the ASF. For all the shoots, stress status was analysed using the PAM fluorometer at the beginning and end of the experiment. For the treatments, 3 seagrass shoots were taken at 24 h and 3 at 48 h of antibiotic incubation, while controls were taken only after 48 h. After collection, shoots were rinsed in ASF and processed for cell viability assay. A small sample of approximately 1 cm^2 was taken from tip and the bottom part of each seagrass leaf using a sterile scalpel, and placed within the 8-well chamber. Samples were processed with the LIVE/DEAD® BacLight™ kit, as described above, and analysed under confocal microscopy in order to check for: (i) the effectiveness of the antibiotic action on microbial viability,

and (ii) choice of the correct timeframe (i.e. 24 h vs. 48 h) for antibiotic incubation prior to subsequent DO^{15}N incubation (Appendix A).

(iv) For the enriched experiment, at each incubation time interval and shoot collection (0.5, 2, 6 and 12 hours) seagrass sections for the treatment 'without microorganisms' were collected for cell viability assay from parts of the seagrass leaf immediately adjacent to the areas used for NanoSIMS analysis. Seagrass sections were placed within 8-well chambers and processed using the LIVE/DEAD® BacLight^{TM} kit, as described above. Once the tissue was fixed, slides were stored in the dark at 4 ºC and analysed using confocal scanning laser microscopic imaging within 48 h from the end of the experiment.

To date, the use of the kit to assess microbial viability has always been confined to the study of microbial biofilms cultured in laboratories. This is the first study, to our knowledge, which tested for the application of LIVE/DEAD® BacLight^{TM} kit in the analysis of complex environmental biofilm samples. As shown in Fig. 4.1 – A & B, I was able to easily discriminate between alive (green fluorescent) and dead (red fluorescent) *E. coli* cells, thus inferring the reliability of LIVE/DEAD® BacLigh^{TM} reagents. Fig.4.1– C & D represent images of seagrass leaf sections analysed for the pilot study 'without microorganisms' at 24h (C) and 48h (D) of incubation with antibiotic mixture. Based on the cover percentage of residual microorganisms, no significative difference was found between the two incubation times ($p=0.142$) with incubation at 24h presenting a microbial cover average of 0.31 ± 0.13 and, 0.40 ± 0.26 at 48h. Thus, I chose to incubate seagrass leaves with the antibiotic mixture for 24h prior ^{15}N amino acid spike during the experiment.

For the enriched ^{15}N experiment, the images acquired under confocal microscopy showed the efficacy of scraping to eliminate bigger epiphytic organisms together with the use of the antibiotic mixture to target a variety of microorganisms, including bacteria gram+ and gram-, archaea and fungi (Fig. 4.1 - E). Where epiphytes were

present, microbial cells and epiphytic algae were visualised clearly and images were not influenced by plant background epifluorescence (Fig. 4.1- F). Moreover, I was also able to visualise a high number of microbial cells harbored by epiphyticalgae (Fig. 4.1 - G) present on seagrass leaf surface. A clear difference was detected between the percentage cover of microorganisms on seagrasses incubated with and without antibiotics ($p<0001$). While for seagrass treated with antibiotics the microbial cover percentage was less than 1% (on average 0.24 ± 0.09) of the leaf surface analysed, for seagrass with epiphytic organisms the average cover percentage was estimated at 6.3 ± 1.3.

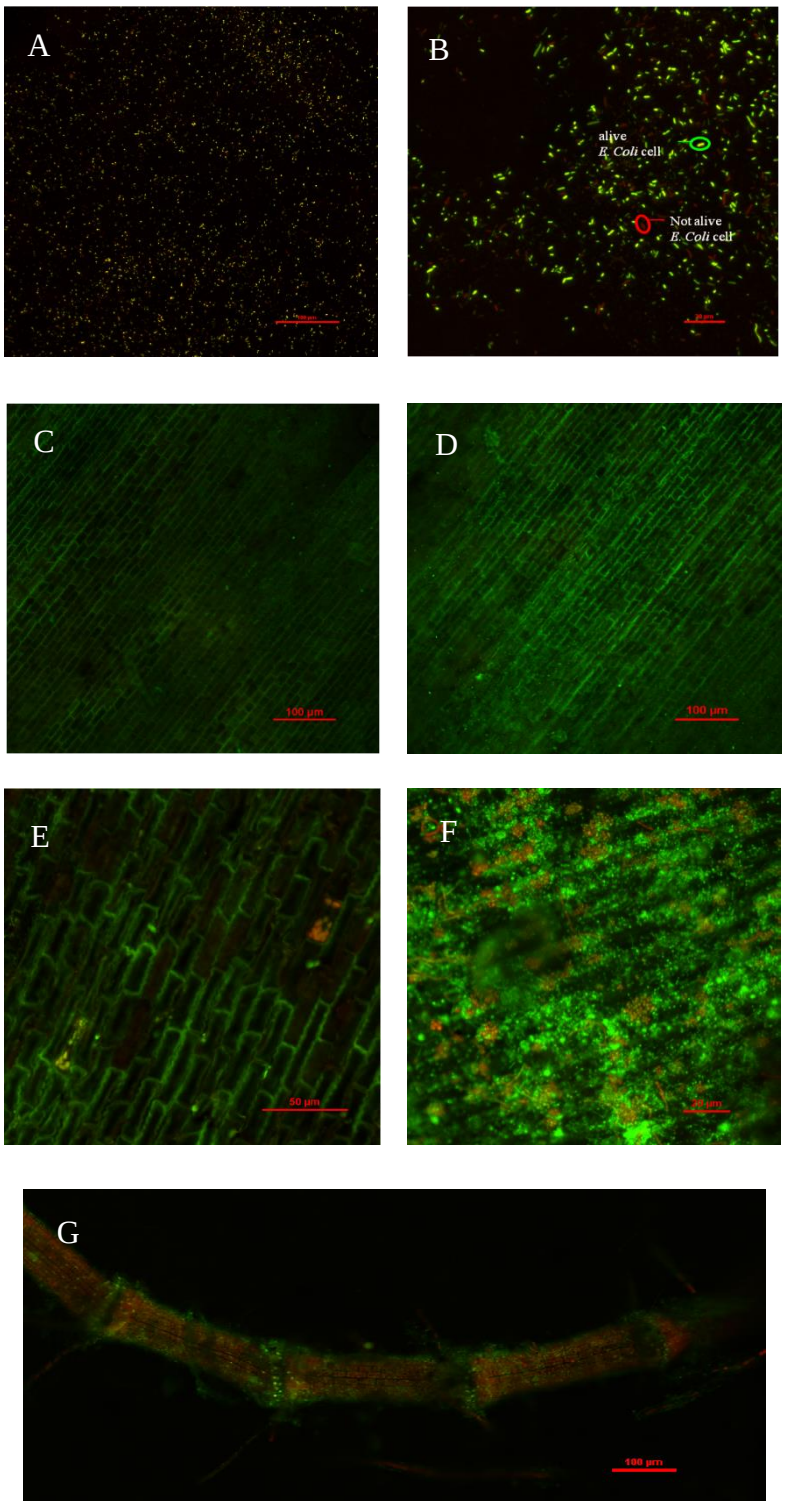


Fig. B1 - Scraping and antibiotic incubation efficiency visualised using the LIVE/DEAD® BacLigh™ kit under confocal microscopy. *E. coli* alive (green) and dead cells (red; A & B) proved the kit reagents were working. Antibiotic incubation at 24h (C) and 48h (D) were not significant different. On the contrary, a clear difference is visible between images of seagrass leaves deprived of epiphytic algae and microorganisms (E), and seagrasses covered with epiphytes (F). High number of microbial cells are present not only on *P. sinuosa* leaf surface but also as epiphytes of algae inhabiting seagrass leaves (G).

Table B2 Pulse-amplitude modulater (PAM) chlorophyll fluorometer values for seagrass leaves at the beginning and the end of experiment (dark yields adaptation). Optimal values are expected to be over 0.60 yields (Y) (Westphalen et al., 2005).

Incubation Time (h)	Samples	PAM (Y)
0	R1	0.79
	R2	0.78
	R3	0.70
	R4	0.79
	R5	0.81
	R6	0.79
0.5	TRT+BF R1	0.80
	TRT+BF R2	0.78
	TRT+BF R3	0.70
	TRT-BF R1	0.79
	TRT-BF R2	0.71
	TRT-BF R3	0.60
	CTL+BF	0.78
	CTL+BF	0.78
	CTL+BF	0.79
	CTL-BF	0.69
	CTL-BF	0.75
	CTL-BF	0.76
2	TRT+BF R1	0.87
	TRT+BF R2	0.64
	TRT+BF R3	0.80
	TRT-BF R1	0.65
	TRT-BF R2	0.81
	TRT-BF R3	0.82
	CTL+BF	0.69
	CTL+BF	0.73
	CTL+BF	0.77
	CTL-BF	0.78
	CTL-BF	0.80
	CTL-BF	0.68
6	TRT+BF R1	0.70
	TRT+BF R2	0.75
	TRT+BF R3	0.76
	TRT-BF R1	0.91
	TRT-BF R2	0.86
	TRT-BF R3	0.65
	CTL+BF	0.85
	CTL+BF	0.75
	CTL+BF	0.76
	CTL-BF	0.81
	CTL-BF	0.69
	CTL-BF	0.73
12	TRT+BF R1	0.74
	TRT+BF R2	0.66
	TRT+BF R3	0.82
	TRT-BF R1	0.80
	TRT-BF R2	0.74
	TRT-BF R3	0.71
	CTL+BF	0.80
	CTL+BF	0.65
	CTL+BF	0.77
	CTL-B	0.78
	CTL-B	0.82
	CTL-B	0.72

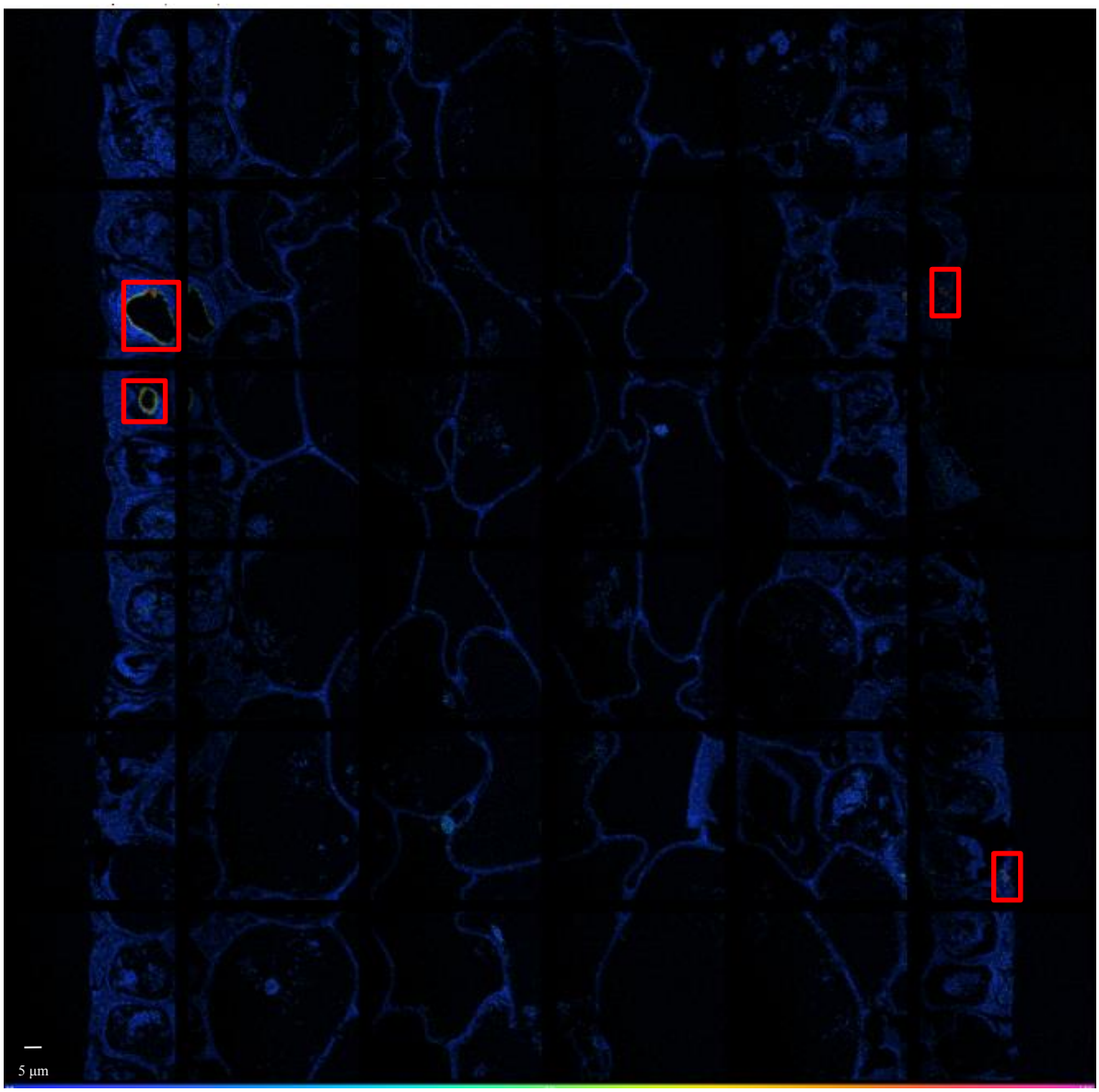


Fig. B2 - Chain image of seagrass cells after 12h form the $DO^{15}N$ spike for the treatment 'without microorganisms' (Chapter4). The image is obtained by 'stiching together' 36 single images together. On the right side, two images were few remaining bacteria are highlighted. On the left side, two seagrass cells which present enrichment are highlighted. The remaining cells are characterised by the absence of ^{15}N-enrichment