Characterisation of Marine Gel Particles and Associated Bacterial Communities

Aisha Salim Mohamed Al-Wahaibi

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School of Natural and Environmental Sciences

Newcastle University

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Abstract

Gel particles including biopolymers, microgels, transparent exoploymeric particles (TEP), and other classes of exopolymeric substances (EPS) are ubiquitous in the marine environment. Despite the different names for these gel particles in the literature they represent closely related materials and will be referred to simply as marine gel particles (MGPs). MGPs have a natural ability to aggregate, forming large particles of 'marine snow' that play an important role in the carbon flux to the ocean floor and the biogeochemical cycling of carbon in the sea. MGP aggregates are also a habitat for bacteria and their metabolic activities can affect the structure and dynamics of the MGPs. Extracellular DNA (eDNA) as an abundant element in the marine environment. eDNA is a key component in the structural integrity of some biofilms, and likewise could have similar effect on the structure of MGP aggregates. Hence, it is hypothesized here that extracellular DNA (eDNA) is present in MGPs as a result of bacterial growth and lysis. Therefore, the aim of this study was to investigate the presence of eDNA in MGPs. In addition, to characterise the bacterial community associated with MGPs and to examine their functional potential focusing on the occurrence of nuclease genes. A further aim was to investigate the production of deoxyribonuclease (DNase) by marine bacteria isolated from free living and attached bacteria. Seawater samples collected from the North Sea and filtered through a 100-µm sieve and a 0.4 µm polycarbonate filters to collect MGPs. eDNA occurrence was probed by bioimaging with cell-impermeant fluorescent DNA dyes YOYO-1 and TOTO-3. For the bacterial community structure and function analysis, MGPs were subjected to total DNA extraction and sequencing of the V4 region of the 16S rRNA gene using illumina MiSeq. Marine bacterial isolates were also investigated for DNase secretion on methyl green DNase test agar, with genome sequencing being carried out for the most productive DNase isolate. Results of the bioimaging analysis and quantification of the MGP composition demonstrated for the first time the presence of eDNA in MGPs. Proteobacteria dominated the bacterial community structure within MGPs, where *Pseudoalteromonas* and *Vibrio* were the most abundant genera. The bioinformatics based functional predictions of the bacterial community using KEGG analysis also affirmed the presence of numerous nuclease genes. The results from studies of isolated strains reported for the first time DNase secreting bacteria associated with MGPs in the North Sea. Additionally, there are 43 nuclease genes present in the genome of the prolific DNase producer *Serratia marcescens*. In conclusion, the co-

occurrence of eDNA and DNases in the MGPs indicate important implications for understanding the dynamics and properties of MGP in the world's oceans. This work can contribute to a further understanding of the role of the bacterial activities in MGPs formation, degradation and sedimentation processes.

Dedication

To My Parents with Sincere Love and Appreciation.

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Abbreviations, Acronyms and Units

ANOVA	Analysis of Variance
α-diversity	Intra-community diversity
BCC	Bacterial Community Composition
β-diversity	Inter-community diversity
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CLSM	Confocal Laser Scanning Microscopy
CSP	Coomassie Stainable Particles
Con A	Concanavalin A
DNA	Deoxyribonucleic Acid
DNaseI	Deoxyribonuclease
DOM	Dissolved Organic Matter
eDNA	Extracellular Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
FL	Free Living
iDNA	Intracellular Deoxyribonucleic Acid
KEGG	Kyoto Encyclopedia of Gene and Genome
KO	KEGG Orthology
MNase	Micrococcal Nuclease
MGP	Marine Gel Particles

NCBI	National Centre for Biotechnology Information
NucB	Nuclease B
PA	Particle Attached
PC	Polycarbonate filters
PCR	Polymerase Chain Reaction
PCA	Principal Component Analysis
ppt	Part per thousand
OTUs	Operational Taxonomic Units
SDS	Sodium Dodecyl Sulfate
STAMP	Statistical Analysis of Metagenomic Profiles
TEP	Transparent Exopolymeric Particles
QIIME	Quantitative Insight into Microbial Ecology
rRNA	Ribosomal Ribonucleic Acid
rpm	Rotation per minute
µL	Microliter
µm	Micrometer

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Chapter 1. Introduction

1.1 Background

Carbon dioxide is the main driver of global climate change. Oceans are a natural and active carbon sink on earth. About 50% of anthropogenic CO_2 remains in the atmosphere, whereas about 25% enters the oceans and about 25% is absorbed by land vegetation (Passow and Carlson, 2012; Ciais *et al.*, 2014). The ocean provides a dynamic cycling system where inorganic carbon is processed through three main vertical pumps; the solubility pump, the carbonate pump and the biological pump (for reviews Legendre *et al.*, 2015 and references therein). These processes drive carbon sequestration and eventually settled on the ocean's floor via sedimentation of organic matter (OM) and particulate organic matter (POM) sinking aggregates. This process is attenuating the carbon from the atmosphere and therefore alleviating the global climate problem. Moreover, dissolved organic matter (DOM) and POM are both the vehicle by which the carbon is transported from the surface to the deep of the oceans. The aggregation of the POM is facilitated by sticky gels made up of exopolymeric materials (EPM) that holds the particles. The cycling of POM by marine microbial communities largely drives its degradation and is thus a process in helping to define carbon fluxes to the deep sea. Hence, bacteria are mediating the transformation processes of the sinking aggregates by altering the OM transport dynamics through the continuous cycles of aggregation and degradation the particles undergoing until it reaches the sea floor. This uncharacterised process is raising important questions about what are the factors controlling the fate of these sinking particles? And what are the causes of the continuous aggregation and disintegration processes of the DOM and POM? Yet these questions have not been fully addressed.

It is of paramount importance to understand the stability of gel particles and therefore, it is necessary to fully understand the composition of the gel particles. Despite the importance of extracellular DNA (eDNA) as an abundant element in the marine environment and as a key structural component in three-dimensional biofilms (Whitchurch *et al.*, 2002). The eDNA component has been overlooked in the gel particles of the oceans. Similarly, eDNA could affect the structural integrity of gel particles as it is made up of microbial extracellular polymeric substances (EPS), which can form both biofilms and suspended aggregates. EPS is a component that could drive aggregation and by means of harbouring bacterial communities that release enzymes by which disintegration can occur.

1.2 Gel particles in the ocean: origin and various terminologies

Gel particles are ubiquitous in the ocean in various shapes and forms with an estimated abundance of around 10^6 -10^8 particles l^{-1} (Cisternas-Novoa *et al.,* 2015). Generally, gel particles are defined as a hydrated porous gel polymer matrix made up of EPS (Verdugo *et al.*, 2004). Gel particles are formed by biotic and abiotic processes. Biotic formation occurs following EPS release from bacteria, phytoplankton mainly diatoms, and macroalgae (Decho and Moriarty, 1990; Kiørboe and Hansen, 1993; Passow, 2002a; Thornton, 2004; Chen and Thornton, 2015; Li *et al.,* 2015; Decho and Gutierrez, 2017). In contrast, abiotic formation involves the spontaneous assembly of dissolved organic matter (DOM), from small precursors to larger particulate organic matter (POM) (Chin *et al.,* 1998; Passow, 2000; Engel *et al.,* 2004). The sticky property of gel particles facilitates the annealing process that forms bigger aggregates such as marine snow and sinking aggregates, as it enables the adherence of organisms, detritus and other materials (Azetsu-Scott and Passow, 2004).

Gel particle production occurs in the surface ocean and is found to be highest following algal blooms (Engel, 2004; Verdugo, 2012). The gel particles are less dense than seawater when not attached to other particles or organisms. Consequently, gel particles tend to migrate vertically upwards to the sea surface, accumulating in the sea surface microlayer (SML) to concentration typically 40 times higher than in underlying water (Cunliffe *et al.*, 2013; Galgani and Engel, 2013; Wurl *et al.*, 2016). The SML occupies the uppermost few hundred μm of the ocean surface in direct contact with the atmosphere. Its physicochemical and biological properties are distinct from those of the underlying water and it is the interface through which ocean-atmosphere exchange of gases and particles takes place (Cunliffe *et al.*, 2011).

Gel particles in the ocean have been reported in the literature using different terms to describe particles larger than 0.4 μm in diameter, including gel organic matter (GOM) or polymer gels, marine microgel, transparent exopolymeric particles (TEP), extracellular polymeric substances (EPS), microbial polymers, biopolymers, marine flocs, marine colloids and other classes of exopolymeric materials (Alldredge *et al.*, 1993; Chin *et al.*, 1998; Hatcher *et al.*, 2001; Sheng *et al.*, 2010; Verdugo and Santschi, 2010). However, these terms describe virtually the same things that are all part of the POM pool (Xu *et al.*, 2019 and references therein). For the reason that all these gel polymers are by definition made up of EPS, they may have similar composition (Samo *et al.*, 2008; Engel, 2009). Regardless of the different terminologies and classification of these gel particles, it is challenging to differentiate between them when in their natural state and unstained. Gel particles will henceforth be referred to here as marine gel particles (MGPs),

to include all hydrated gel biopolymers made up of EPS, consisting of polysaccharides, proteins, and nucleic acid (Sheng *et al.*, 2010; More *et al.*, 2014; Flemming, 2016). MGPs appear under the microscope in different irregular forms and shapes, for example filamentous and sheet like (Simon *et al.*, 2002; Verdugo *et al.*, 2004).

1.3 The composition of marine gel particles (MGP)

The composition of small MGP reported in the literature has traditionally been based on a colorimetric method that classifies them into two discrete types, based on the staining dye to which they bind (Engel and Passow, 2001). Gel particles composed predominantly of polysaccharides are called transparent exopolymeric particles (TEP) and can be stained with Alcian blue, an acidic polysaccharide stain (Alldredge *et al.*, 1993). The other class of gel particles is proteinaceous, (Figure 1.2), which can be stained with Coomassie Brilliant Blue and are termed Coomassie stainable particles (CSP) (Long and Azam, 1996; Cisternas-Novoa *et al.*, 2014). The overlap of the colour grade of the Alcian blue and Coomassie brilliant stains remains a subject of debate as neither TEP nor CSP can be distinguished separately if stained simultaneously (Engel *et al.*, 2004). However, currently, TEP and CSP are the only categories recognised in marine gel particle research (Figure 1.2) (Cisternas-Novoa *et al.*, 2014; Cisternas-Novoa *et al.*, 2015; Thornton, 2018). This classification limits the understanding of MGP composition to polysaccharides and proteins and therefore neglects other components that might be present and contribute to MGP structure. Consequently, the detailed composition of MGP is not yet fully characterised. However, the small MGP pool in the ocean evidently is heterogeneous (Verdugo and Santschi, 2010), and further investigations are therefore required to fully understand the full range of compositions. In comparison, large marine gel aggregates (e.g. marine snow) have been investigated using fluorescence dyes and microscopy (Holloway and Cowen, 1997a; Flintrop *et al.*, 2018).

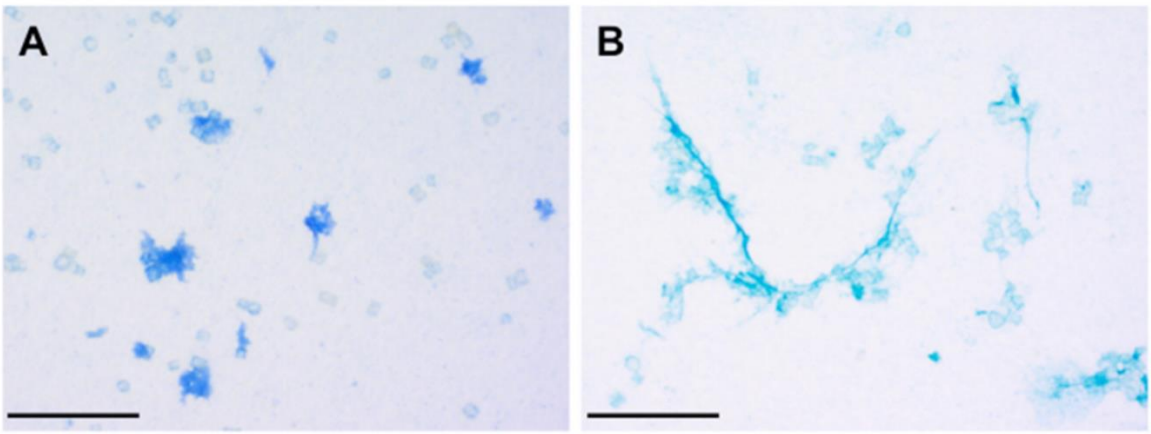

Figure 1.1 Images of stained particles from a culture of the diatom *Thalassiosira weissflogii* collected simultaneously. Coomassie stainable particles (CSP) (A) and Alcian blue stained transparent exopolymeric particles (TEP) (B). Scale bars 100 µm (Thornton, 2018).

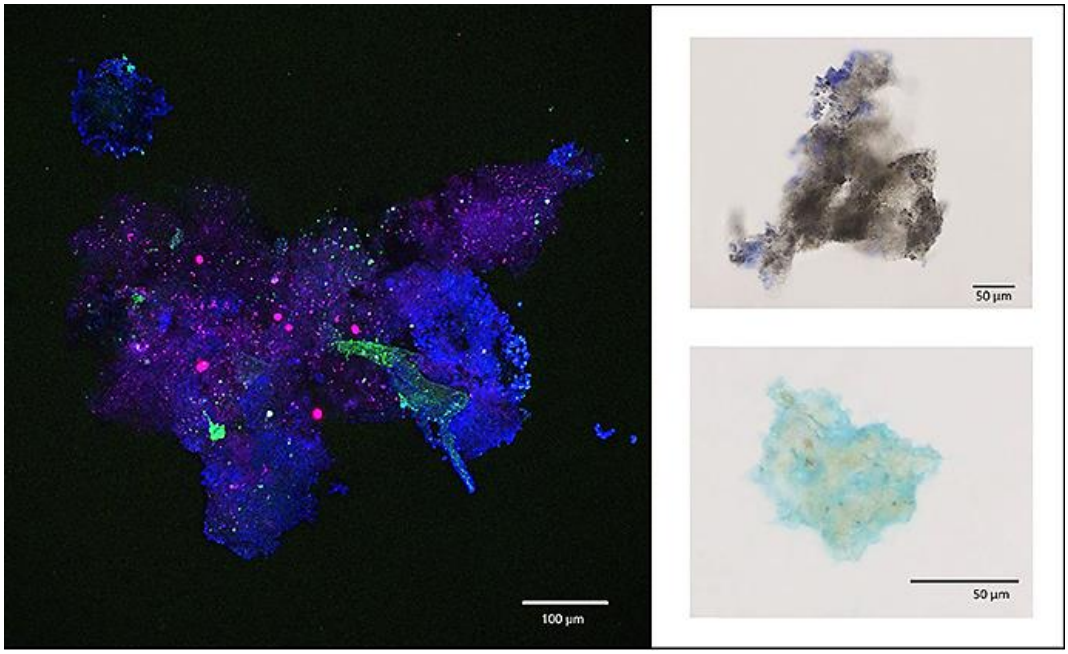

Figure 1.2 Marine gel particle image (marine snow aggregate) obtained by confocal laser scanning microscopy: blue, polysaccharide-containing structures; green, structures containing nucleic acids; red, structures containing chlorophyll. Top right: Marine snow aggregates stained with Coomassie Brilliant Blue G (staining of CSP) and lower right marine snow aggregate stained with Alcian blue (staining of TEP) (Busch et al., 2017).

1.4 MGP and associated bacteria

The natural ability of MGP to aggregate and form flocs makes them a favourable microenvironment for diverse bacterial communities (Ploug and Grossart, 2000; Simon *et al.*, 2002). Typical MGP bacterial abundances may be 10,000-fold, higher than in the surrounding seawater (Ploug and Grossart, 2000; Grossart *et al.*, 2006) making them intense "hot spots" of microbial and biogeochemical activity (Alldredge *et al.*, 1986; Herndl and Peduzzi, 1988; Mari *et al.*, 2007). For heterotrophic bacteria MGP are especially rich in nutrients and surface-bound extracellular enzymes that can be used in processing MGP components. The interaction of bacterial communities with MGP thus plays a crucial role in the formation and degradation of organic matter in the ocean (Logue *et al.*, 2016). Consequently, this has a large impact on marine biogeochemical cycles and the dynamics of sinking marine-sinking aggregates (Jiao *et al.*, 2014).

A number of studies have reported the diversity of bacterial communities associated with MGP, marine picoplankton particles, and bacterial communities attached to particles (DeLong *et al.*, 1993; Rath *et al.*, 1998; Ganesh *et al.*, 2014; Busch *et al.*, 2017; Mestre *et al.*, 2017; Mühlenbruch *et al.*, 2018). The MGP attached microbial communities are phylogenetically distinct, with different functional capabilities compared to free living microbes in surrounding waters (Mével *et al.*, 2008; Ortega-Retuerta *et al.*, 2013; Rieck *et al.*, 2015). There is an agreement on some specific groups that are enriched in some particle communities, such as Proteobacteria, Bacteroidetes and Planctomycetes (Bižić-Ionescu *et al.*, 2015; Salazar *et al.*, 2016). However, spatial and temporal variations mean there is no consistent pattern. Furthermore, diverse bacteria are reported in association with different size classes of MGP (Mestre *et al.*, 2017).

Many studies have focused on larger marine particle sizes like marine snow (> 500 µm) so that smaller MGP size classes are relatively understudied. Therefore, the focus of this study is on the smaller MGP fractions (0.4- 100 µm). Fortunately, developments in high throughput next generation sequencing (NGS) of 16S rRNA gene, have greatly enhanced understanding of the bacterial community structure living in association with both these smaller MGP and larger marine snow particles (Sunagawa *et al.*, 2015).

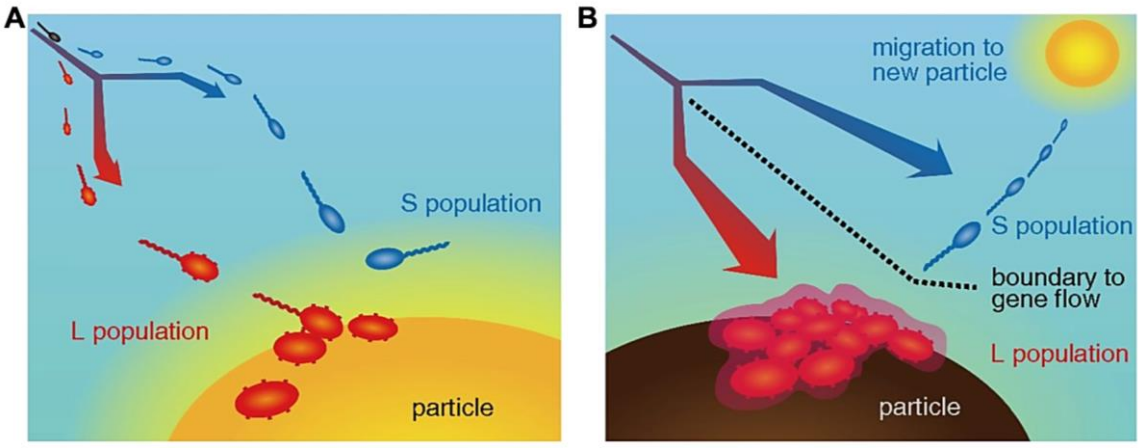

Figure 1.3 Bacterial utilisation of MGP employ different ecological behaviours motility, chemotaxis, surface attachment and biofilm formation. Populations S (Blue) and L (Red) are competing to reside the particle (A). Population L settled and formed a biofilm on the particle, while population S migrated hunting another particle (Yawata et al., 2014).

Bacteria-MGP interactions are very important to carbon cycling and the fate of sinking organic matter in the ocean by acting as a vehicle for vertical carbon transport (Azam, 1998; Passow, 2002b; Arnosti, 2011). Moreover, bacterial activities and the release of extracellular enzymes within MGP regulate the rate of marine carbon sequestration (Smith *et al.*, 1992; Smith *et al.*, 1995; Lechtenfeld *et al.*, 2015). In this thesis, special attention is given to the role of bacterial extracellular material and enzymatic activities within the MGP, as these intense interactions contribute to the formation and degradation processes.

A recent study by Mestre *et al.* (2018) revealed how sinking marine particles promote the transport of the microbiome from the ocean top sunlit zone to the deep; the microbiome associated with larger ocean surface marine aggregates was found to be similar to the deep ocean microbiome.

1.5 Extracellular materials in the ocean

Extracellular materials are abundant in the marine environment, being discharged from organisms through lysis or apoptosis (Okshevsky and Meyer, 2015). The role of extracellular substances in the ocean was reviewed (Decho, 2010; Decho and Gutierrez, 2017and references therein). Here, extracellular DNA (eDNA) and extracellular enzymes (ECE) are discussed in the context of their interaction with marine bacteria and MGP.

1.5.1 *Extracellular DNA (eDNA)*

DNA is an abundant molecule in all environments, and its concentration in the ocean is about 0.2 to 19 µg l^{-1} (Deflaun *et al.,* 1987; Paul *et al.*, 1987; Tani and Nasu, 2010). Extracellular DNA (eDNA), the DNA that is not enclosed in cells, is the most abundant form of DNA found in the oceans in high concentrations, mainly on the surface of deep-sea sediment (the largest reservoir on the planet), at a concentration of 0.31g of total DNA m^{-2} (Dell'Anno and Danovaro, 2005). The release of eDNA in the marine environment is mostly from dead and live organisms through several mechanisms, such as active secretion from living cells, passive release from the cells of dead organisms, autolysis, and viral phage-induced lysis, or through association with extracellular membrane vesicles (Ibáñez de Aldecoa *et al.*, 2017; Nagler *et al.*, 2018a). Marine sediments are a huge reservoir of eDNA that can be a repository of genetic material and a diversity recorder (Corinaldesi *et al.,* 2014; Corinaldesi *et al.*, 2018). The high turnover rates of eDNA make it a valuable source of carbon, nitrogen, and phosphorus for bacterial consumption. In addition, eDNA adsorption onto organic material in sediments has been reported to play a role in the phosphorus cycle in the ocean (Dell'Anno *et al.,* 2002).

The cycling of dissolved DNA in the ocean (Figure 1.4) has been attributed to bacterial extracellular enzymatic activities, for example extracellular nuclease utilisation of eDNA as a resource for bacterial consumption (Lennon, 2007). Additionally, bacterial utilisation of eDNA is important at the physiological, evolutionary, and ecological levels (Vorkapic *et al.*, 2016 and references therein). Despite eDNA being an important component in the biofilm matrix and having functions that mainly support and strengthen the biofilm matrix structure (Whitchurch *et al.,* 2002; Dominiak *et al.,* 2011; Jakubovics *et al.,* 2013), little is known about eDNA in ocean MGPs. As MGPs are made up of EPS that are the main component of biofilms, it is reasonable to assume that the two are compositionally similar. It can therefore be hypothesised that the eDNA in MGPs is a product of the intense bacterial activity. Therefore, as eDNA has been widely demonstrated as a key role player in the structure biofilms it is likely to be important to the composition of MGP and marine flocs (Alawi *et al.*, 2014; Das *et al.*, 2014; Nagler *et al.*, 2018b).

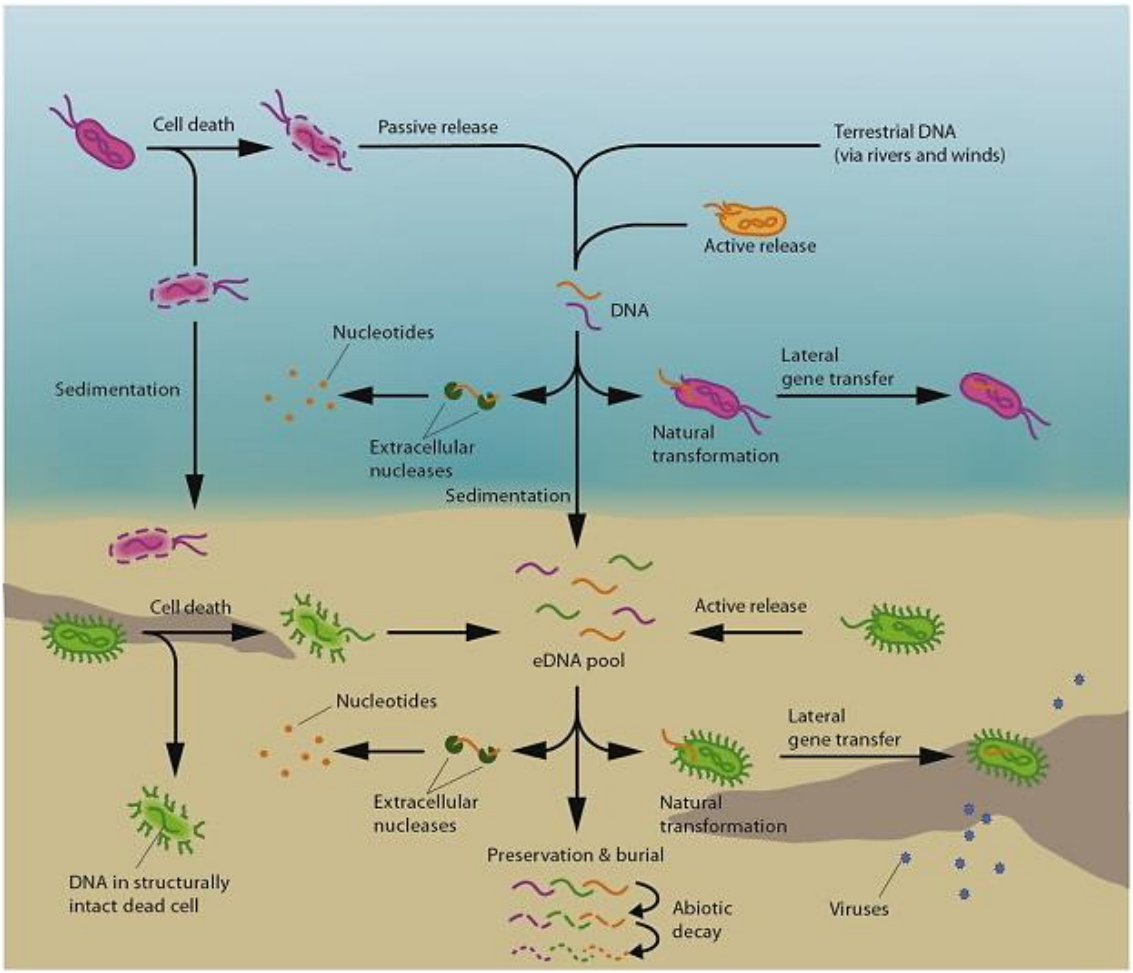

Figure 1.4 The processes by which eDNA originates, accumulates, sinks, and is ultimately buried in sediments on the ocean floor (Torti et al., 2015).

1.5.2 *Extracellular enzymes (ECE)*

Extracellular enzymes (ECE) are prevalent in the ocean, either attached to cell surfaces or truly free, i.e. dissolved in seawater (Chróst, 1990; Kamer and Rassoulzadegan, 1995; Baltar *et al.*, 2010; Baltar, 2018). Free-living enzymes importantly contribute to hydrolysis in coastal seawater (Allison *et al.*, 2012; Liu and Liu, 2018). The involvement of ECE in interactions between microbes and the particles to which they attach results in changes to the particle structure (Huston and Deming, 2002) that may also affect biogeochemical processes in the surrounding water, which has resulted in increased attention on ECE in relation to microbial hotspots on aggregates (D'ambrosio *et al.*, 2014), especially MGP-associated aggregates like marine snow (Karner and Herndl, 1992; Smith *et al.*, 1992; Grossart *et al.*, 2007; Arnosti *et al.*, 2012; Kellogg and Deming, 2014; Balmonte *et al.*, 2016). In addition, extracellular enzymatic activity in marine snow is regulated by quorum sensing (QS) (Gram *et al.*, 2002; Jatt *et al.*,

2015; Krupke *et al.*, 2016) that is thought to impact the hydrolysis of the sinking aggregates (Hmelo *et al.*, 2011).

In addition to catalysing the process of organic matter degradation and consequently impacting carbon fluxes to the deep sea (Azam and Malfatti, 2007), ECE have a role in determining the lifetime of DOM in the ocean (Traving *et al.*, 2015). In the euphotic zone, about 80% of POC is hydrolysed enzymatically (Kellogg and Deming, 2014). In the initial stages of POM degradation, microbial ECE hydrolysis converts high-molecular-weight to low molecular weight (<800 Da) organic matter, enabling it to pass through the bacterial cell membrane for uptake (Nikaido, 2003). This hydrolysis and the associated nutrient cycling provide food for heterotrophic bacteria and other organisms, thus ECE hydrolysis plays a vital role (Arnosti, 2003; Arnosti, 2011; Balmonte *et al.*, 2016). In addition, aggregate formation is likely to cause an alteration in the profile and rate of hydrolysis in enzyme activities (Ziervogel *et al.*, 2010).

Deoxyribonucleases (DNases) are hydrolytic enzymes targeting the phosphodiester bonds of DNA. DNases are produced naturally by bacteria for nutrient metabolism, defence, and genetic evolution (Dubnau, 1999; Chen and Dubnau, 2004; Vorkapic *et al.*, 2016; Veening and Blokesch, 2017). DNases are generally found in bacterial biofilm dispersion through the breakdown of DNA (Whitchurch *et al.*, 2002). These studies demonstrate that nucleases from marine bacteria have a promising potential for biofilm removal and NucB is a good example (Nijland *et al.*, 2010; Shakir *et al.*, 2012; Shields *et al.*, 2013). However, DNases have not been studied within MGP or marine snow to date (Al-Wahaibi *et al.*, 2019). The availability of eDNA in the oceans might trigger the production of extracellular DNases, and if so, the presence of nucleases secreted by floc-associated bacteria could impact the structural integrity of marine particles.

1.6 Dynamics of MGP in the ocean

Marine gel particles are inseparable from all interactions with bacteria and other organisms they encounter, the processes they are involved in, and the dynamics of aggregation and dissolution they go through in the ocean environment. Therefore, the implications of the interaction between bacteria and MGP for ocean processes are likely to be substantial.

1.6.1 *MGP in surface microlayer*

Marine gel particles accumulate in the SML, forming a biogenic layer that is implicated in air-sea gas exchange (Cunliffe *et al.*, 2011; Galgani and Engel, 2013; Engel and Galgani, 2016). As aggregation and formation occur in the photic zone, MGP expand in size and tend to adhere

to organic detritus and other organisms and materials, forming bigger sinking aggregates. Gel particles undergo continuous formation and degradation in the marine environment due to several factors: physical, chemical, and biological. However, not all MGP transform to bigger aggregates in the ocean, which poses the question, what other factors can inhibit the process of MGP formation in the ocean? This question is important because MGP contribute to wider, climate-related processes. For example, airborne MGP from aerosols generated in the SML can initiate the formation of cloud condensation nuclei (CCN) and ice nucleating particles (INP), leading to cloud formation (Orellana *et al.,* 2011; Wilson *et al.,* 2015; DeMott *et al.,* 2016; Quinn *et al.,* 2017; Rastelli *et al.,* 2017). Recent findings by Rastelli *et al.* (2017) showed that marine aerosols produced experimentally from North-east Atlantic Ocean surface water contained a range of organic components, including proteins, carbohydrates, cell elements, microbes and, for the first time, eDNA. Generally, these components reflect those of marine sinking aggregates, although to date there is no clear evidence for the presence of eDNA in marine aggregates.

Nonetheless, the well-known fate of MGP in the marine environment is either to attach to surfaces to initiate biofilms formation, or to continue aggregating into larger aggregates that then sink into the deep ocean (Figure 1.5).

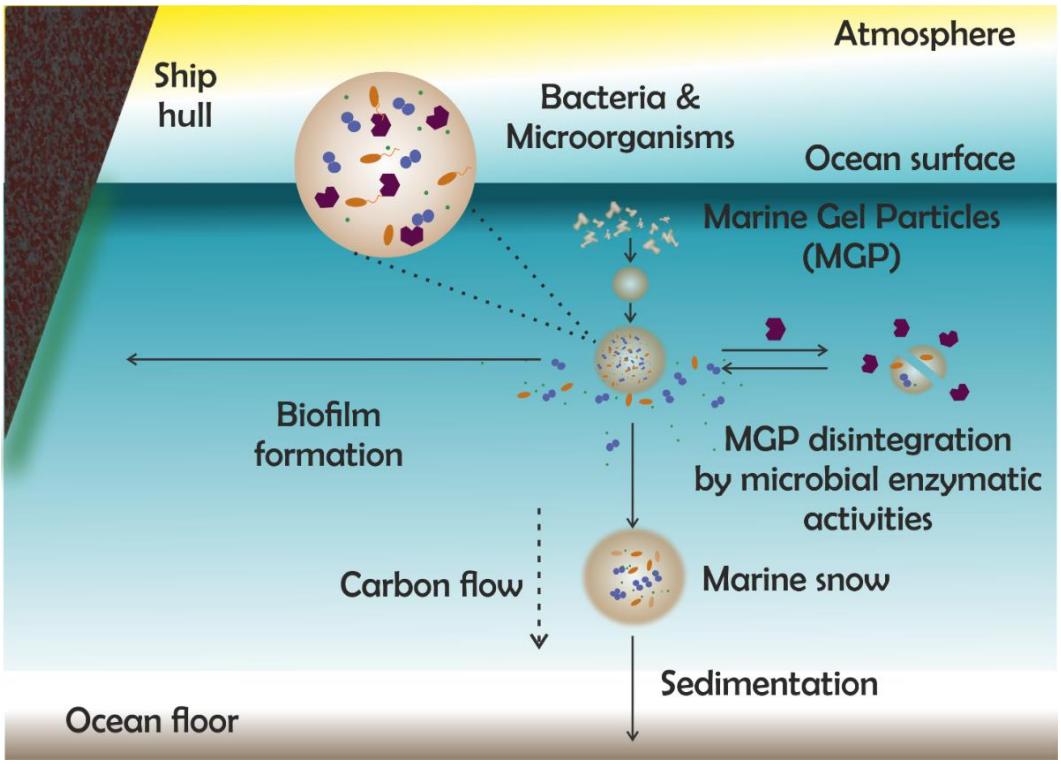

Figure 1.5 The dynamics of MGP in the ocean, showing continuous formation and degradation with an impact on the associated bacterial community.

1.6.2 *The bioflocculation of MGPs*

Flocculation is a natural behaviour of MGPs (Chin *et al.,* 1998; Engel *et al.,* 2004). Marine bacteria mediate the flocculation process (Ding *et al.,* 2008, Yamada *et al.,* 2013, Yamada *et al.,* 2016), transferring POM to the deep ocean (Fowler and Knauer, 1986; Alldredge and Silver, 1988). However, the precise mechanisms involved are unclear, although a few studies have addressed this. One found that bacterial EPS facilitate aggregation through specific chemical signalling (Mori *et al.,* 2017). A study of cultures of the marine photosynthetic bacterium *Rhodovulum* sp., isolated from a shrimp farming facility in Thailand, revealed that its flocculation ability was enhanced by the presence of NaCl, RNA, and DNA but hampered by the addition of RNase and DNase (Watanabe *et al.,* 1998). In another study nucleic acid produced in *Rhodovulum sulfidophilum* cultures resulted in floc formation and integrity maintenance (Suzuki *et al.,* 2009).

In addition, EPS enhance the flocculation of *Rhodovulum* sp. in the presence of divalent cations like Ca^{2+} and Mg^{2+}, that assist fisheries' wastewater treatment (Watanabe *et al.,* 1999). Another marine bacterium, *Sagittula stellata,* has been reported to stimulate DOM aggregation and gel particle formation (Ding *et al.,* 2008). A recent study used *Pseudoalteromonas* spp. to examine gel particle coagulation and showed that these marine bacteria enhanced the aggregation of chitin model gel particles into larger sizes within 24-96 hours (Yamada *et al.,* 2016). Interestingly, another study on marine sinking particles and the phage's associated with them suggested that cell lysis induced by the associated viruses could enhance the aggregation of the sinking particles (Guidi *et al.,* 2016), although the precise nature of this process was not investigated. This lack of mechanistic information shows the clear limitation of these studies.

Bacterial attachment of marine snow is thought to be facilitated by chemical signalling of QS cell-to-cell communication (Gram *et al.,* 2002; Mori *et al.,* 2017), which is in agreement with bacterial behaviour in consortium-like structured biofilms (Bassler, 1999). However, the specific mechanism by which this occurs is poorly understood, as is whether the eDNA expelled by cells could play a role in MGP aggregation as in biofilms (Whitchurch *et al.,* 2002). In addition, the extracellular vesicles (EVs) that are produced by microbes and which contain lipids, nucleic acid, extracellular enzymes, and microbial info-chemicals, have been emphasised recently as having an impact on the ecological processes of the surrounding environment (Schatz and Vardi, 2018).

Bacterial EPS is attributed in enhancing flocculation in aquatic systems. A recent study investigated the role that polyunsaturated aldehydes, produced by phytoplankton and proliferate in post-algal bloom, play in the flocculation of TEP. It reported a significant increase in the size

of aggregates >200 µm by the end of the bloom period (Bartual *et al.,* 2017). Another study reported that sunlight affected the flocculation process, in that EPS transformed to TEP, stable flocs were formed by exposure to solar radiation, and with UV, unstable flocs were formed (Shammi *et al.*, 2017; Sun *et al.*, 2017a; Sun *et al.,* 2019).

The flocculation process has been widely used to eliminate solid waste in industrial and biotechnological applications such as water pollution management, seawater desalination, wastewater treatment, aquaculture systems, microalgae harvesting, and biofuel production as it is economical and eco-friendly. These processes require the formation of activated sludge that resembles the natural formation of marine snow (Shahadat *et al.*, 2017; Tansel, 2018 and references therein). Mainly, flocculation in these industries is initiated by the addition of, for example, $FeCl_3$, Mg^{2+}, Na^{2+} and Ca^{2+} (Chin *et al.,* 1998; Kawato *et al.,* 2016; Meng and Liu, 2016). Therefore, bacterially- induced flocculation may have future applications as an environmentally friendly solution for water quality applications in the marine environment.

Recently, the agglomeration of marine microplastic has been attributed to EPS flocculation (Summers *et al.,* 2018). Similarly, polyethylene microplastic was reported to be coated by biofilms and to coagulate with marine gel particles and enhance aggregate sinking to the deep ocean (Galloway *et al.,* 2017; Michels *et al.,* 2018). Therefore, the natural process of flocculation initiated by EPS may stimulate self-cleaning of the aquatic environment (for reviews Santschi, 2018).

1.6.3 *MGP in biofilm formation*

Figure 1.5 shows how MGPs are important to biofilm initiation in the ocean. Aggregation forms a floating biofilm or 'Protobiofilm' (Bar-Zeev *et al.,* 2012) that can, for example, adhere to ship's engines and hulls, to industrial pipelines, and to reverse osmosis membranes in desalination plants (Meng *et al.*, 2013; Bar-Zeev *et al.*, 2015; Discart, 2015; Lee *et al.*, 2015; Levi *et al.*, 2016; Li *et al.*, 2016). All these studies reported that MGPs are causing biofilms in desalination plants. Therefore, by understanding the role of MGP in the formation of biofilm development, it may be possible to reduce the problem of MGPs causing biofilm on marine structures.

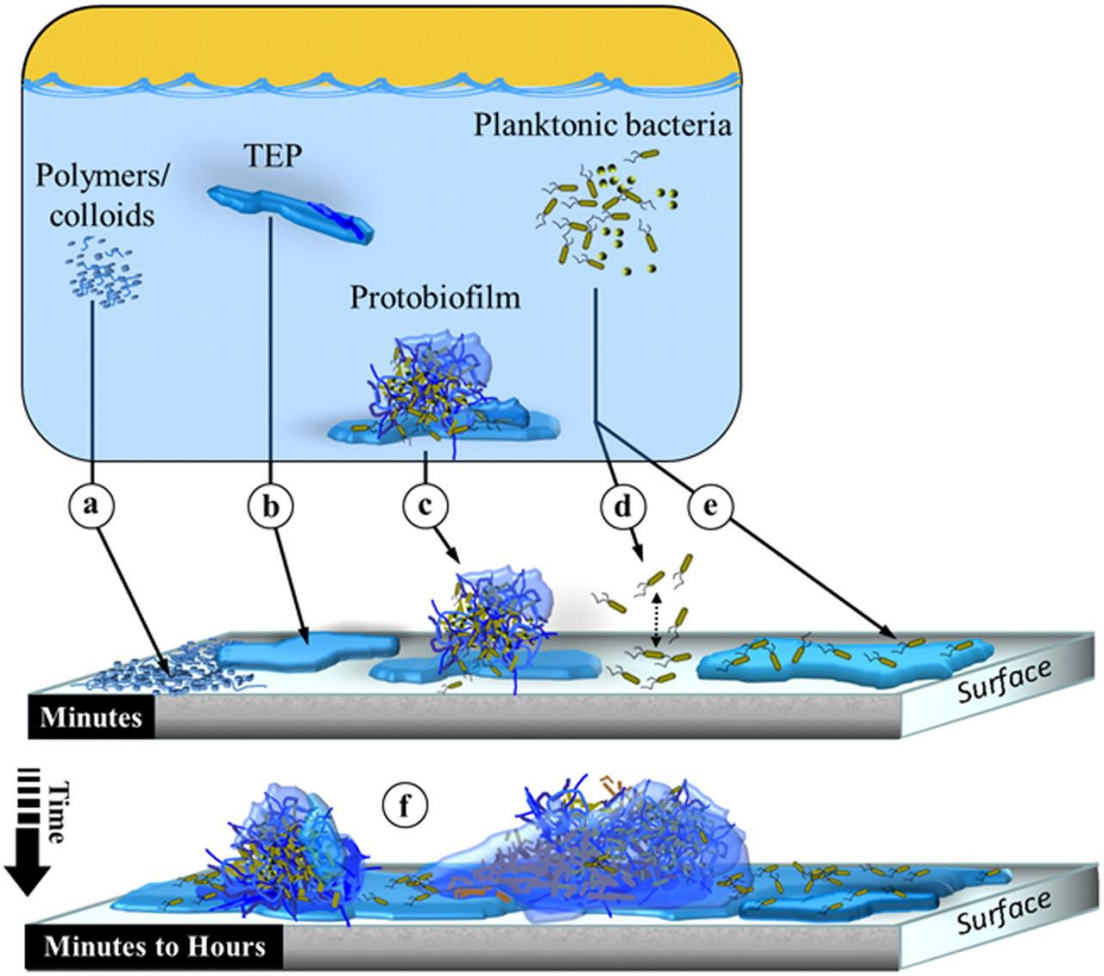

Figure 1.6 Schematic illustration of the process by which transparent exopolymeric particles (TEP) attached to bacteria develop a biofilm once attached to surfaces (Bar-Zeev et al., 2012).

1.6.4 *MGP in ocean biogeochemical cycles: carbon cycling, sequestration and sedimentation*

The oceans absorb around 25% of atmospheric CO_2, by which help in reducing carbon effect on global climate change. The deep ocean sequestration of carbon and its ultimate burial in deep sea sediments occurs by three vertical carbon pumps: the solubility pump; the carbonate pump; and the biological pump (BCP) (Legendre *et al.*, 2015). In the BCP marine autotrophs transform CO_2 into organic carbon via photosynthesis and the resulting particulate organic carbon (POC) is exported vertically to the deep sea (Figure 1.7). Initially the BCP was thought to be the only driver of this downward carbon flux (Boyd and Trull, 2007). However, the microbial carbon pump (MCP), proposed by Jiao *et al.* (2010), also plays a crucial role that also contributes to the large ocean reservoir of DOM (Hedges, 2002; Jiao *et al.*, 2014). This has put into context the key role of microbes in transforming carbon in the ocean and in its long-term storage (Azam, 1998; Jiao *et al.*, 2010; Jiao and Azam, 2011), particularly the role of the MCP as a major

contributor to the global carbon cycle. Based on the ability of DOM to resist remineralisation, DOM is classified as labile, semi-labile, semi-refractory, or refractory (Hansell *et al.*, 2012). Labile DOC has a <100 years lifetime and refractory (or recalcitrant) DOC has >100 years lifetime (Jiao *et al.*, 2010; Jiao and Azam, 2011; Jiao *et al.*, 2011; Jiao and Zheng, 2011; Jiao *et al.*, 2014; Robinson *et al.*, 2018). However, little is known about the mechanisms by which bacteria contribute to the formation and long-term storage of these carbon fractions in the deep oceans (Robinson *et al.*, 2018; Wang, 2018). However, during the export process, the heterotrophic bacteria associated with MGP utilise the carbon and convert it to organic carbon form via photosynthesis, thus, making it available for the associated microbial communities and other organisms, enabling it to enter the food web.

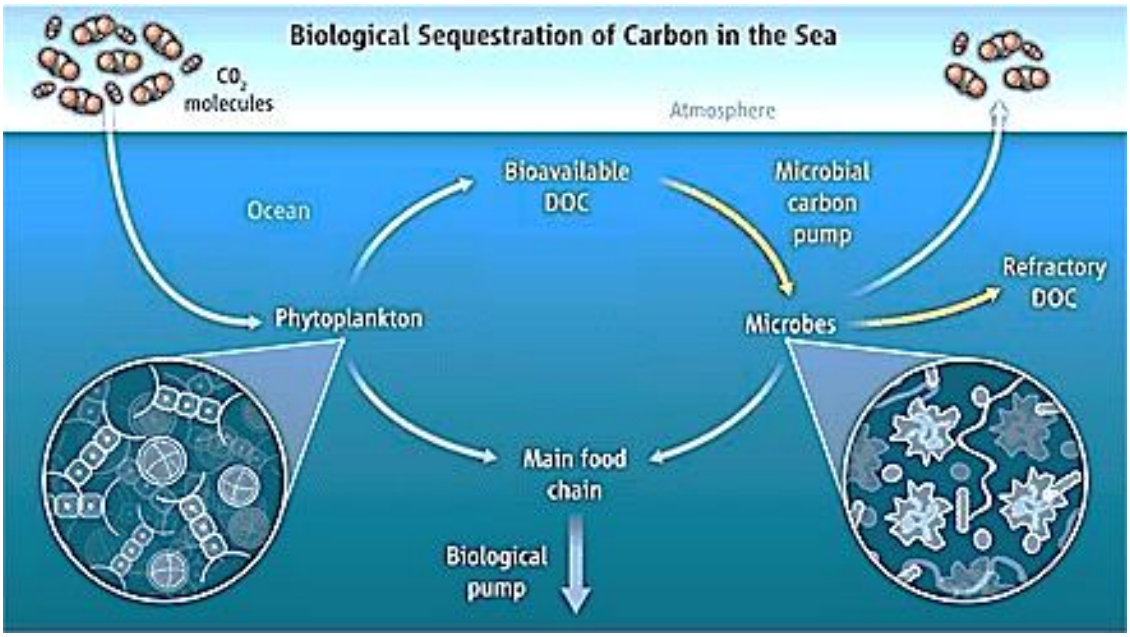

Figure 1.7 Carbon sequestration by the biological carbon pump in the ocean (Stone, 2010).

Marine gel particles function as a carbon carrier between the surface and the bottom of the ocean and they play a vital role in the carbon cycle in the ocean through the vertical transport of sediments to the ocean floor (Passow, 2002c; Christina and Passow, 2007; Xavier *et al.*, 2016). Regarding sinking particles, their eventual fate, notwithstanding remineralisation, is sedimentation in the deep ocean. Where marine sinking aggregates and marine snow are mediating the export of the carbon flux to the ocean floor (Fowler and Knauer, 1986; Shanks, 2002). These vehicles are a form of DOM that is produced in the euphotic zone of the ocean and POM. The carbon cycling and sequestration by MGP-associated communities play an important role in carbon cycling and particle dynamics (Azam, 1998). Hence, it is important to

understand the fate of the carbon in the ocean and what is the role that bacterial interaction with MGPs play in the mechanisms involved in the aggregation and dissolution of the sinking particles in the ocean.

1.7 Features of the sampling location

The sampling area of this study is the North Sea offshore the North East coast of England. The North Sea is a large semi enclosed shallow sea of the European continental shelf with depth less than 100 m (Paramor *et al.*, 2009). The Atlantic Ocean is the main water influx into the North Sea through the English Channel and northern North Sea. The North Sea is high in nutrients mainly nitrates and phosphates, where higher concentrations are found in the coastal waters due to the effluents of the rivers. The surface water temperature generally ranges between 0-20^{o}C with seasonal variation and average salinity is 32-34.5 ppt of coastal areas (Walday *et al*., 2002).

1.8 Biology and ecology of *Pseudoalteromonas atlantica*

P. atlantica is gram-negative marine motile bacteria that belongs to Gammaproteobacteria. It is found in water column and living in association to seaweed and crabs (Copeland *et al.* 2006; Costa-Ramos and Rowley, 2004). *P. atlantica* produces acidic polysaccharides, EPS and forms biofilms (Perepelov *et al*. 2005) and various extracellular compounds including proteolytic enzymes (Hoffman and Decho, 2000) and extracellular alginate lyase (Matsushima *et al.* 2010).

1.9 Importance of this study

MGPs are clearly integral to the formation of sinking aggregates and marine snow and the interaction of MGP with bacteria involves the production of extracellular enzymes. To understand this in more detail requires an understanding of bacterial community composition and functional capabilities. Therefore, the aim of this work is to generate new insight into MGP composition in terms of its association with eDNA, in particular the presence of marine bacterial extracellular DNases in MGPs.

Therefore, this thesis addresses the following questions:

1. Does eDNA present in the MGP matrix?

2. What is the structure and the potential function of the bacterial community associated with MGP (in the North Sea), in the size fraction 0.2-100 µm size range of initial microbial attachment that has not been well studied?
3. Is the production of DNase enzyme common among marine bacteria and MGP-associated bacteria in particular?

Considering that MGP-associated bacterial communities can affect MGP aggregate integrity through growth, nutrition, and interactions involving the expulsion of intracellular material to the surrounding MGP microenvironment, leading to MGP aggregation and/or disintegration. Therefore, the key hypothesis of this study is that eDNA is a component of MGPs, and DNase secretion probably common within MGP-associated bacterial communities.

1.10 Aim and objectives

The overarching aim of this study is to characterise the MGPs structural composition and the bacterial communities associated with them.

The objectives of this study are as follows:

1. Develop appropriate methods for isolating MGP from seawater to allow their further characterisation.
2. Investigate the presence of extracellular DNA in the MGP.
3. Set up a bacterial gel particle as a floc model of MGP, from a single bacterium of *Pseudoalteromonas atlantica*.
4. Study the structure and function of the microbial community associated with MGP by total DNA extraction and 16S rRNA metabarcoding for community analyssis.
5. Investigate the production of nuclease in bacteria isolated from the marine environment free living (FL) and particle attached (PA) of MGP.
6. Examine how DNase affects MGP bacterial particle degradation.

1.11 Thesis outline

To accomplish the aim and objectives, this thesis is structured as followings. Chapter 1 has reviewed current knowledge on the origins of MGP and the factors that affect MGP dynamics in the ocean in its relation with bacterial interactions. Chapter 2 highlights the compositional components of MGP using a method of MGP isolation based on the resuspension of MGP isolated from small liquid sample volumes collected on polycarbonate filters. It also investigates the presence of eDNA in natural MGP and by marine bacterial EPS of the

Pseudoalteromonas atlantica MGP bacterial floc model. Chapter 3 explores the composition and function of the bacterial community associated with MGP focusing on nucleases. Chapter 4 studies the diversity of free living (FL) and particle attached (PA) marine bacteria producing deoxyribonucleaese, using culture-dependent methods. Finally, Chapter 5 provides a general discussion and conclusions on the overall results from this work and discusses future directions and recommended work.

Chapter 2. Isolation and staining reveals the presence of extracellular DNA in marine gel particles

2.1 Abstract

Methods for examining the biochemical composition of marine gel particles (MGPs) have been limited to the detection of acidic polysaccharides and proteins, in transparent exopolymeric particles (TEP) or Coomassie stainable particles (CSP). The dynamic ecological interactions between microorganisms in the oceans within the micro-niche of MGPs can results in the secretion of extracellular polymeric substances (EPS) which can influence the structure and stability of these particles. Although extracellular DNA (eDNA) is well known as a component of bacterial EPS and biofilm it has not been previously recognised in MGPs. Here, it is hypothesised that eDNA is present in MGPs due to the presence of bacteria in these protobiofilm like particles. Therefore, the aim of this chapter was to isolate MGPs and use *Pseudoalteromonas atlantica* EPS as MGP model, to investigate the presence of eDNA in MGP milieu. MGPs were isolated from North Sea surface seawater using polycarbonate (PC) filters and gentle vacuum filtration. The gel particles that were retained after filtration through these PC filters ranged in size from 0.4 to 100 µm in size. A new method for MGP isolation in liquid suspension was developed here for the first time as previous studies of MGPs have all studied the particles on filters. Isolating MGPs in suspension enabled them to be studied as they occur naturally in the environment. To investigate the occurrence of eDNA in MGPs, fluorescent microscopy was employed using two different eDNA dyes: YOYO-1 with Nile Red (targeting cell membranes) as a counterstain and TOTO-3, which was also stained with ConA for localising glycoproteins and SYTO-9 for live/dead bacterial nucleic acid. Stained MGPs were visualised using confocal laser scanning microscopy (CLSM). Staining indicated the presence of proteins and polysaccharides. eDNA was localised throughout the natural MGPs and also in artificial flocs of *P. atlantica*. Collectively, these results provide the first evidence which supports the widespread presence of eDNA throughout MGPs. This has significant implications for our understanding of the dynamics and fate of the MGPs in the ocean.

2.2 Introduction

MGPs are organic materials that are released by bacteria and diatoms in the ocean; they are mainly made up from EPS that assembles naturally (Chin *et al.*, 1998; Verdugo *et al.*, 2004; Verdugo and Santschi, 2010). These gel materials are sticky and they have been studied only on filters (Alldredge *et al.*, 1993). Moreover, their adhesiveness enables MGPs to form suspended clumps (flocs) or biofilms when attached to surfaces. Many studies evidence the involvement of MGPs in biofilm formation in the marine environment, mainly on reverse osmosis (RO) membranes of desalination plants (Bar-Zeev *et al.*, 2009; Lee *et al.*, 2015; Li *et al.*, 2015). It is clear that the structures and behaviour of MGPs and biofilms are similar and MGPs have also been referred to as protobiofilms (Bar-Zeev *et al.*, 2012). This suggests that they may share the same structural constituents. The biochemical composition of attached biofilms is well studied, while conversely the composition of MGPs is understudied. Hence, it is important to fully understand the structure and biochemical composition of MGPs and the way they occur naturally in seawater in liquid suspension, rather than on filters (Alldredge *et al.*, 1993; Villacorte *et al.*, 2015). This can make the study of natural MGPs quite challenging and it requires a proper isolation technique. Prior studies have all investigated MGPs based on filters or fixed to microscope slides, that has hindered comprehensive vision into the particles natural existence as three-dimensional structure (Hewes and Holm-Hansen, 1983; Engel, 2009). A recent study has employed in-situ FlowCAM for online quantification of TEP that can give a real time results (Thuy *et al.*, 2017). However, this method is not-feasible for all studies in particular those that require transfer to laboratories and depend on analysis by other instruments. Therefore, the main driver for the development of a new isolation technique in this study was to separate MGPs from natural seawater to get access to intact particles in three dimensions for consequent characterisation.

The biochemical composition of MGPs has been based on their binding to Alcian blue and Coomassie brilliant blue dyes, subsequently designated two classes of particles: acidic polysaccharides (TEP) and proteinaceous particles (CSP). These categories are an over simplification and their continued use limits current understanding of MGPs. Moreover, MGPs are a rich source of nutrients that are a niche for bacterial attachment and colonisation that also involves the formation of biofilm-like communities on these particles (Yawata *et al.*, 2014; Pelve *et al.*, 2017). To this end, the excretion of bacterial EPS co-occurs with eDNA flux that is released during the bacterial life cycle via active or passive cell lysis or the active transport of DNA (Vlassov *et al.*, 2007; Suzuki *et al.*, 2009; Ibáñez de Aldecoa *et al.*, 2017).

Consequently, the released eDNA may possibly play a role in MGPs' structure and integrity, as in biofilms (Whitchurch *et al.*, 2002; Okshevsky and Meyer, 2015). Furthermore, as eDNA is recognised as a glue for bacterial adhesion in biofilms, it is reasonable to hypothesise that if present, it may have the same function in the MGP matrix (Das *et al.*, 2010). However, the occurrence and function of eDNA in MGPs have not yet been studied. Despite few studies on the composition of marine snow aggregates that has reported cell enclosed DNA in the microbial colonisers of the particles (Holloway and Cowen, 1997a; Flintrop *et al.*, 2018). However, the composition of MGPs with regard to eDNA has not been characterised previously. Moreover, the smaller size MGPs are the building blocks of marine snow and sinking aggregates. Nonetheless, the precise characterisation of eDNA in the composition of MGPs is scant, although it is vital to comprehend the aggregation behaviour and the bacterial colonisation of these particles.

The identification of the original source of the MGPs in the ocean is challenging due to the various possible producers, including diatoms, bacteria and other organisms (Stoderegger and Herndl, 1999; Passow, 2002a; Ortega-Retuerta *et al.*, 2010; Deng *et al.*, 2016; Iuculano *et al.*, 2017). Added to that, the rapid development, adherence and dispersal behaviour of these particles make the composition of the MGP pool undistinguishable. Therefore, to overcome the constraints of the heterogeneity and structural complexity of natural MGPs, the production of gel particles by a single bacterial species as a model for MGPs was developed here. Given that MGPs are produced by many marine bacteria, *Pseudoalteromonas atlantica* is a well-studied marine bacterium which known for high EPS production, initiate biofilms and contributes to MGP aggregation (Li *et al.*, 2016; Roca *et al.*, 2016; Yamada *et al.*, 2016). This single species *P. atlantica* MGP model system would allow more careful experimentation and understanding of the nature and composition of bacterially derived artificial MGPs which could give deeper insights into our understanding of natural MGPs.

Studies of the presence of eDNA in biofilms often employ fluorescent staining and microscopy, mainly with Confocal Laser Scanning Microscopy (CLSM) (Okshevsky and Meyer, 2014; Yuan *et al.*, 2015; Reichhardt and Parsek, 2019). CLSM with fluorescent dye labelling is an efficient microscopy technique used to gain information on the composition of hydrated matrices like EPS and marine snow (Cowen and Holloway, 1996; Holloway and Cowen, 1997a; Zhang *et al.*, 2015; Schlafer and Meyer, 2017). Sensitive labelling and bioimaging techniques are becoming more widely used in the imaging of organic matter and marine snow (Holloway and Cowen, 1997a; Flintrop *et al.*, 2018). To allow insights into eDNA in the microscale environments of MGPs and a more precise understanding of the composition of MGPs.

Fluorescent intracellular DNA (iDNA) dye are used in this study for the visualisation of marine bacteria associated with particles, as this dye can penetrate cell membranes and bind to DNA allowing differentiation between iDNA and eDNA (Qin *et al.*, 2007; Samo *et al.*, 2008; Hu *et al.*, 2012; Gallo *et al.*, 2015). However, previous reports have not looked at the occurrence of eDNA in the MGP matrix. It is hypothesised here that eDNA is expelled into the MGP matrices as a result of bacterial interactions with and within the MGPs like; cell lysis due to growth, reproduction and death, defence and grazing on the matrix components. Therefore, the aim of this chapter was to investigate the presence of eDNA in natural and artificial MGPs.

2.2.1 Aim and objectives

The aim of this study was to isolate marine gel particles from seawater and to investigate the presence of eDNA in the MGP composition.

Objectives

1. To isolate MGPs on filters and to concentrate the particles by recovering them in a small volume of filtered sterile seawater.
2. To stain MGPs with Alcian blue and Coomassie brilliant blue for a conventional EPS composition test.
3. To investigate the presence of eDNA in natural MGPs by a combination of fluorescent staining techniques, visualised using bioimaging CLSM.
4. To investigate the presence of eDNA in artificial MGPs composed of flocs of *Pseudoalteromonas atlantica*.

2.3 Materials and methods

2.3.1 Seawater collection

Seawater samples from the North Sea were collected on board the Princess Royal research vessel from approximately one mile offshore from the Northumberland coast (55°06.972 N, 1°25.600 W) on 23/03/2015, 23/02/2016, 18/07/2016, 31/10/2016, 13/02/2017 and 25/03/2018. Surface seawater was transferred to the laboratory in Nalgene bottles of 5 and 10 L volume and stored at 4°C in a cold room.

2.3.2 Isolation of MGPs from seawater by filtration

The seawater was pre-filtered through a 100 µm mesh sieve to remove larger particles and other organisms. Then the 100 µm filtered seawater was vacuum filtered onto pore size 0.4 µm polycarbonate (PC) filters (Whatman, GE, USA) with gentle vacuum filtration carried out using a glass vacuum filtration device (47/50 mm, Sartorius, UK, https://www.sartorius.co.uk/), which was used to collect the filtered particles. For MGP collection the vacuum filtration was set up with a vacuum pump (Sartorius) with a pressure controller set to minimum < 150 mmHg (Engel, 2009). PC membrane filters (GE Healthcare Whatman™, UK, https://www.fishersci.co.uk) were chosen because of their smooth surface, which enables easy particle removal. PC filters with different pore sizes were used throughout this work for different independent experiments, as will be mentioned later in each chapter. However, here the smaller size of 0.4 µm was used as particulate gel particles ranged between 0.4 and 100 µm in diameter (Passow and Alldredge, 1995). To avoid clogging of the filters, each PC filter was replaced with a new one for every 1L of seawater filtered.

2.3.3 Isolation of concentrated MGPs in suspension

In order to concentrate the particles collected, after each filtration process as described in section 2.2 the PC filters were suspended in 50 ml falcon tubes containing 5 ml of 0.2 µm filtered, sterilised autoclaved seawater (Millipore Syringe Filters, Cole-Parmer). The particles on the PC filters were pooled in the liquid medium with gentle shaking to allow the particles to be released from the filters. The particles in liquid suspension is mimicking the natural

occurrence in the aquatic environment. Particles were stored at 4ºC until required for further experiments.

2.3.4 Bacterial strains and growth conditions

Single species of bacteria were used to set up model systems for the study of artificial MGPs. Three marine bacterial strains, *Pseudoalteromonas atlantica* (DSM-No. 6840), *Pseudoalteromonas citrea* (DSM-No. 8771) *and Idiomarina homiensis* (DSM-No. 17923), were obtained from DSMZ- German Collection of Microorganisms and Cell Cultures (DSMZ) (https://www.dsmz.de/). They were used for the production of exopolymers to be used as an artificial MGP model.–These bacteria were chosen based on their ability to produce gel polymers, as reported in previous studies (Li *et al.*, 2016; Roca *et al.*, 2016; Yamada *et al.*, 2016). These strains were first cultivated in marine broth (Difco 2216, Becton Dickinson, UK) according to the DSMZ instructions and they were preserved in 50% glycerol stock at -80ºC. Each bacterial isolate was then grown on marine agar plates (Difco marine agar 2216, Becton Dickinson, UK) and incubated at 20ºC for *P. atlantica*, 26ºC for *P. citrea* and 28ºC for *I. homiensis*. *P. atlantica* showed best polymer production and was therefore selected here as the most suitable species for further study.

2.3.5 Production of gel particles by marine Pseudoalteromonas atlantica cultures

In sterile Erlenmeyer flasks of 50 ml volume, a single colony of *P. atlantica* was inoculated into 20 ml of 0.2 µm filtered artificial seawater (ASW; 33 g/L, Sea salts: Sigma-Aldrich, UK) at a salinity 33-34‰ to mimic natural seawater salinity. Then the bacterial inoculum was incubated with agitation at 150 rpm using an orbital shaker at room temperature.

2.3.6 Compositional analysis of MGPs produced by P. atlantica

To investigate the composition of the artificial MGPs, multiple staining techniques were used. The filtered particles were stained for acidic polysaccharides following Alldredge *et al.* (1993) with a modification in the washing procedure. Particles were stained on filters with 0.2 µm-filtered 0.02% Alcian blue (Sigma-Aldrich) dissolved in 0.06% (v/v) acetic acid (pH 2.5) for 1 minute and washed with MilliQ water three times to eliminate excess dye, followed by gentle vacuum filtration as described previously in section 2.3.2. Coomassie brilliant blue G (CBBG, Sigma-Aldrich), was used (Long and Azam, 1996), with modification in the washing step. The

particles were washed with MilliQ water three times to eliminate excess dye, followed by gentle vacuum filtration as described previously.

2.3.7 MGP staining with eDNA probes

Two staining techniques were used to probe eDNA presence in natural MGPs and *P. atlantica* EPS particles in the same manner. Initially, a combination of fluorescent stains, YOYO-1 (Life Technologies) and Nile red (Sigma-Aldrich), was used, as previously described by Rostami *et al.* (2016) with modification as it is applied here for particles. YOYO-1 is an ultrasensitive cell-impermeant dye and it is a high affinity nucleic acid stain targeting eDNA. Nile red is a lipophilic stain for intracellular lipid used widely as a membrane dye. It was applied as a counter stain to differentiate between the bacterial cell membranes and the eDNA found in the MGPs. In 2 ml sterile tubes, 500 µl of the suspended particles were stained with YOYO-1 (Rostami *et al.*, 2016) at a concentration of 2.4 nM for 10 minutes at 20°C covered in foil to avoid light. Nile red was then added at a concentration of 1 µg/mL for 15 minutes, covered with foil and stored in dark prior to microscopy.

Another set of tri-stain combining Concanavalin A (Con A, Tetramethylrhodamine Conjugate A, C860, Life Technologies) a lectin stain used to characterise glycoproteins and carbohydrates, TOTO-3 (T3604, Life Technologies) a cell-impermeant DNA stain used for eDNA probing and SYTO-9 (Life Technologies) a cell permeant DNA stain for nucleic acid of live and dead bacterial cells. TOTO-3 and SYTO 9 are commonly applied together to distinguish between intracellular and extracellular DNA (Pakkulnan *et al.*, 2019). Furthermore, the selection of dyes combination in both of the sets used have considered the elimination of the overlap between excitation and emission wavelengths to ensure clear contrast of the fluorophore.

In 2000 µl sterile tubes, 500 µl of the suspended particles were stained with 10 µM Con A (stock concentrations 1 mM), 2 µM TOTO-3 (stock concentrations 1 mM) and 20 µM SYTO 9 (stock concentrations 5 mM). Stains were added to 500 µl of the filtered MGPs or *P. atlantica* particles in the same manner. A control of unstained particles was used from the same filtered particles or culture. The labelled samples were incubated in the dark for 15 minutes at room temperature prior to bioimaging.

MGP visualisation by microscopy

Samples were placed in a pre-sterile µ-Slide with 8 wells and a glass bottom (Ibidi, https://ibidi.com/). A volume of 250-300 µl of each sample was placed in each well. This

volume was chosen to minimise the sample thickness inside the wells and to unify the focal plane. A Leica TCS SP2 fluorescent microscope (Leica Microsystems CMS GmbH, Germany, www.leica-microsystems.com) was used for the initial observations of MGPs. The observations of the natural MGPs were made using inverted microscopy and the MGP particle samples were visualised at different magnifications. Direct observation was done under microscopy. Images were captured at different time intervals and durations. The fluorescent dye SYTO 9 was used to visualise the microorganisms attached to the particles.

Bioimaging

A CLSM at the bioimaging unit, Newcastle University, was used for the bioimaging of the MGPs. Images were acquired with a Leica SP8 (www.leica-microsystems.com), which is a high-resolution fluorescent inverted microscope with water immersion. The lens used was model HCPL APO CS2, objective 63x/1.20 numerical aperture (NA). The CLSM was used to visualise the MGPs (Ploeger *et al.*, 2008) as it emits an intense light and is able to make thin optical slices for three-dimensional reconstruction, which was needed to visualise the relatively large three-dimensional particles. The fluorophore was stimulated by a white light laser (WLL), where each fluorophore was excited and emitted (acquired) at certain suitable wavelengths as described in Table 2.1. The aggregate matrix staining was evaluated based on the binding and visibility of the stains.

Table 2.1 List of fluorophores and the excitation and acquisition wavelengths of visualisation

Fluorophore	Target	Excitation (nm)	Aquisition (nm)
YOYO-1	eDNA	491	501-532
Nile Red	Cell membranes	553	567-781
Concavalin A (TMR)	Glycoproteins	552	560-643
TOTO-3	eDNA	642	652-784
SYTO 9	DNA	483	490-591

2.3.8 Quantification of the fluorescent dyes intensity

The fluorophores in the CLSM-acquired images were quantified by LAS X software (Leica microsystems), where the mean values of the fluorescent intensity were measured by quantifying the area of the particle. The mean intensity values of each channel (fluorophore

acquisition) were compared to the control, i.e. the unstained particles. The averages of five particle replicates were measured and the experiments were repeated three times. The average mean values of the fluorescence intensity of 15 particles were quantified per stain, repeated in three experiments (mean ± SD; $p<0.05$) and compared between stained and unstained particles using unpaired t-test.

2.4 Results

2.4.1 Filtered MGP observations

The implemented technique for filtering and re-suspending MGPs from the PC filters in a 5 ml volume of 0.2 µm filter sterile ASW enabled a new method to be developed to obtain concentrated MGPs in liquid suspension, rather than the more widespread method used in classical studies where MGPs are attached to filters (Alldredge *et al.*, 1993; Engel, 2009; Villacorte *et al.*, 2015). A range of MGP sizes from 0.4 to 100 µm was obtained from the PC filter suspension. MGPs were visualised at different magnifications, as presented in Figure 2.1 (A-D). The concentrated particles obtained in the test tubes appeared by microscopy visualisation in various heterogeneous sizes and shapes, namely; porous, sheet-like, and filaments-like. The particles (unstained) appeared under the microscope in a bright field with different gradients of grey to dark black as it seems that they contained different materials like faecal pellets and organism residues. The particle in Figure 2.1 A was observed to be emitting some auto-fluorescence that can be due to natural pigments within the particles that could absorb and emit microscope light.

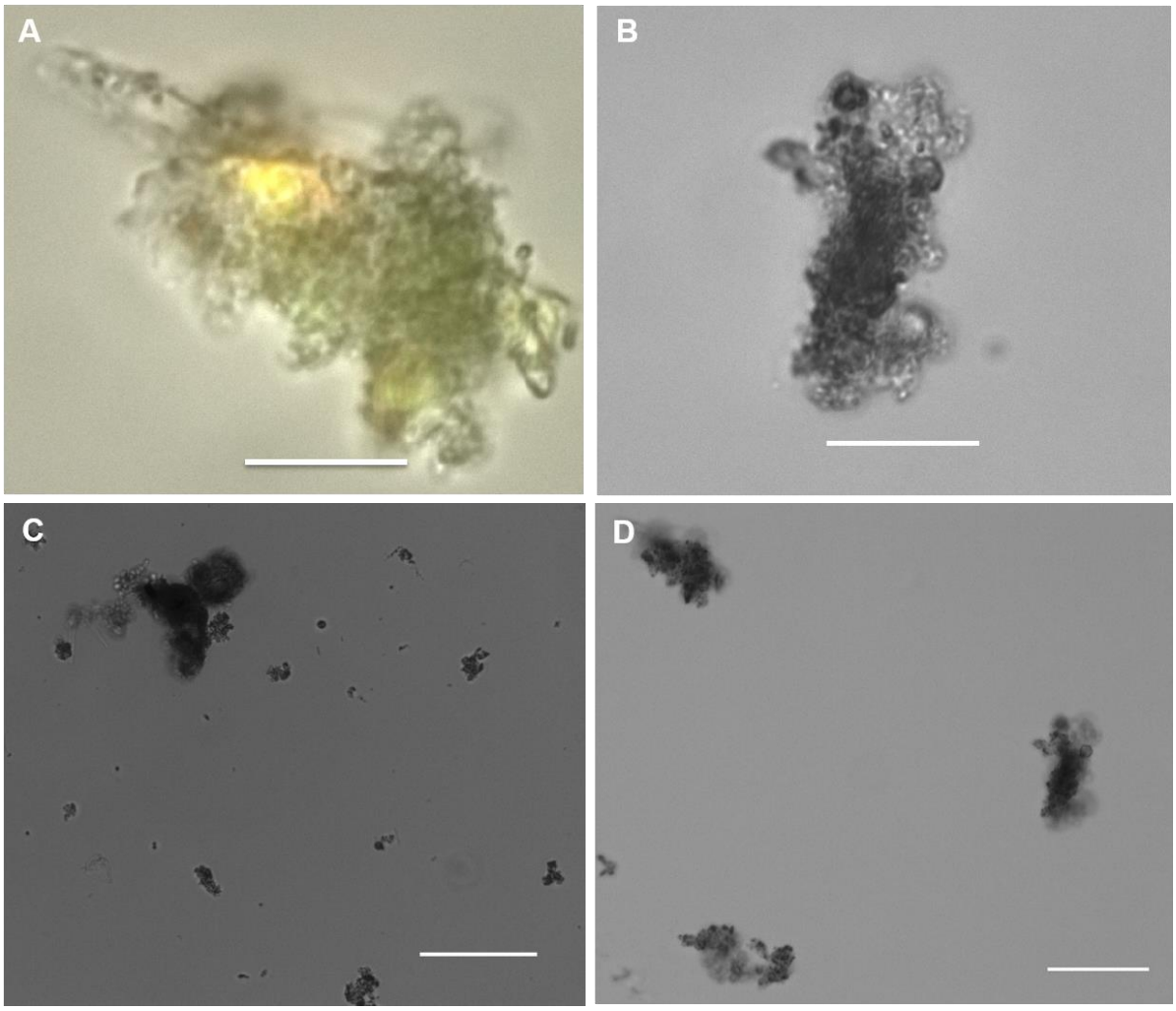

Figure 2.1 Marine gel particles recovered from PC filters after re-suspension in 0.2 µm filter sterilised seawater. Scale bar = 20 µm, image captured at 63x objective and visualisation with phase contrast (A). Scale bar = 50 µm (B, C and D), image captured at 40x objective for B and 20x objective for C and D.

Visulisation of EPS in the MGP matrix

Alcian blue stained EPS in the MGP matrix effectively on the PC filters as shown in Figure 2.2 (A and B). The MGP appeared to bind more to Alcian blue than to Coomassie brilliant blue (Figure 2.2 C and D).

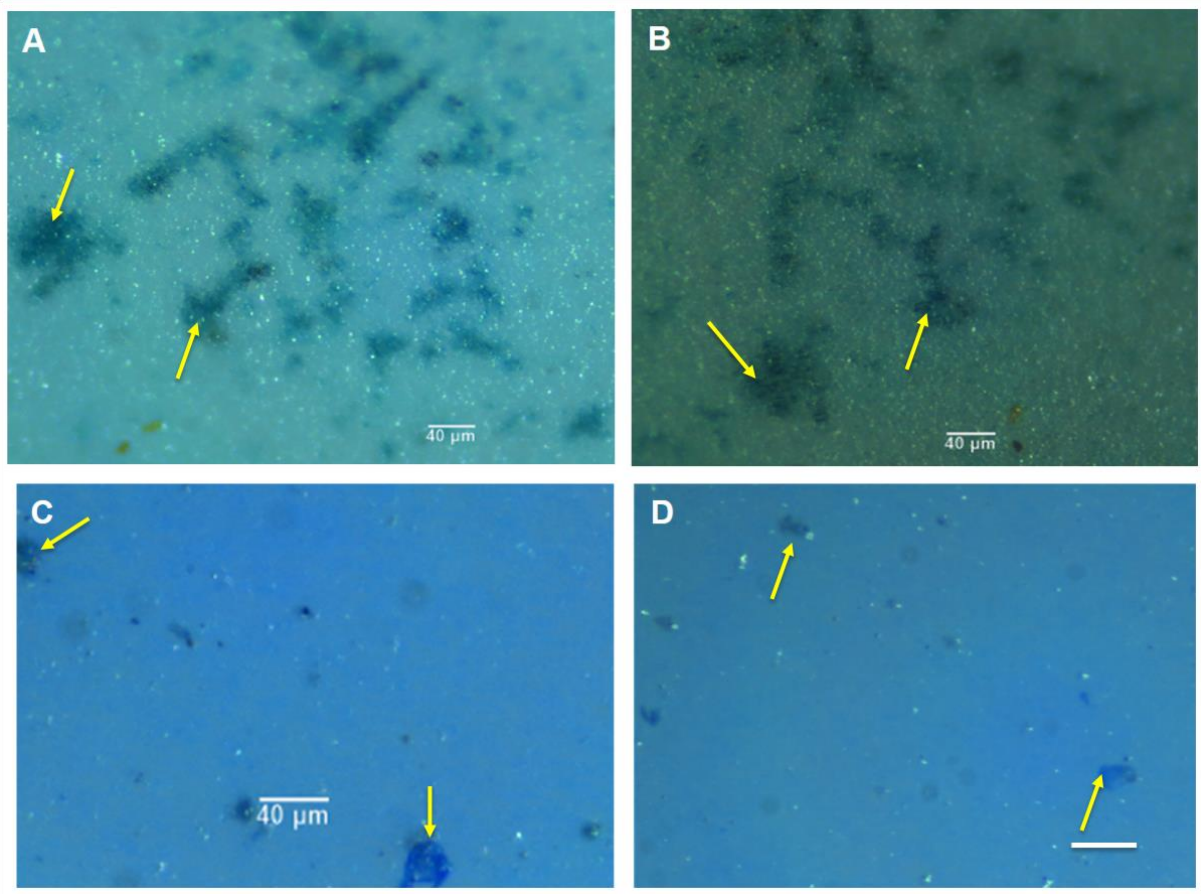

Figure 2.2 MGP from off the Northumberland coast (the North Sea), visualised using bright-field microscopy with fibre light. The MGPs were stained with Alcian blue dye (A and B; yellow arrows) and with Coomassie Blue (C and D; yellow arrows). Scale bar = 40 µm. Images captured using upright Leica microscope and fibre light with 10x objective.

The visualisation of purified MGPs in a bright field is presented in Figure 2.3 A. The MGPs visualised have different particle sizes range from 0.8 µm to 200 µm. Staining MGPs with SYTO 9 (green), a live/dead bacterial DNA stain, has shown that the MGP particles contain microorganisms, as revealed by the bright green DNA stain (Figure 2.3 B). Large aggregates are intensively colonised by microorganisms. Bacteria are associated with particles and some appeared in single bacterial cells suspended in the sample.

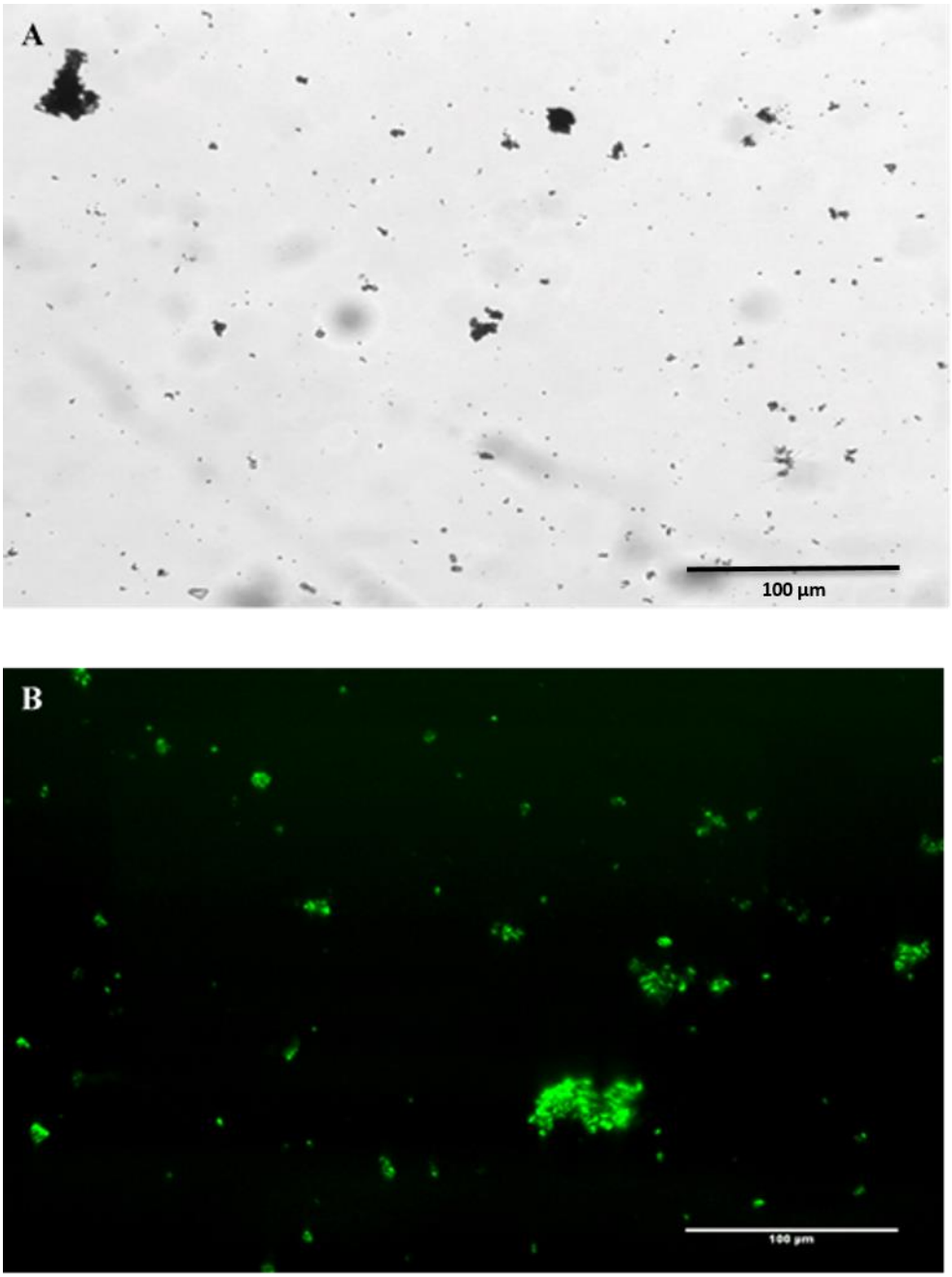

Figure 2.3 Natural MGPs of different sizes from the North Sea. Bright field microscopy images of MGPs (A) Nucleic acid SYTO 9 green stain of MGP revealing bacterial presence. Fluorescent staining with DNA dye SYTO 9 (1 nM) (B). Scale bar = 100 µm. Image capture usingt 20x objective.

2.4.2 Visualisation of eDNA in natural MGP matrix

Confocal laser scanning microscopy (CLSM) images clearly demonstrated the localisation and presence of eDNA in the matrices of natural MGPs collected from the North Sea. This has been confirmed via two sets of fluorescence staining that were applied separately to investigate the occurrence of eDNA in MGPs (Figure 2.4 A). Moreover, quantification of the average mean

value of the fluorophore intensity using Leica software, as depicted in Figure 2.4 B of YOYO-1 (green; that represent eDNA occurrence) mean value 7.28 (SD ± 4.33) vs. unstained mean value 0.91 (SD ± 0.76), $p = 0.06$ revealed no significant differences (> 0.05) between the stained and unstained MGPs. With Nile Red mean value 5.75 (SD ± 1.33) vs unstained 1.29 (SD ± 1.57), $p = 0.03 < 0.05$ showing a significant difference between the stained and unstained MGPs.

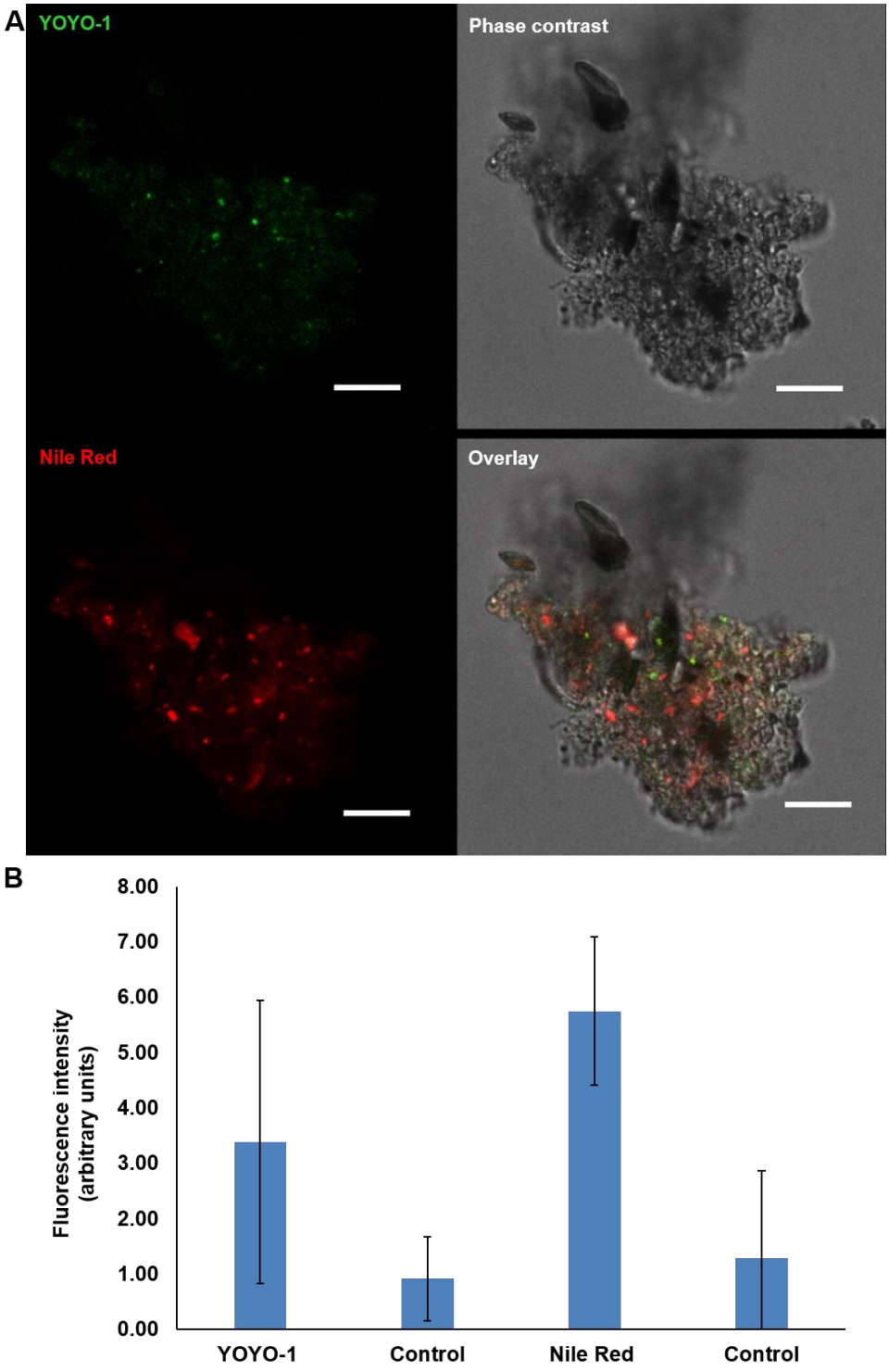

Figure 2.4 CLSM images acquired by Leica SP8 of natural MGPs stained with YOYO-1 to localise the presence of eDNA (green). Nile Red (red) was used as a counter stain for bacterial cell membranes. Scale bar = 5 µm (A). Image captured using 63x objective. Quantification of the fluorescence intensity of each fluorophore against the control (unstained MGPs) (B). The graph presents the mean ± SD of 15 CLSM micrographs. Error bars represent the standard deviation of the data set (n = 3), $p<0.05$. Nile Red intensity showed significant difference between stained and control.

The 3D image of the natural MGP showed a wide distribution of eDNA (YOYO-1) as presented in Figure 2.5. The YOYO-1 stain (green) appeared at different levels of brightness showing the spread of eDNA across the particle areas. On the other hand, the cell membrane stained with Nile Red (red) bound only to limited regions of the particle, representing cell membrane that appeared to occupy larger patches.

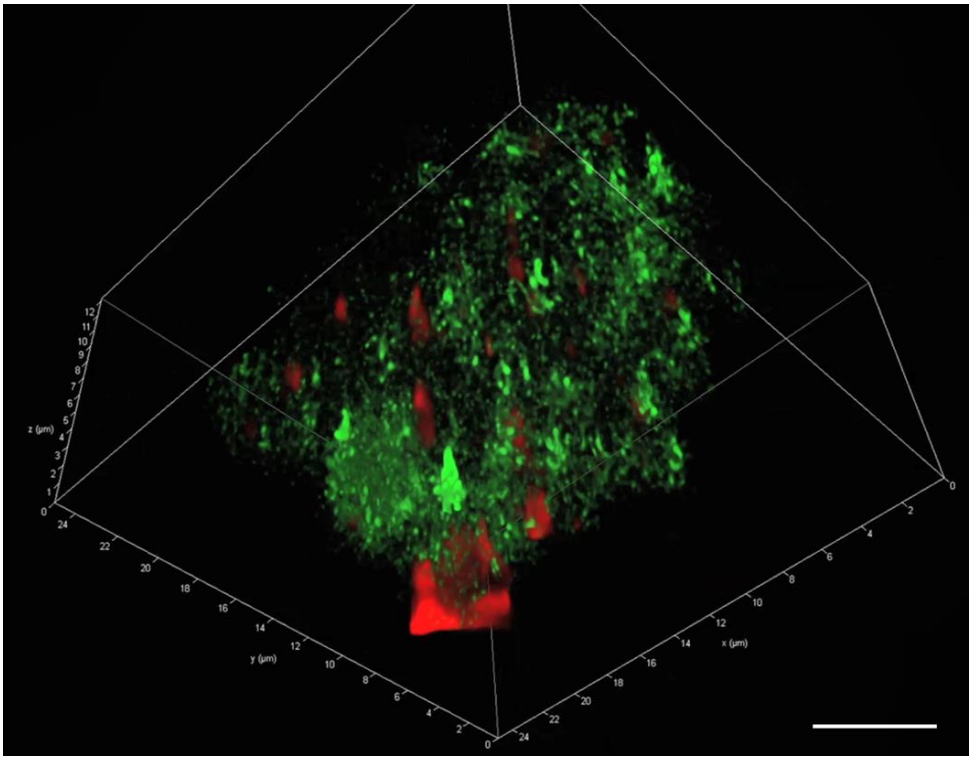

Figure 2.5 A 3D deconvolution image of a single natural MGP stained with YOYO-1 (green = intracellular DNA) and Nile red (red = eDNA). Scale bar = 5 µm. Image captured using Leica SP8 CLSM.

Visualisation and quantification of MGP matrix components using CLSM showed that TOTO-3 (red) has the highest fluorescence intensity of the triple staining set (Figure 2.6A). The TOTO-3 eDNA stain showed the highest occurrence mean value 4.14 (SD ± 1.30) vs unstained mean value 0.94 (SD ± 0.04), displaying a significantly different $p = 0.013$. Con A mean value 3.52 (SD ± 1.31) vs unstained mean value 0.90 (SD ± 0.51) $p = 0.032$. Intracellular DNA stained by SYTO 9 2.24 (SD ± 0.85) vs unstained mean value 0.02 (SD ± 0.01) $p= 0.011$ (Figure 2.6B). The mean fluorescence intensity of all three stains demonstrated significant differences ($p < 0.05$) in comparison to the unstained mean values.

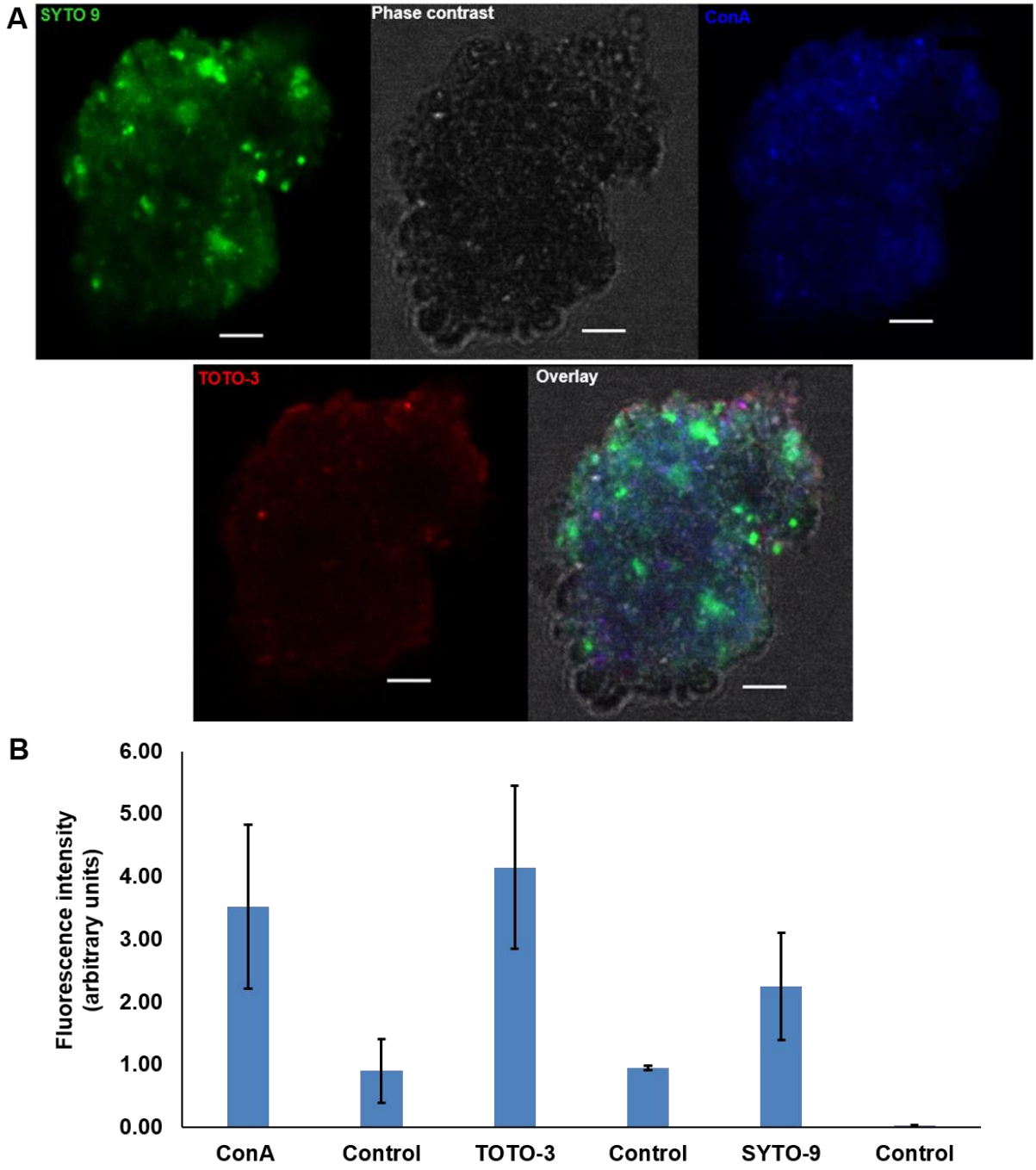

Figure 2.6 CLSM images acquired by Leica SP8 of natural MGPs stained with TOTO-3 (red) to localise the presence of eDNA, SYTO 9 (green) for intracellular DNA and with ConA (blue) for glycoproteins. Scale bar = 5 µm (A). Image captured using 63x objective. Quantification of the fluorescence intensity of each fluorophore against the control (unstained MGP) revealed high mean value of TOTO-3 indicating the presence of eDNA (B). The control is unstained particles. The graphs present the mean ± SD of 15 CLSM micrographs. Error bars represent the standard deviation of the data set (n = 3), $p<0.05$.

The 3D deconvolution image of a single MGP particle stained with ConA, SYTO-9 and TOTO-3 is shown in Figure 2.7, displaying the distribution of the glycoproteins (blue), iDNA (green) and eDNA (red) respectively. The coverage of glycoproteins was less than that of iDNA and

eDNA. The eDNA is spreading out into the larger area of the particle, exceeding the iDNA distribution.

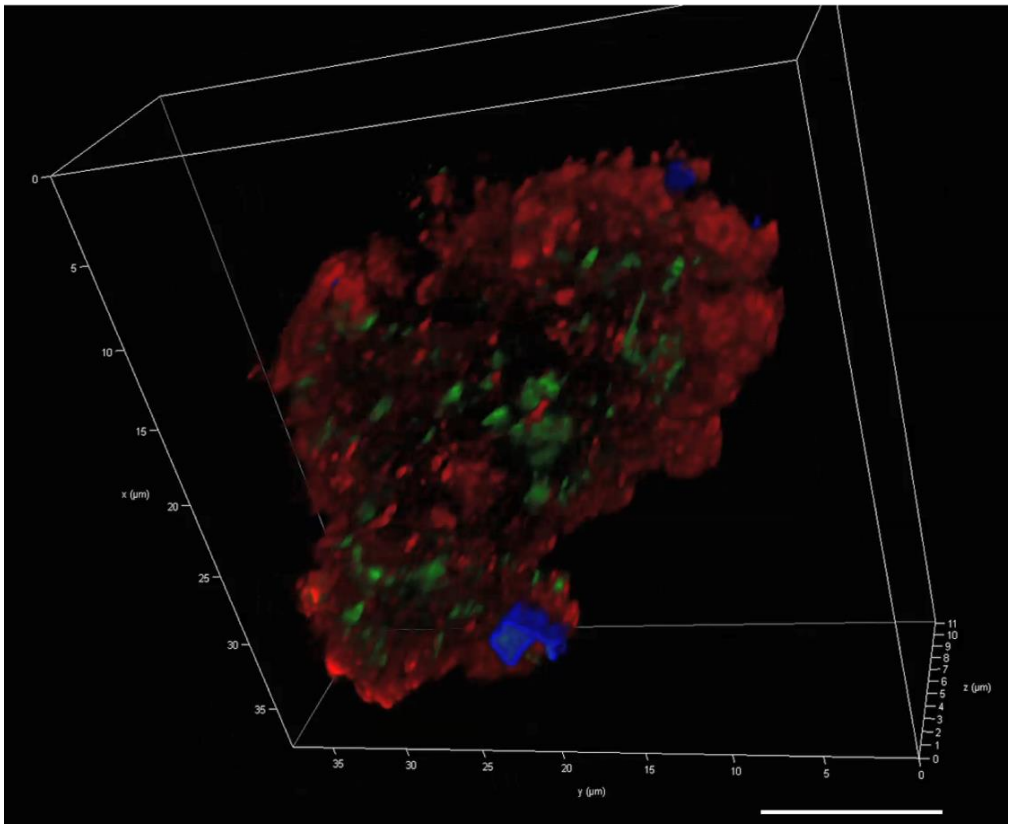

Figure 2.7 3D deconvolution image of a single natural MGP stained with ConA (blue), TOTO-3 (red) stained eDNA and SYTO 9 (green) stained iDNA. Scale bar = 10 µm.

2.4.3 Pseudoalteromonas atlantica EPS

P. atlantica ($281x10^4$ CFU/ml) showed EPS formation in ASW medium. The EPS particles were clearly observable after 96 hours of incubation as demonstrated in Figure 2.8 A, showing aggregation and growing into larger clumps over time (Figure 2.8 B). In the single particle image in a dark field (Figure 2.8C), it looks like the particles have a clear matrix because of the single culture EPS.

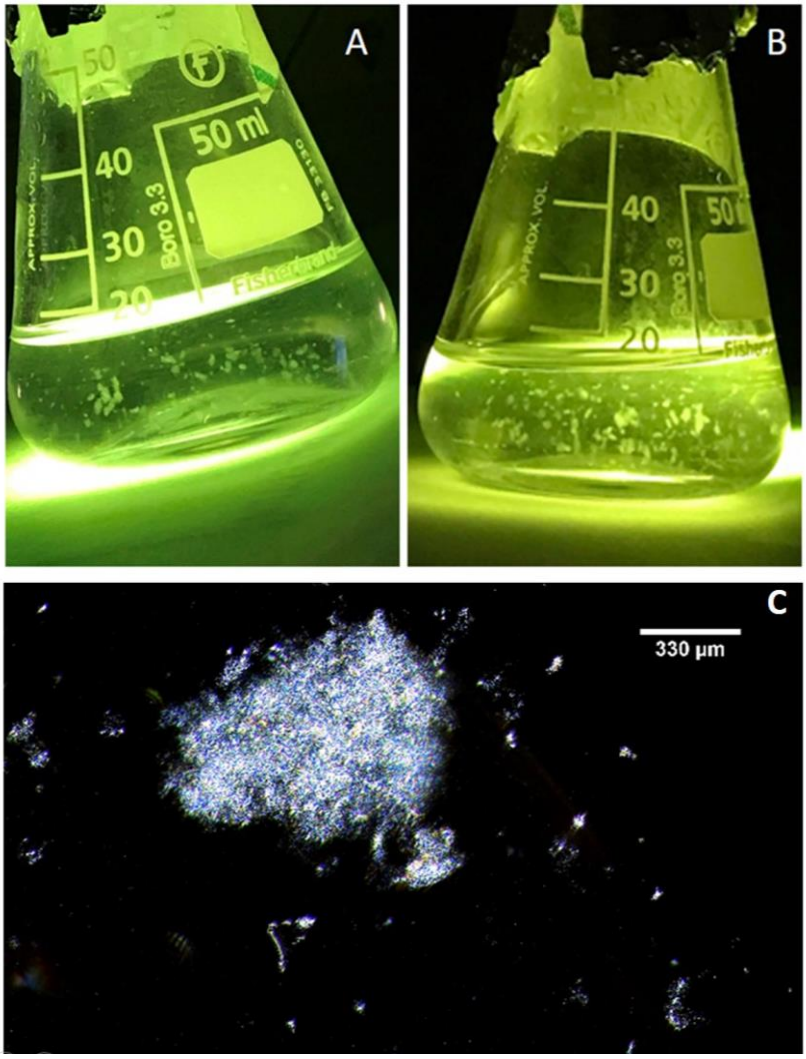

Figure 2.8 *Pseudoalteromonas atlantica* biopolymers as MGP representative grown in artificial seawater after 96 hours (A) and after 168 hours (B). Inverted microscope image captured at 20x of *P. atlantica* particles. Scale bar = 330 µm.

2.4.4 Visualisation of EPS in the P. atlantica matrix

Staining the *P. atlantica* EPS matrix on the PC filters with Alcian blue displayed particles of different sizes bound successfully to the dye, as shown in Figure 2.9 (A&B). Coomassie brilliant blue staining showed visible particles, as presented in Figure 2.9 (C&D). However, the Alcian stained particles here have a distinct shape compared to CSP.

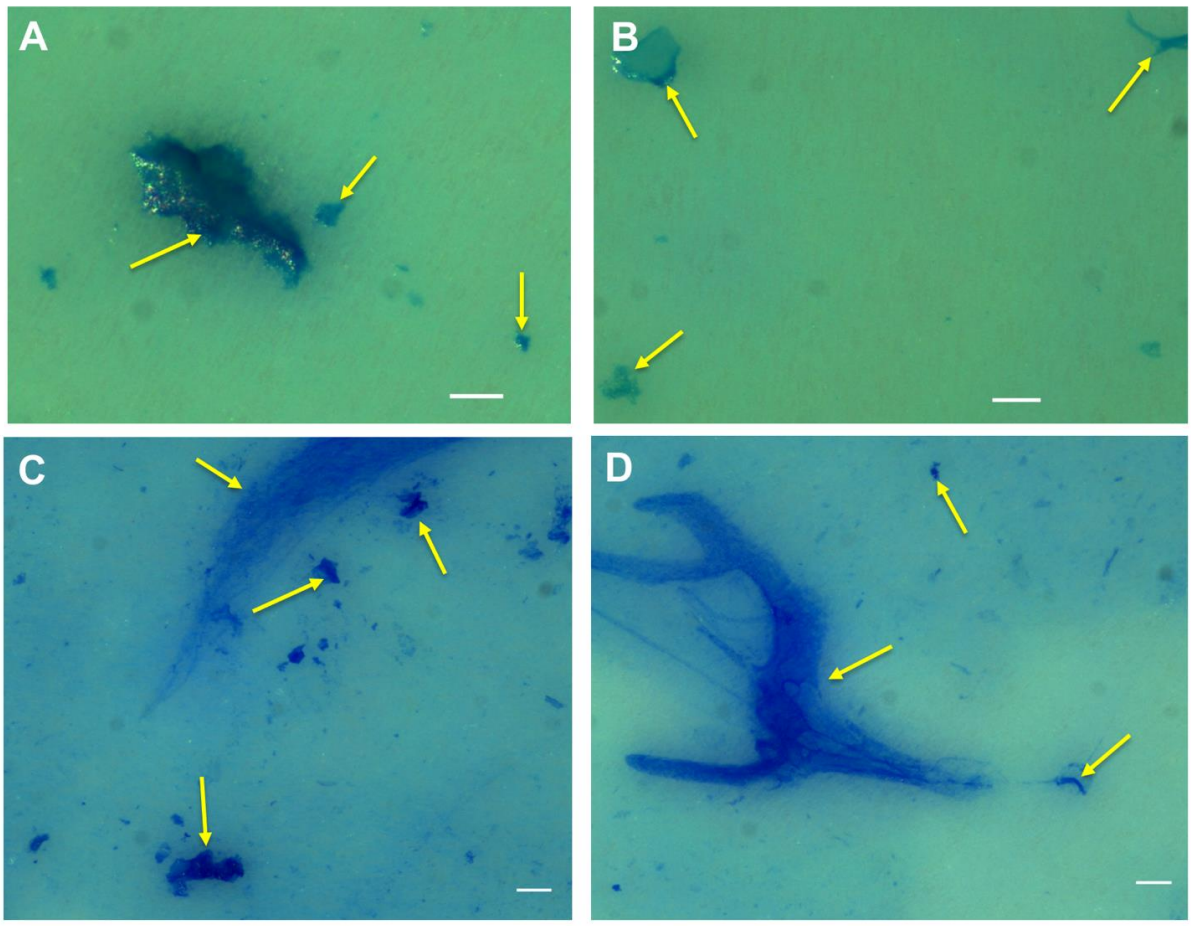

Figure 2.9 *P. atlantica* EPS stained with Alcian blue dye (A and B) and Coomassie Blue (C and D) as indicated by yellow arrows. Images captured using upright Leica microscope with fibre light at 10x objective. Scale bar = 50 µm.

2.4.5 Visualisation of eDNA staining in Pseudoalteromonas atlantica EPS (MGP model)

To assess YOYO-1 staining for the detection of eDNA (Figure 2.10 A), quantification of the average mean fluorophore intensity of YOYO-1 6.99 (SD ± 4.94) vs unstained 0.47 (SD ± 0.08), $p = 0.08 > 0.05$ showed that there is no significant difference between stained and unstained particles. On the other hand, Nile Red 5.69 (SD ± 1.57) vs unstained 1.57 (SD ± 1.62) $p = 0.03 < 0.05$ showed a significant difference between stained and unstained particles (Figure 2.10B). There is a variation between the stained particles. The majority of the YOYO-1 stain was obviously external to the cells spreading in the matrix at different regions and it did not overlap with Nile Red, as shown in the overlay image. Compared to YOYO-1, Nile Red covered different areas of the aggregate matrix and stained slightly distinctive features.

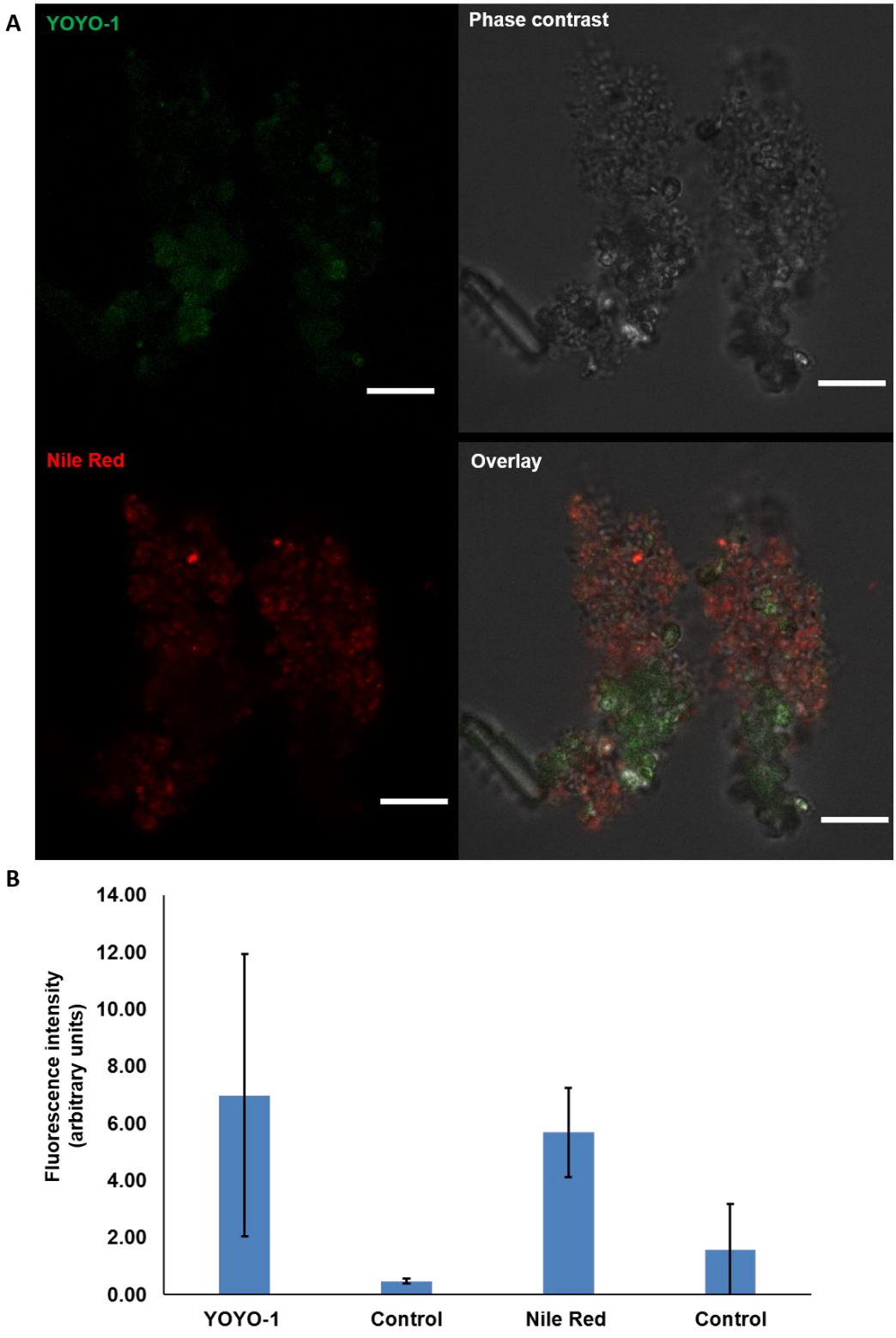

Figure 2.10 CLSM images acquired by Leica SP8 at 63x of *P. atlantica* EPS MGP model stained for eDNA with YOYO-1 (green); Nile Red (red) as counter stain for bacterial cell walls to localise the presence of eDNA. Scale bar = 5 µm (A). image captured using 63x objective. Quantification of the fluorescence intensity of each fluorophore revealed high mean value of YOYO-1 indicating the presence of eDNA. The control is unstained particles. The graphs present the mean ± SD of 15 CLSM micrographs. Error bars represent the standard deviation of the data set (n = 3), $p<0.05$.

A 3D image of *P. atlantica* single particle matrix (Figure 2.11) shows that the distribution of the stains seems to be spreading into more regions in the stained sample (Figure 2.11A) while appearing in smaller spots in the unstained sample (Figure 2.11B).

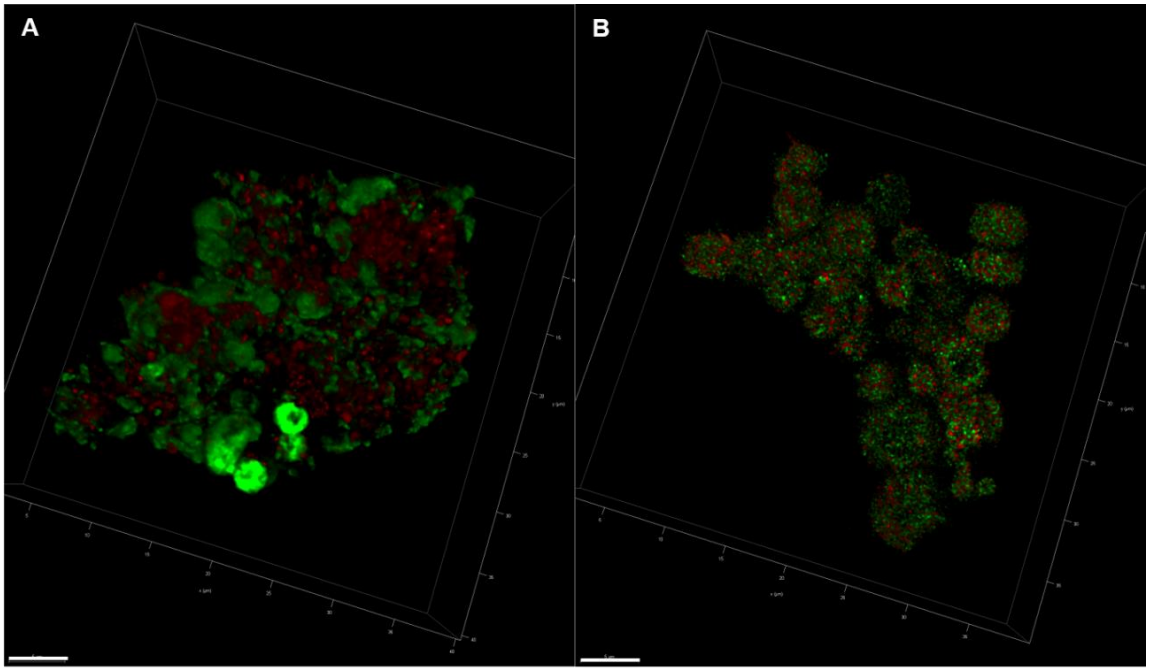

Figure 2.11 3D images of *P. atlantica* particles stained with YOYO-1 (green) for eDNA and Nile Red (red) (A). Unstained particle of the same culture collected at the same time (B). Scale bar = 5 µm. Image captured using 63x objective.

Visualisation and quantification of *P. atlantica* particle matrix components using CLSM are depicted in Figure 2.12 A, showing that Con A has the highest fluorescent intensity of the triple staining set. Con A showed the highest presence in the *P. atlantica* matrix 18.17 (SD ± 10.65) vs unstained 0.25 (SD ± 0.21) significantly different $p = 0.043$. TOTO-3 for eDNA (red) 4.30 (SD ± 1.90) vs unstained 0.18 (SD ± 0.15) $p = 0.019$ significant difference. Intracellular DNA stained by SYTO 9 7.29 (SD ± 3.34) vs unstained 0.02 (SD ± 0.01) $p = 0.019$ as presented in Figure 2.12 B.

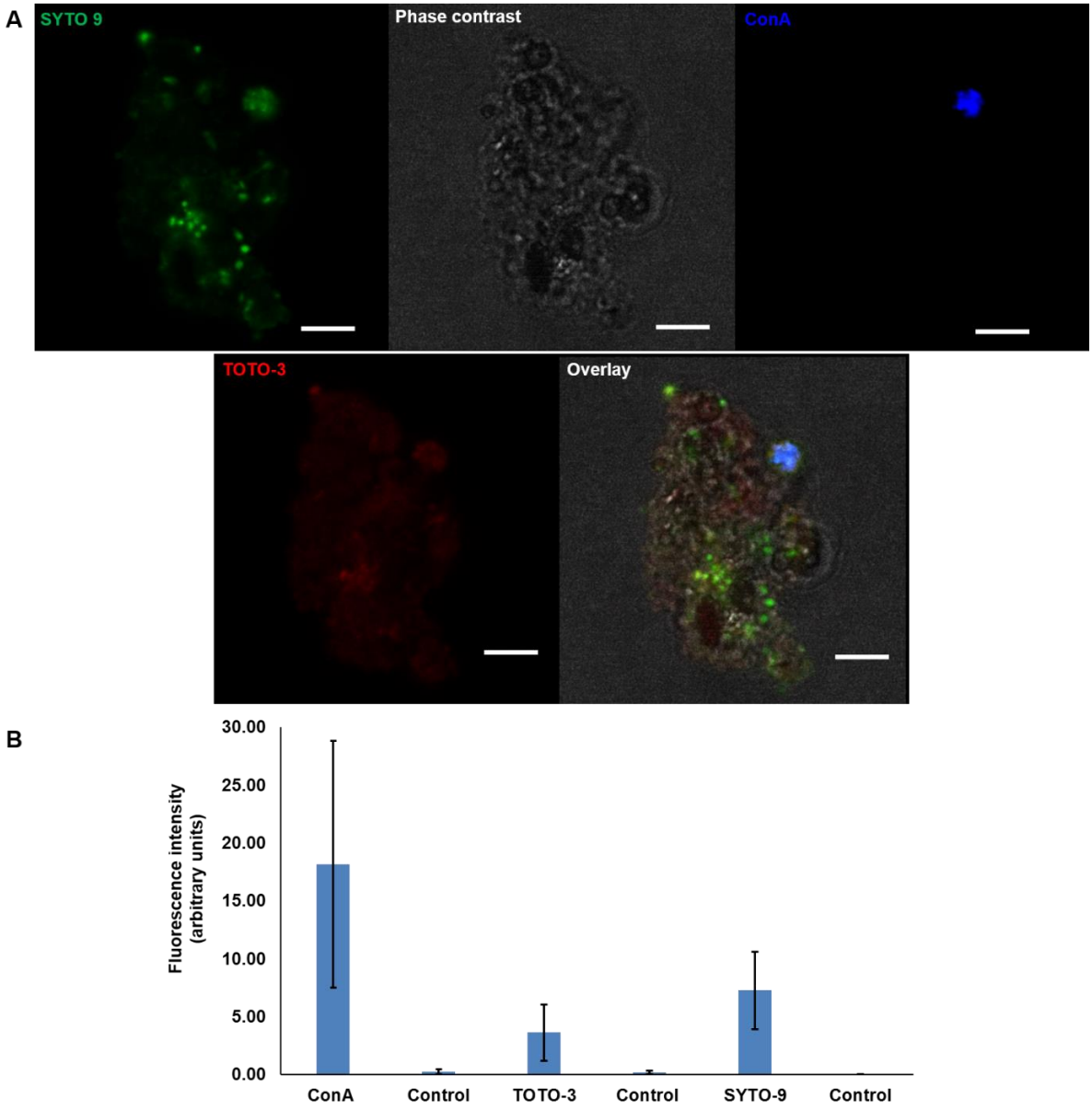

Figure 2.12 CLSM images acquired by Leica SP8 of *P. atlantica* EPS as MGP model stained for eDNA with TOTO-3 (red) and SYTO9 as counterstain (green), and ConA for glycoproteins (blue). Scale bar = 5 µm. Image captured using 63x objective. Quantification of the fluorescence intensity of each fluorophore revealed a high mean value. The control is unstained particles. The graphs present the mean ± SD of 15 CLSM micrographs. Error bars represent the standard deviation of the data set (n = 3), $p<0.05$.

Visualisation of *P. atlantica* particles and eDNA using CLSM in a 3D image (Figure 2.13) showed a clear distribution of the fluorophores with dominance of Con A (blue). TOTO-3 (eDNA, red) appeared scattered in association to the matrix, whereas SYTO 9 (green) appeared in bigger spots.

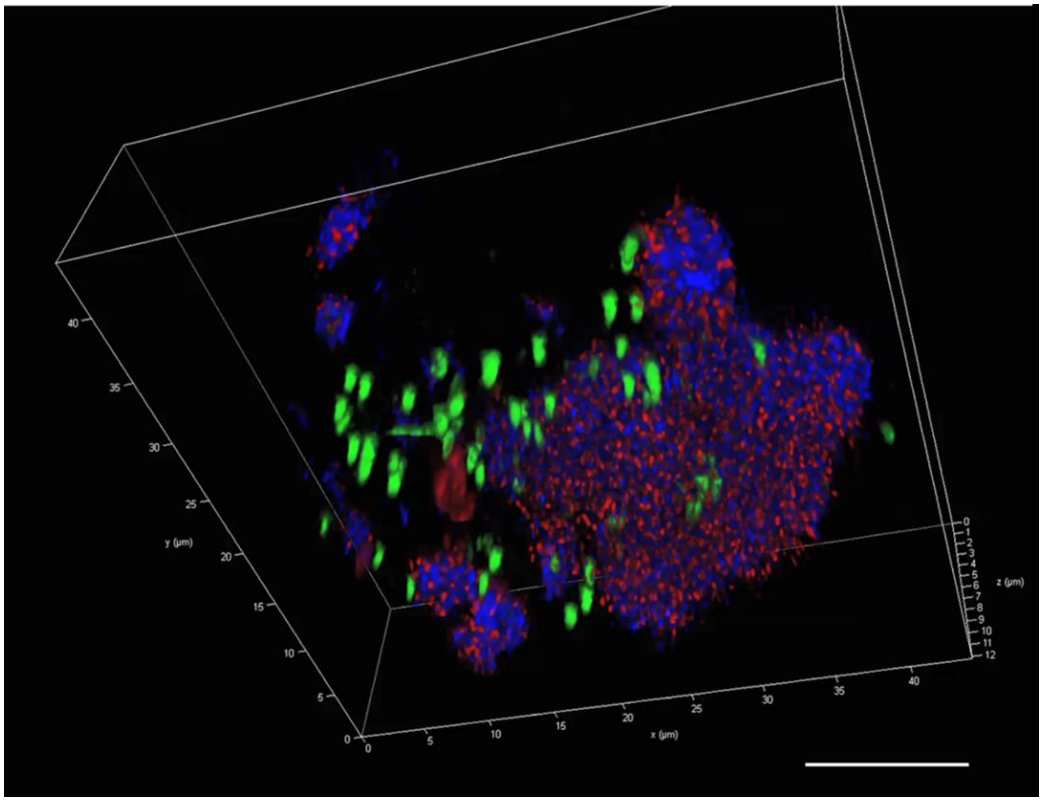

Figure 2.13 3D deconvolution image of a single particle of *P. atlantica* particles stained with ConA (blue), TOTO-3 (red) and SYTO 9 (green). Scale bar = 10 µm. Image captured using 63x objective.

2.5 Discussion

This study shows the isolation of MGPs on the filters by re-suspending the particles in a smaller volume of artificial seawater. This method allowed the maintenance of the MGPs' structure to be studied as it occurs naturally, suspended in seawater, without the need to fix the particles for microscopy visualisation. The approach used here is the first example for MGPs of 0.4-100 µm, as previously they have been studied on filters (Alldredge *et al.*, 1993; Passow and Alldredge, 1994; Villacorte *et al.*, 2015). A similar method has been used to investigate marine snow >500 µm (Flintrop *et al.*, 2018). However, the current study investigated a smaller size range of 0.4-100 µm that makes up the marine snow sinking aggregates. In addition, this work aimed to understand the initial composition of the natural MGPs prior to any further structural complexity arising during growth and/or through sticking to other materials or organisms. Since EPS is the basic common material of MGP's and *P. atlantica* a model particles of *P. atlantica* EPS were used in this study. Furthermore, this study presents for the first time the presence of eDNA in natural MGPs of the North Sea and in the EPS of *P. atlantica* as a model of MGP. The in-vitro staining of both particle types with specific eDNA stains YOYO-1 and TOTO-3 visualised using CLSM endorsed the occurrence of eDNA here for the first time. With these two staining approaches it was possible to differentiate between intracellular and extracellular DNA in the MGP matrices. Despite the possibility that impermeant stains might penetrate damaged cells attached to the MGPs, the staining was effective for the labelling of eDNA.

2.5.1 Purification of MGPs in suspension

Here was developed for the first time a method for MGP particle isolation and concentration in a smaller volume of seawater, where the particles were recovered in 5 ml of 0.2 µm filtered sterile seawater. This enabled the MGPs to be recovered in the same way as occurs in nature. The fragile features of MGPs are problematic throughout filtration and processing, but potential damage was minimised by gentle handling. Conversely, this can be reversed as these particles are sticky by nature and have the ability to promote spontaneous reassembly. Although this may hinder the recovery of the particles from the filters, it was shown here that the particles could be retrieved by using PC filters. As PC filters are smooth, they make it easy to capture particles and they have negligible adsorption to filtrate molecules, enabling the retrieval of particles and re-suspension in artificial seawater in test tubes (Alldredge *et al.*, 1993; Arruda Fatibello *et al.*, 2004; Discart, 2015; Villacorte *et al.*, 2015). Obviously, the PC filter method is the most used

technique and therefore it was selected here for these features. The different methods and tools used to sample MGPs have permitted different investigations and enabled a further understanding of the ocean's gel particles. There are no methods in the literature to isolate MGPs in liquid suspension.

2.5.2 EPS staining

The conventional staining protocols of the MGP (Alldredge *et al.*, 1993; Long and Azam, 1996) have shown that MGPs and *P. atlantica* here can be seen as both TEP and CSP. These two colorimetric-based methods are the only known valid protocols for marine gel particles identification. MGPs would be a heterogeneous mixture of particles that includes the classical type of particles known TEP or CSP (Thornton, 2018). Investigations of the MGPs' matrix composition is challenging due to their heterogeneity and structural complexity. There is a suggestion that such environmental heterogeneity is important in microbial ecology, diversity and biogeochemistry (Long and Azam, 1996; Long and Azam, 2001; Kiørboe *et al.*, 2002). Thus, the assumption that eDNA is a component of MGP composition would be important to illustrate the MGPs' precise composition.

2.5.3 The presence of eDNA in MGPs and P. atlantica EPS

The application of CLSM imaging has enabled the exploration of marine and freshwater aggregates and sludges (Thill *et al.*, 1998; Neu, 2000; Waite *et al.*, 2000; Böckelmann *et al.*, 2002). Marine snow > 500 µm has been investigated by fluorescent dyes and CLSM (Holloway and Cowen, 1997b; Flintrop *et al.*, 2018). Holloway and Cowen (1997a) characterised the compositional structure of marine snow in terms of investigating the presence of polysaccharides using concanavalin A, proteins using [5-(4,6-dichlorotriazin-2-yl] amino) fluorescein, and for DNA using propidium iodide. However, the prior studies did not explore eDNA. Here it was possible to study smaller gel particles of size range 0.4-100 µm that have not been studied yet for eDNA composition. eDNA is a considerable component in EPS matrices generally and a key structural component in biofilms specifically (Dominiak *et al.*, 2011; Okshevsky and Meyer, 2015; Deng *et al.*, 2018).

The presence of eDNA in marine particles and sinking aggregates like marine snow has not been reported previously. Here the application of YOYO-1 and TOTO-3 has validated the

occurrence of eDNA in the MGP milieu, although there are variations in the intensity of the fluorophore signals. Previously it has been reported that eDNA is labelled in biofilms and bacterial aggregates by these two stains (Rostami *et al.*, 2016; Pakkulnan *et al.*, 2019). The 3D image illustrated a wider distribution of eDNA than iDNA. Assuming that eDNA accumulates through time in the MGP matrices, this suggests the release of eDNA from living bacterial cells that inhabit the MGP. The importance of eDNA in MGPs' architecture has not yet been demonstrated in the aggregation of marine gel particles and marine snow.

Moreover, eDNA has been recognised in biofilms and activated sludge by fluorescent dyes and DNase treatment (Whitchurch *et al.*, 2002; Dominiak *et al.*, 2011). Trials in which DNase I and MNase were added to the MGP and *P. atlantica* particle suspension using 18 hours time-lapse imaging did not result in particle dispersion or particle size alteration as expected (Appendix A). This may be due to the fact that nuclease enzymes are susceptible to high salinity seawater.

The bacterial MGP model of *P. atlantia* EPS used in this study revealed similar labelling patterns to natural MGPs. Morever, the findings provide proof of the presence of eDNA in the natural MGP matrix and the *P. atlantica* particle model. The fluorescent staining protocols successfully stained and localised the eDNA in the MGP matrix and the *P. atlantica* EPS. Regardless of the variation in the signals of the fluorophore in some samples, the mean values of the fluorescent intensity were higher in the stained particles than the control (unstained particles), which showed mostly weak signals.

The challenging issue of auto-fluorescence with regard to brightness and visibility, which could interfere with the fluorescent stains, was unavoidable (Thiele *et al.*, 2015). In addition, due to their buoyant nature these particles tend to travel up the well chamber, causing the particles to be untraceable. Therefore, the strategy for choosing particles to be imaged was based on a uniform focal plane and avoiding super bright particles as they could result in more variation in fluorescent intensity.

Flintrop *et al.* (2018) recently demonstrated a new method to characterise the compounds that make up marine snow in situ by implementing a soft-embedding and sectioning microbial diversity by hybridisation (FISH)-probe labelling. Nevertheless, the current study employed a combination of fluorescent stains that had not previously been used together for studying marine particles or marine snow.

This study tested the hypothesis that eDNA is present in the MGP structure. The current staining methods, however, do not stain lipid, cationic or uncharged polysaccharide, humic acids,

materials that may also be important components of the MGPs. Additionally, some particles may be too small to be recognised by optical microscopy.

2.6 Conclusions

This study set out to isolate MGPs and to investigate their composition in terms of eDNA. In addition, it used a bacterial EPS model used to mimic MGP to understand their composition of eDNA. The aims were achieved and the most notable findings to emerge are as follows:

1. The conventional components of acidic polysaccharides and proteins of MGPs have been found in both natural MGPs and the *P. atlantica* model EPS. This finding endorses the heterogeneity of MGPs' composition. However, these findings uphold the question of the ability of these two stains to differentiate between two types of gel particles, or they might imply that all gel particles can be stained by the two dyes equally.
2. This work enabled resolved natural MGPs from the North Sea of size range 0.4-100 µm to be visualised as individual aggregates by labelling their eDNA components using fluorescent stains for the first time. This is vital for studying the gel particles.
3. For the first time, YOYO-1 and TOTO-3 have localised the presence of eDNA in the matrices of MGPs of the North Sea and the EPS of *P. atlantica* as MGP model particle. This is an important consideration as it will put into context eDNA as a significant component of MGPs (that co-occur with bacteria in the matrices) and its role in the make-up of marine snow and sediment aggregates. The implication of eDNA in the aggregation is a key research issue in the marine gel particles domain that is important in answering the bigger questions of marine snow formation into labile and recalcitrant forms and eventually carbon sequestration via sedimentation.

Chapter 3. Characterisation of bacterial communities associated with marine gel particles

3.1 Abstract

Marine gel particles (MGPs) are inhabited by diverse bacteria that belong to various taxonomic groups and which also show a range of metabolic functions. The interaction of bacteria within its community and with MGPs results in the expression of numerous enzymatic activities that influence MGP dynamics and consequently impact marine biogeochemical cycles. It is important to understand microscale MGP-bacterial interactions as these can determine both the structure and functions of the bacterial community and the stability and fate of MGPs in the ocean. To aid understanding of bacteria-MGP interactions, this study sought insights into the taxonomic composition and potential metabolic functions of MGP-associated bacterial communities. Seawater samples were collected from the North Sea in spring and summer (2017) and filtered through a 100 µm sieve and a 0.4 µm polycarbonate filter by gentle vacuum filtration. MGP size fractions were then separated into five groups: 0.4 µm, 1 µm, 5 µm, 12 µm and 100 µm by serial filtrations on polycarbonate filters (PC). Bacterial communities of MGPs were characterised by total DNA extraction, where the 16S rRNA gene were sequenced for community analysis using high-throughput illumina MiSeq sequencing. In addition, the functional predictions of the metabarcoding profiles of these communities were studied for the presence of nuclease enzymes using Tax4Fun and identified with Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis. Results showed that bacterial communities associated with different MGP size groups during spring and summer are notably different. In addition, the bacterial community composition of the MGPs is mainly dominated by Bacteroidetes, Firmicutes, Proteobacteria and Cyanobacteria, and that variations between MGPs are size related. The prediction of community function profiles showed that the bacterial community exhibits diverse metabolic pathways where purine and pyrimidine pathways showed significant abundance in summer. In additions, there are 75 diverse nuclease encoding genes present within the bacterial community. These observations suggest the potential for DNA turnover in MGPs which may affect their dynamics.

3.2 Introduction

A large proportion of the organic carbon in the ocean is exported to the deep sea by sinking aggregates that are made up of MGPs. MGPs are a favoured niche for bacterial colonisation, which leads to the development of protobiofilms which can have intense metabolic activity. Moreover, the repletion and succession of bacterial communities on MGPs could determine the particles' ecological function, impacting aggregation dynamics (Mari *et al.*, 2017), fate and sinking rates of the aggregates to the ocean floor, and consequently affecting marine biogeochemical cycles, principally marine carbon sequestration (Lechtenfeld et al., 2015; Enke et al., 2018). Hence, marine bacterial communities play a pivotal role in the carbon cycle through the microbial carbon pump (Worden *et al.*, 2015; Landa *et al.*, 2016). The interactions of MGP with bacterial communities are the main driver of enzymatic activities in the microscale MGP environment. As MGPs are dense, they harbour bacteria with intense microbial activities and these bacterial metabolites could influence the aggregation and degradation process of the MGPs (Alldredge et al., 1986; Smith et al., 1992; Ziervogel et al., 2010). Not much is understood about the interaction of the bacterial community and the MGPs, and little is known about the role of the bacterial community enzymes on the dynamics and stability of MGPs (Enke *et al.*, 2018; Enke *et al.*, 2019).

Understanding the fundamental microbial processes that govern the aggregation and disintegration of microbial communities is key to interpreting the ecology of microbial-aggregate interaction. Therefore, to enhance our understanding of these microscale interactions the functional abilities of the bacterial community first need to be mapped. The complexity of this quest lies in the complications of plotting the functional capabilities and roles of the particle-attached bacterial communities as they contain enormous taxonomic diversity (Cordero and Datta, 2016).

Traditionally, bacterial communities in the ocean are classified based on their lifestyle, being referred to as free-living (FL) or particle-attached (PA) bacteria (Rieck et al., 2015; Pelve et al., 2017) the latter being responsible for the majority of organic matter degradation (Lyons and Dobbs, 2012). The occurrence of eDNA in MGPs and *P. atlantica* flocs was discussed in Chapter 2, and these observations suggest that the associated bacterial community has the metabolic capacity to utilise eDNA. The presence of eDNA in the MGP micro-niche proposes the coexistence of the nuclease enzymes within the bacterial community as a requirement for the breakdown and utilisation of the particle components by bacterial communities. Therefore,

due to the presence of eDNA in the MGP matrices it is hypothesised that the MGPs-associated bacterial communities encode nuclease genes.

To address the interrogation of eDNA and nuclease co-occurrence in the MGP milieu it is essential to understand the bacterial community structure and function since bacteria – MGP interactions are likely to be the primary driver of MGP dynamics in the ocean. These aggregates are a habitat for microorganisms and the enzymatic activities associated with them may affect the aggregation and disintegration dynamics of these gel particles (Karl and Knauer, 1984; Crump *et al.*, 1999; Bochdansky *et al.*, 2010; Smith *et al.*, 2013). Moreover, bacteria have been reported to promote the aggregation of MGPs (Ding et al., 2008; Yamada et al., 2016). However, not much is known yet about the precise role played by bacterial enzymes and the factors that control the structural integrity, or how the co-occurrence of the eDNA and the extracellular nucleases in the MGPs may have antagonistic functions affecting the structural integrity of the particles. Hence, an understanding of the role of bacteria in MGPs would be aided by understanding potential metabolic functions of the community.

Many studies have reported the structure of bacterial communities associated with sinking particles (DeLong et al., 1993; Fontanez et al., 2015; Bachmann et al., 2018), most of them focusing on marine snow and sinking aggregates. Mestre et al. (2017) reported individual bacterial community structures related to five discrete size fractions (0.2-200 µm), but they did not examine the functional diversity linked to these communities. Therefore, in this study the structure and function of MGP bacterial communities in the size range 0-2-100 µm was investigated to understand the potential bacterial metabolic functions. Assuming that this size range determine the newly produced gels with less detritus and attached materials and therefore, to get better insight into bacterial attachment to pure marine gels.

Through the advancement of next-generation sequencing and high-throughput molecular technologies, the development of metagenome sequencing and the advance in bioinformatics tools has allowed a better understanding of the uncultured bacterial communities. Hence, there have been increasing efforts to understand the microbial communities attached to marine particles and marine snow to expand and deepen the knowledge of the predicted functions of the marine microbiome (Louca *et al.*, 2016; Mendes *et al.*, 2017; Ferrer *et al.*, 2018). Therefore, a bioinformatics-based approach was applied to understand the structure and function of the bacterial communities associated with MGPs.

3.2.1 Aim and objectives

The aims of this study were to: (i) characterise the composition of the bacterial community associated with different size groups of MGPs (0.4-100 µm) from the North Sea; and (ii) to gain an insight into the putative metabolic capabilities of the MGP bacterial communities. Therefore, the following objectives were identified:

1. To study the total bacterial community of MGPs in the range 0.4-100 µm.
2. To separate MGPs into five discrete size fractions 0.2-0.4 µm, 0.4-1 µm, 1-5 µm, 5-12 µm and 12-100 µm from polycarbonate filters, in order to understand the bacterial community associated with each.
3. To isolate bacterial community total DNA through extraction from MGP, followed by 16S rRNA sequencing for community analysis using the Illumina MiSeq platform.
4. To investigate bacterial community functional profiles through the Tax4Fun R-package, and to localise the presence of nuclease genes within the bacterial community of MGPs.

3.3 Materials and Methods

3.3.1 Pilot study

A pilot study was initially conducted to understand the structure of the overall bacterial community associated with MGPs from the North Sea.

Seawater collection

Coastal seawater was collected on 31st March 2016 from Cullercoats (55^{o}0 N, 1^{o} W) in a clean sterile bucket at 0.5 m depth. Seawater were transferred in five-litre carboys to the laboratory at Newcastle University and stored in a cold room at 4^{o}C until it was needed for processing within 24 hours.

Sample preparation

The filtration of the seawater was carried out on 0.4 µm pore-size polycarbonate filter papers as described in Chapter 2. For a second size group with upper limit of 100 µm, to result in 0.4-100 µm filter, the seawater was first passed through a 100 µm stainless steel sieve (Fisherbrand™) to get rid of large particles and debris. Therefore, the filter sizes filter size contain particle > 0.4 µm, the second 0.4-100 µm and a third < 0.4 µm.

Total DNA extraction and sequencing

The total DNA of MGPs was isolated using an Invitrogen™ protocol (PureLink™ Genomic DNA Mini kit) following the manufacturer's instructions. The DNA concentration of the samples was measured using the Nanodrop and the samples were then sent to Mr DNA labs (Shallowater, Texas, USA: http://www.mrdnafreesoftware.com) for metabarcoding of the 16S rRNA gene. The sequencing was performed using an Ion PGM ™ platform. The 300 bp 16S rRNA gene V4-V5 hypervariable region was targeted with 515F/806R primers (Caporaso et al., 2011). The operational taxonomic units (OTUs), which are a group of similar DNA sequences (Blaxter et al., 2005), were defined at 97 percent similarity and taxonomically classified using BLAST against a database derived from Green Genes, RDPII and NCBI databases.

Data format

Raw data were received in FASTA format, a text-based format representing the nucleotide sequences. The data were then converted to FASTQ files using software from www.mrdnafreesoftware.com.

3.3.2 Marine gel particles size fractions study

To isolate different MGP sizes, a size fractionation of the particles was carried out by serial filtration of pre-filtered surface seawater through 100 µm steel mesh sieve as previously described in chapter 2. The pre-filtered seawater was then again passed over different pore sizes of PC filters (GE Healthcare, UK), as described in Mestre et al. (2017), with modification of the filter sizes and range of particle sizes. Fractions were obtained by sequential fractionation, as presented in Table 3.1.

Table 3.1. Fraction labels and corresponding size ranges in µm.

Label	Size range (µm)
0.4 µm spring 0.4 µm summer	0.2-0.4
1 µm spring 1 µm summer	0.4-1
5 µm spring 5 µm summer	1-5
12 µm spring 12 µm summer	5-12
100 µm spring 100 µm summer	12-100

Sample collection

The seawater was collected on-board the Research Vessel Princess Royal from approximately one-mile off the Northumberland coast on 2nd May 2017 for the spring samples and 20th July 2017 for the summer samples (55^{o}06.972 N, 1^{o}25.600 W). The offshore site is preferred because it avoids the anthropogenic influence from land effluent. Seawater was collected from a depth of one metre using a clean sterile bucket, with approximately 30 litres of seawater collected in total. The samples were stored in 5-10-litre carboys (sterilised by autoclave) and transported

immediately to the Newcastle laboratory, and stored at 4°C in a cold room until processing within 24 hours.

Sample preparation

In the laboratory samples were first pre-filtered through a sterilised 100 µm mesh sieve to remove larger debris, followed by a sequential filtration to isolate MGPs as described by Mestre et al. (2017), with modified filter sizes. Polycarbonate filter (GE Healthcare) sizes were of 12 µm, 5 um, 1 µm, 0.4 µm and 0.2 µm. To avoid filter clogging, each filter was used to filter one litre of seawater using gentle vacuum filtration (Sartorius, UK) (Padilla *et al.*, 2015). The PC filters were then stored at -20°C in individual falcon tubes until DNA extraction.

Total DNA extraction and sequencing

The DNA from five replicates of each size fraction was isolated with the MO BIO Power Soil® DNA isolation kit (QIAGEN), using 2 ml of sucrose lysis buffer (0.75M Sucrose, 0.02M EDTA, 0.05M Tris-base, 0.4M NaCl, pH 9.0) and 50 µl of 20 mg/ml lysozyme, both added to a falcon tube containing 2 mm glass beads and the PCTE filters, previously prepared and briefly vortexed. The tubes were incubated at 37°C for 30 minutes with periodic vortexing, before adding 500 µl of Proteinase K/SDS solution (187.5 µl 20 mg/ml proteinase K, 375 µl 20% SDS, made up to 1 ml with sucrose lysis buffer) to each, followed by t, vortexing and incubation at 55°C for two hours, with further periodic vortexing. Next, 1 ml of lysate was added to a microcentrifuge tube and the DNA purified/extracted according to the Power Soil® DNA Isolation Kit protocol (12888) (https://mobio.com/media/wysiwyg/pdfs/protocols/12888.pdf).

The DNA samples were sequenced by Illumina MiSeq sequencing refs at NUOMICS (Northumbria University, UK). The V4 hypervariable regions were PCR-amplified utilising the Caporaso primer set (515F/806R) according to manufacturer PCR conditions, with subsequent PCR reactions using the suitable Illumina adapters and 15bp barcodes that allow for demultiplexing (Caporaso et al., 2011). Amplicons were then indexed according to sample, normalised using the SequalPrep™ Normalisation 96-Well Plate Kit (Invitrogen), and pooled prior to sequencing.

Data format

Raw data were received in paired-end, demultiplexed FASTQ file format.

3.3.3 Clustering and phylogenetic analysis of the bacterial community

Pilot study

The sequencing supplier MrDNA provided complete analysis of the sequences. First, the sequence barcodes, primers and sequences less than 150bp were all removed. Sequences with uncertain base calls and homopolymer runs exceeding 6bp were also removed. Sequences were de-noised, OTUs generated and chimeras removed. OTUs were defined by clustering at 97 percent similarity. Final OTUs were taxonomically classified using BLAST against a curated database derived from GreenGenes, RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov) (DeSantis et al., 2006).

Data files were received in tab delimited text (txt) format with all taxonomy assigned. These txt files were initially imported into Excel and rearranged to be suitable for analysis as per the software requirements (.csv files for R and .txt files for Statistical Analysis of Metagenomics Profiles (STAMP)).

MGP size fractions study

The raw Illumina MiSeq sequencing data were analysed using the QIIME (Quantitative Insights into Microbial Ecology) pipeline http://qiime.org/. QIIME version 1.9.1 with SILVA database was employed for the bacterial community structure analysis. Each of the five replicates of each size fraction in each season condition were pooled separately into a single FASTQ file. The reason was to prevent loss of sequence while processing the sequences reads as the reads of the 12 μm sample have a minimum number of sequences.

The following QIIME workflow was used: QIIME's multiple_join_paired_ends.py script was used to combine the paired-end reads. For downstream analysis, only reads that were successfully paired were used. QIIME's multiple_split_library_fastq.py script was then used to perform quality control and prepare the data for downstream analysis by creating a single FASTA file with compatible IDs. Open reference OTU picking was performed using QIIME's pick_open_reference_otus.py script with default parameters (including use of the Green Genes v13.8 database). The core diversity analysis was carried out using the core_diversity_analysis.py script, using a sampling depth of 8,500 (based on the number of OTUs for the condition with the fewest identified OTUs). Additional principal coordinates analysis (PCoA) was carried out to explore robustness using the jackknifed_beta_diversity.py script using 100 sequences per sample. The make_2d_plots.py script was then used to visualise these.

3.3.4 Diversity indices

The species richness Menhinick's index (Whittaker, 1977), species abundances, density, species diversity indices (Shannon-Wiener), and evenness (Pilou's evenness index), were all computed in RStudio using commands from http://www.flutterbys.com.au/stats/tut/tut13.2.html for both the pilot study and MGP size fraction studies. The alpha diversity within the sample, diversity metrics for the phylogenetic diversity whole tree (PD), number of species richness (Chao1) and OTUs were observed, while beta diversity was presented as PCoA between fractions based on a Bray-Curtis dissimilarity calculated from the OTUs table in STAMP and R.

3.3.5 Prediction of the functional capabilities of MGP bacterial communities

The role of the bacteria associated with MGPs in different size fractions can only be understood following subsequent functional studies using the Tax4Fun R-package, where the 16S rRNA sequences of the bacterial community presented were annotated in the KEGG database. Based on metagenomics amplicon-based sequencing of the 16S rRNA, the functional predictions of the MGP-associated bacterial communities were made using Tax4Fun, which is an open-source R-package http://tax4fun.gobics.de/. The choice of Tax4Fun for functional communities profiling was made because it matched well with the 97 percent similarity of the taxonomic sequencing data. In addition, it is more accurate at annotating 16S rRNA gene sequences with functional annotation of sequenced prokaryotic genomes (Aßhauer et al., 2015).

Tax4Fun workflow

The overlapped forward and reverse sequence reads of each sample were combined by joining the paired end. The split_library_fastq.py script was used to carry out QIIME's default quality control of the sequences. Two rounds of OTU picking were carried out. The first was open reference OTU picking, which uses the GreenGenes v13.8 database, and is done because it generates a phylogenetic tree that includes *de novo* OTUs. The second was closed reference OTU picking, which uses the 97 percent identity clustered in the SILVA (release 119) database. These files were used in downstream Tax4Fun analysis. The BIOM tables generated from closed-reference OTU picking were used in Tax4Fun to generate, firstly, Tax4Fun profiles; the predicted relative abundance of KEGG orthology (KO) groups present in the sample, based on sequences. Secondly, KEGG pathway profiles were generated, which are the predicted relative abundance of KEGG pathways present in the sample, based on taxonomy. The function of non-

rRNA reads was determined by comparison with the May 2018 version of KEGG (Kanehisa and Goto, 2012).

3.3.6 Statistical analysis

Two sample unequal variance t-tests were used to compare bacterial abundance between spring and summer. A single factor ANOVA was applied to analyse the differences between bacterial abundance and different size fractions. However, sequencing data were statistically analysed using the STAMP software package v2.1 http://kiwi.cs.dal.ca/Software/STAMP, as described by Parks et al. (2014). The phylogenetic table containing abundance profiles at the family level was imported to the STAMP tool. Similarly, the metabolic functional profiles that were generated in Tax4Fun from the metabarcoding data set were imported into STAMP. All Excel files used in STAMP were in .csv format. ANOVA is used in STAMP to study means by the Tukey-Kramer post-hoc test at $P > 0.05$ to detect the significant difference between bacterial community abundance in size fractions and among seasons. In addition, t-tests were employed to compare the means of bacterial abundances during spring and summer.

3.4 Results

3.4.1 Bacterial community of the MGP (pilot study)

The pilot study was conducted to get an overview of the bacterial communities associated with different size groups of MGP (0.2, 0.4-100 and 0.4 µm). Here, nine 16S rRNA libraries were produced, totalling 1,055,426 sequences from the different MGP sizes.

The rarefaction analysis of the obtained sequencing data and OTUs indicates that the sequencing depth at 26029 OTUs per sample was sufficient to reveal low abundance OTUs, as the rarefaction plots of sequences per sample against OTUs flatten out (Figure 3.1).

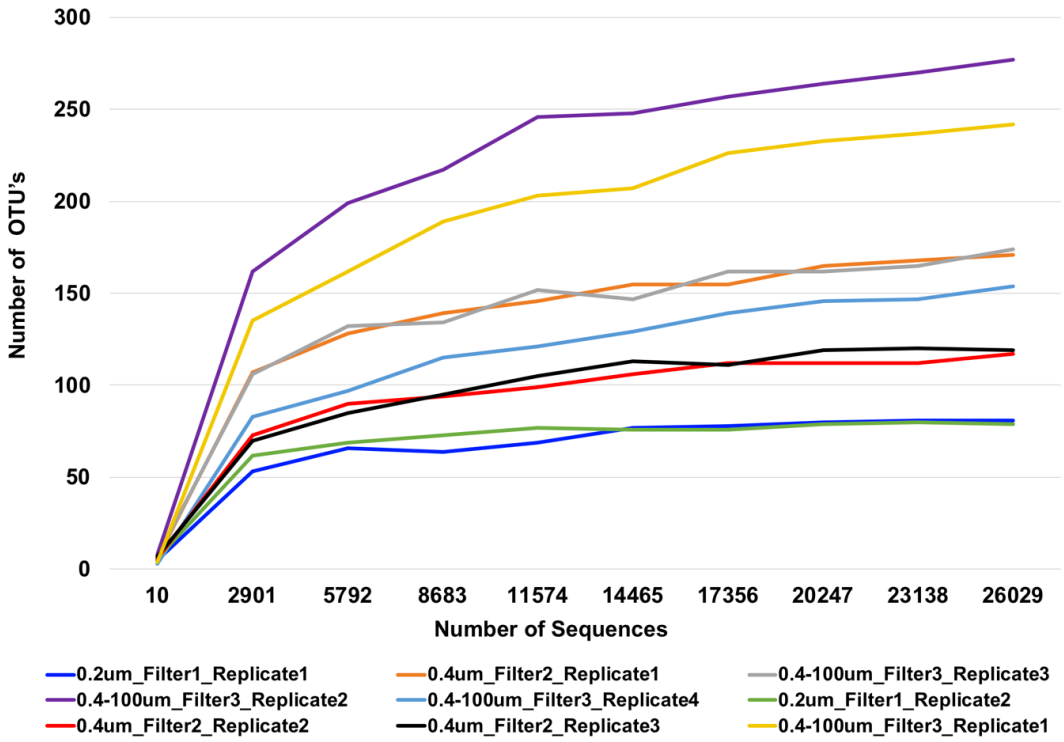

Figure 3.1 Rarefaction curves generated from 16S rRNA sequencing of the different filters (0.2, 0.4-100 and 0.4 µm) of MGP.

The beta diversity presented in PCoA (Figure 3.2) indicates that the samples grouped by one filter size are similar as they appear closer to each other. However, one sample of the 0.2 µm size filters appeared dissimilar, the bacterial community compositions for each different MGP filter size are distinct from each other.

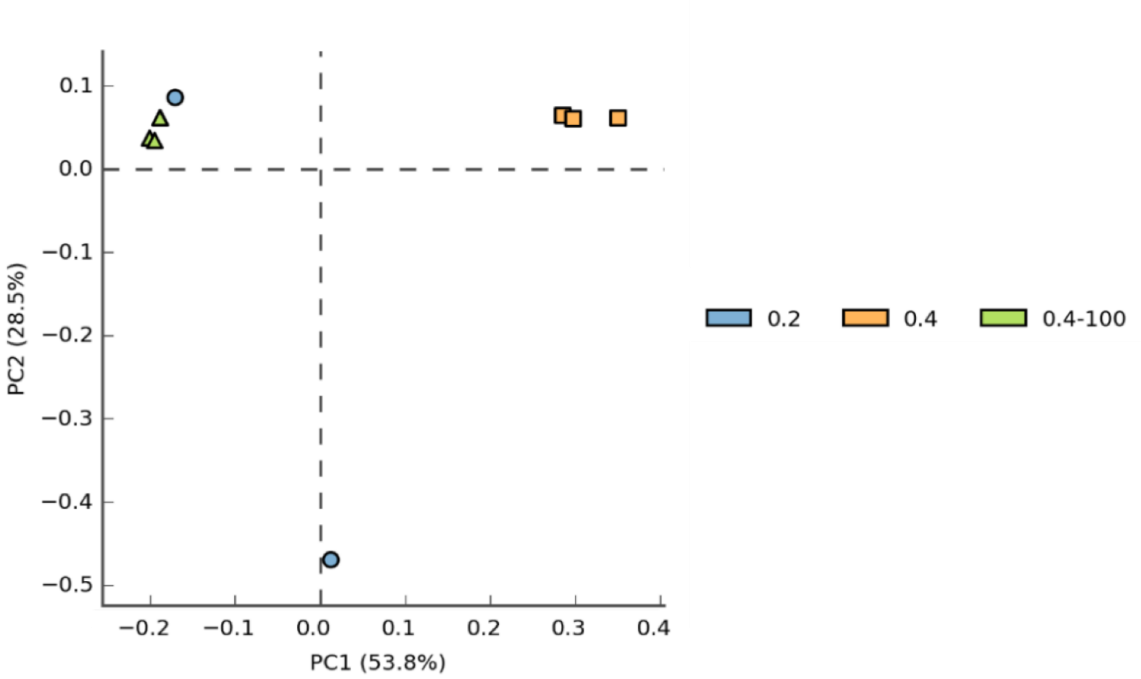

Figure 3.2 Principal Component Analysis of different size fractions: <0.2, 0.4-100 and >0.4 µm.

Diversity of MGP-associated bacterial communities

The number of sequences obtained from 0.4-100 µm are the highest compared to the other size fractions. Interestingly, the number of OTUs observed was also higher in the 0.4-100 µm size fraction compared to the 0.4 µm fraction that was expected to contain greater numbers of large particles and, consequently, more attached bacteria. The alpha diversity represented by species richness (Menhinick), bacterial diversity indices (Shannon-Wiener), evenness (Pilou's) and True Shannon are presented in (Table 3.2). The species richness as indicated by Menhinick is higher in MGP for the size range 0.4-100 µm, followed by 0.4 µm. The bacterial diversity measured by Shannon-Wiener was higher in MGP of filter size 0.4-100 µm compared to MGP of 0.4 µm. The evenness as shown by Pilou's index revealed that the MGP of size 0.4-100 µm contains a heterogeneous community compared to 0.4 µm.

Table 3.2 Statistical summary of the 16S rRNA gene sequencing of MGP-associated bacteria from Northumberland coastal water of different filter sizes (0.2, 0.4-100, and 0.4 µm). Richness, abundance, diversity indices (Shannon, Simpson and True Shannon) were computed in R of the 0.2, 0.4-100 and 0.4 µm filter sizes.

Sample	Number of Sequences	Number of OTUs	Menhinick's (Richness)	Shannon-Wiener	Pilou's (Simpson evenness)
0.2µm_Filter1_Replicate1	48,933	196	0.886	3.333	0.178
0.2µm_Filter1_Replicate2	33,921	299	1.623	4.029	0.169
0.4-100µm_Filter3_Replicate1	151,193	4,801	12.347	6.317	0.117
0.4-100µm_Filter3_Replicate2	121,414	4,664	13.385	6.394	0.118
0.4-100µm_Filter3_Replicate3	148,061	4,517	11.739	6.597	0.118
0.4-100µm_Filter3_Replicate4	145,934	4,622	12.099	6.619	0.118
0.4µm_Filter2_Replicate1	172,756	2,490	5.991	4.724	0.123
0.4µm_Filter2_Replicate2	88,692	2,086	7.004	4.804	0.126
0.4µm_Filter2_Replicate3	144,522	2,431	6.395	4.775	0.123

Bacterial community composition

The taxonomic analysis of the MGP-associated bacterial community composition at the phylum level showed that Proteobacteria is the dominating phylum in all three filter sizes, followed by Bacteroidetes in the filters 0.4-100 and 0.4 µm, as illustrated in Figure 3.3.

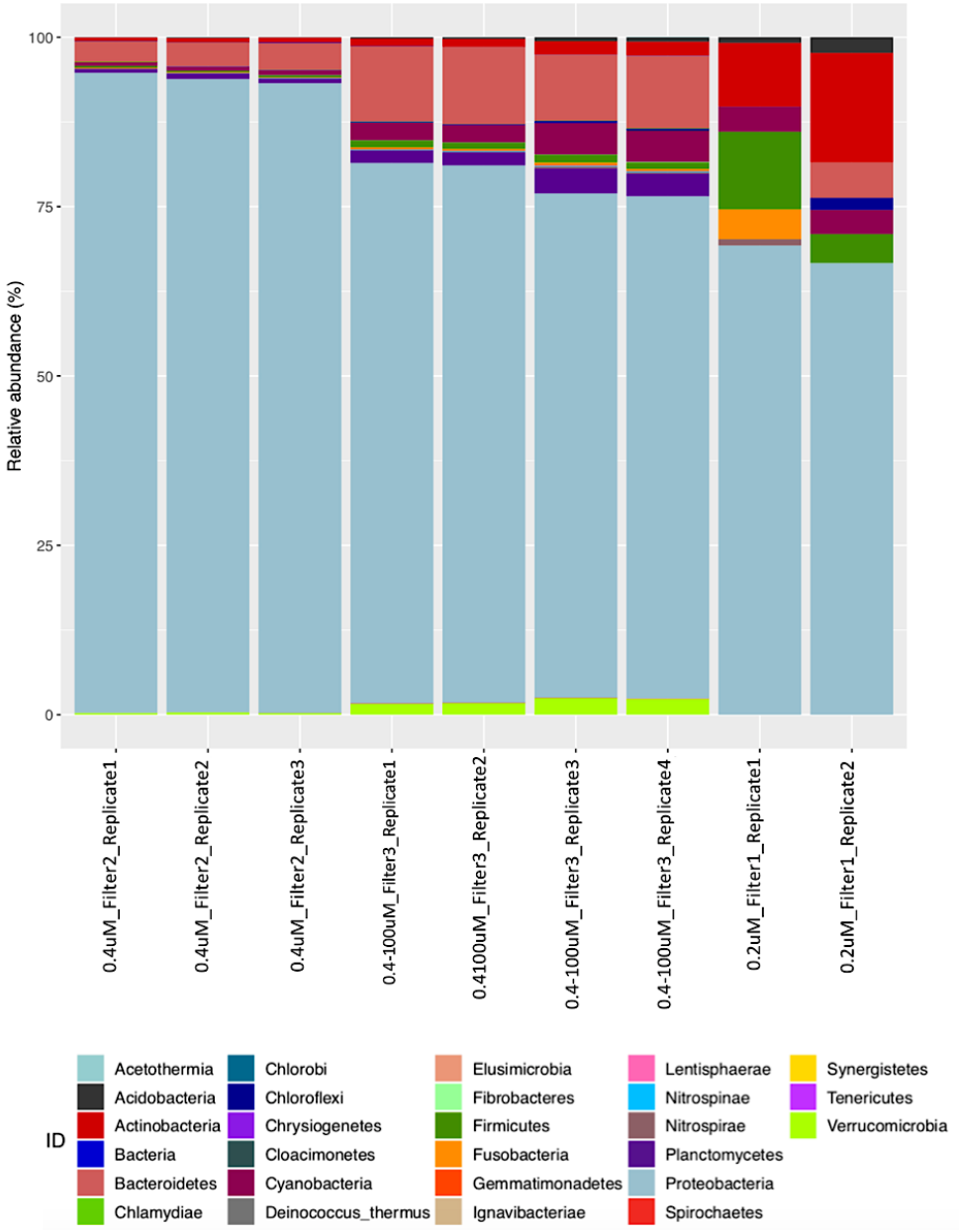

Figure 3.3 The relative abundance of bacterial taxa depicted in stack columns at phylum level of different MGP sizes; <0.2 μm, >0.4 μm and 0.4-100 μm.

To assess the differences in bacterial community composition, sequences were classified to the family level to gain closer insight into the abundance of the MGP-associated bacteria (Figure 3.4).

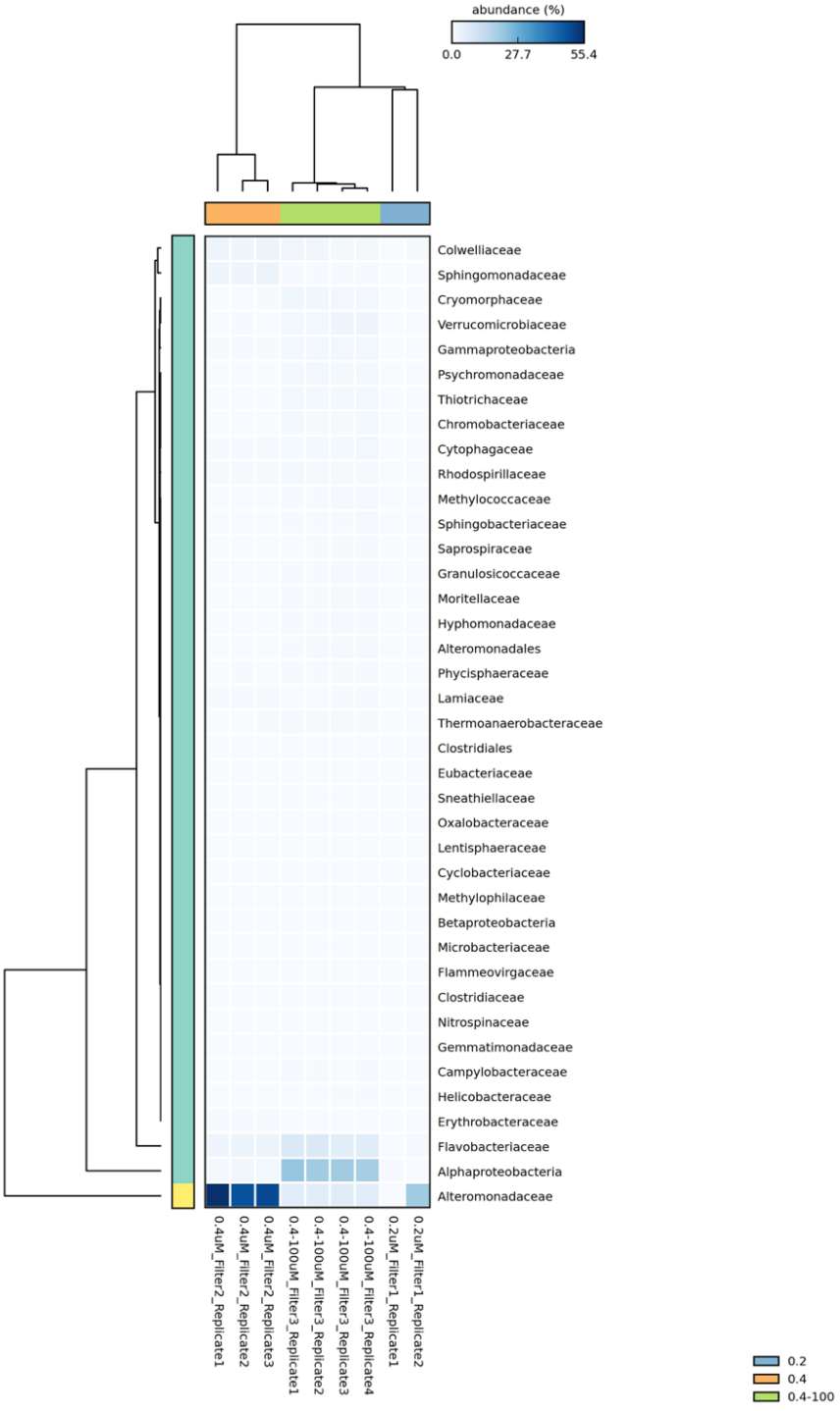

Figure 3.4 Heat map of the various significant bacterial families present in different MGP filter sizes.

3.4.2 Bacterial community composition of different MGP size fractions

MGP separation into five discrete size fractions were successfully resulted into five discrete. A total of 50 samples, comprising of five different size groups of MGP sampled in spring and summer, with five replicates of each size group per season, were all analysed for bacterial community structural composition by 16S rRNA metabarcoding sequencing using the Illumina MiSeq platform. The bacterial community composition associated with different MGP size groups of 0.4, 1, 5, 12 and 100 µm are presented in this section. The five size groups representing different size ranges were tabulated in Table 3.1, which shows that 0.4 and 1 µm groups represent smaller size fractions of the MGP and these clusters may overlap and contain FL bacteria. However, as the size fractions increase to 5, 12 and 100 µm, these three groups designate the PA assemblages.

Diversity of bacterial communities associated with different size fractions of MGP

Species richness, as displayed in the rarefaction curves, shows phylogenetic diversity whole tree diversity (PD) (Figure 3.3 A), Chao1 (Figure 3.3 B) and OTUs (Figure 3.3 C). Despite that the samples with higher OTUs have lost much of their data, the rarefaction analysis indicates that each sample reached a saturation plateau and the depth of 8,500 sequences per sample is sufficient to represent the alpha diversity of taxa within the communities.

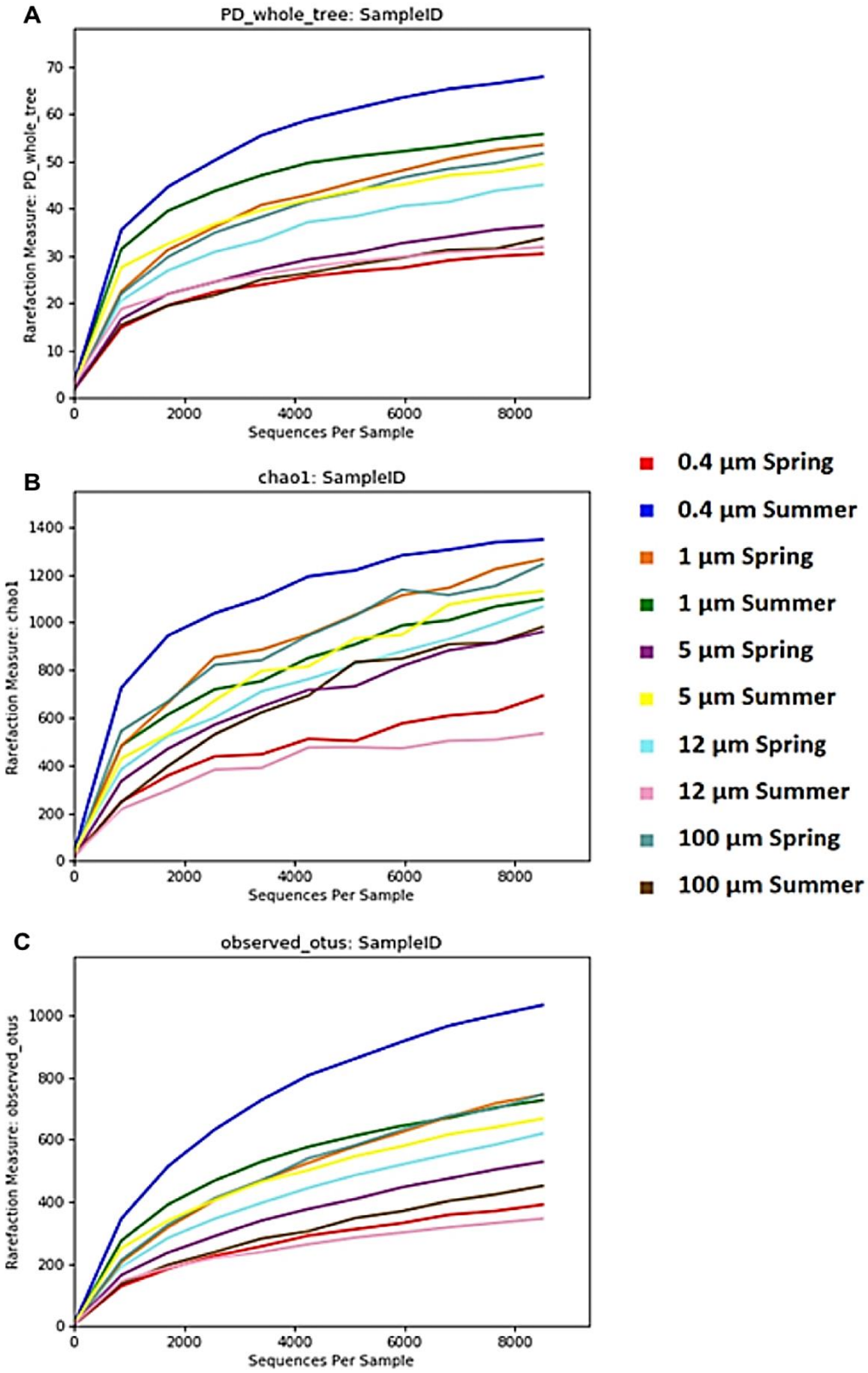

Figure 3.5 Rarefaction curves on (A) phylogenetic diversity (PD), (B) species richness (Chao1) and (C) observed OTUs.

The PCoA based on Bray-Curtis dissimilarity (Figure 3.6A) reveals that bacterial communities of spring and summer samples are dissimilar.

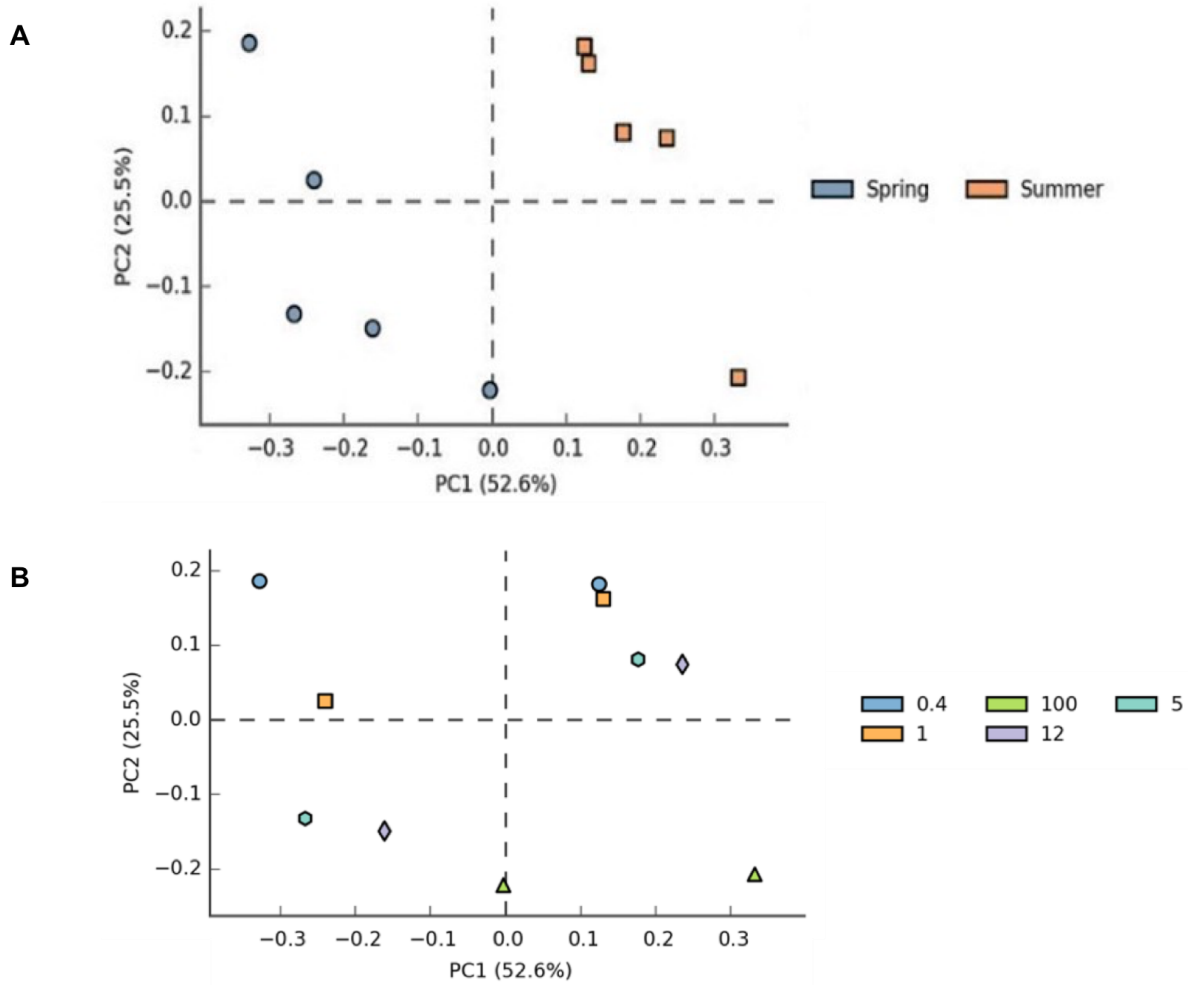

Figure 3.6 Principal Component Analysis showing microbial community dissimilarity among MGP in spring and summer (A), and of different size fractions of MGP: 0.4 µm, 1 µm, 5µm, 12µm and 100µm (B).

Several diversity indices were calculated in R-Studio (Menhinick, Shannon-Wiener, and Pilou's evenness) in order to understand the distribution of the bacterial communities' taxa in association with different MGP size fractions (Table 3.3). Species richness (Menhinick's) tends to increase in the summer samples of 0.4, 1 and 5 µm MGPs size fractions. Conversely, the species richness of the 12 and 100 µm MGP size fractions was lower in the summer samples. In regard to size fractions, the species richness was the highest in the 5 µm summer followed by 100 µm spring and 0.4 summer MGP size fractions.

The Shannon-Wiener index range is between 0 and 5 and it is evenness-sensitive. The bacterial diversity index based on Shannon-Wiener, tends to increase in the summer for the MGPs of 0.4, 1, 5 and 12 µm size fractions, whereas it decreases in the summer for the 100 µm size fraction, as shown in Table 3.3. The highest diversity observed was in the 0.4 µm summer sample (3.555) followed by 1 µm (3.365) and 5 µm (3.185). From these data it appears that the smaller size fractions hold more bacterial diversity in comparison to bigger size fractions as presented in Table 3.3.Thus far, the results indicate diverse bacterial communities associated with different MGP sizes.

The evenness of the taxa within each bacterial community is measured to understand the homogeneity (evenness) of the community. Pilou's evenness (Simpson evenness) was used and the values of it range between zero and one, where values closer to zero represent a more diverse community and the opposite if the values are closer to one. The data had values of Simpson evenness ranging between 0.098 and 0.137, as presented in Table 3.3. There was more variation in the abundances of different taxa observed in the 1 µm size fraction during spring, where Simpson evenness value was 0.098, pointing to a more diverse bacterial community in comparison to other MGP size fractions.

Evidently, the structure of the bacterial community attached to MGP is significantly different for spring and summer. Based on an unpaired t-test, there were significant differences between MGP size fractions in spring and summer; 1 µm ($P_{\text{t-test}} = 0.0003$, $P_{\text{t-test}} < 0.05$), 5 µm ($P_{\text{t-test}} = 0.0042$, $P_{\text{t-test}} < 0.05$), 12 µm ($P_{\text{t-test}} = 0.0227$, $P_{\text{t-test}} < 0.05$), and 100 µm ($P_{\text{t-test}} = 0.0170$, $P_{\text{t-test}} < 0.05$). However, there was no significant difference between the bacterial community of the smaller size fraction of 0.4 µm for spring and summer ($P_{\text{t-test}} = 0.9251$, $P_{\text{t-test}} > 0.05$).

The analysis of variance indicated that the abundance of bacteria was significantly different between size fractions (spring: $P_{\text{ANOVA}} = 0.0004$, $P_{\text{ANOVA}} < 0.05$ and summer: $P_{\text{ANOVA}} = 0.0243$, $P_{\text{ANOVA}} < 0.05$).

Table 3.3 Statistical summary of the 16S rRNA gene sequencing of MGP-associated bacteria from Northumberland offshore water in spring and summer of different filter sizes (0.4, 1, 5, 12 and 100 μm).

Sample	Number of Sequences	Number of OTUs	Menhinick's (Richness)	Shannon-Wiener	Pilou's (Simpson evenness)
0.4μm_Spring	464,102	1,211	1.778	2.501	0.107
0.4μm_Summer	410,663	1,963	3.063	3.555	0.101
1μm_Spring	1,096,024	2,930	2.799	2.984	0.098
1μm_Summer	267,019	1,485	2.874	3.365	0.107
5μm_Spring	575,239	1,743	2.298	2.791	0.103
5μm_Summer	60,443	910	3.701	3.185	0.113
12μm_Spring	271,239	1,443	2.771	2.943	0.106
12μm_Summer	18,303	308	2.277	2.969	0.134
100μm_Spring	256,349	1,706	3.369	3.022	0.104
100μm_Summer	32,348	514	2.858	2.591	0.122

Bacterial community composition

The taxonomic analysis of all the MGP size fractions based on sequencing of 16S rRNA genes matched with Green Genes and SILVA databases, revealing that the dominant bacterial taxa associated with different MGP size fractions (0.4, 1, 5, 12 and 100 μm) generally belong to the phyla Proteobacteria, Bacteroidetes, Firmicutes, and Cyanobacteria with variation in the relative abundances among different sizes and seasons as presented in Figure 3.7. It can be seen from the stack bars that the smaller size fraction of 0.4 μm that represents FL communities in spring is enriched with Bacteroidetes (58 percent), followed by Proteobacteria (38 percent). Proteobacteria are more abundant in spring compared to summer and dominate the 1 and 5 μm size fractions (61 percent and 66 percent, respectively), followed by Bacteroidetes (31 percent and 21 percent, respectively). The relative abundance of Cyanobacteria (mainly family Synechococcaeae) appeared to increase with bigger size fractions (5, 12 and 100 μm) in spring with 11 percent, 23 percent and 37 percent, respectively, and was highest in abundance in the summer in the 100 μm fraction (45 percent). Firmicutes appeared more in summer samples with the higher abundances in the size fractions 0.4 μm (31 percent) and 12 μm (37 percent). Euryarchaeota emerged in the summer samples of all fractions, except 100 μm, with higher abundance of 11 percent in the 0.4 μm size fraction. Shifts in the community structure of the MGP-associated bacteria thus appear to be due to size fractions and season.

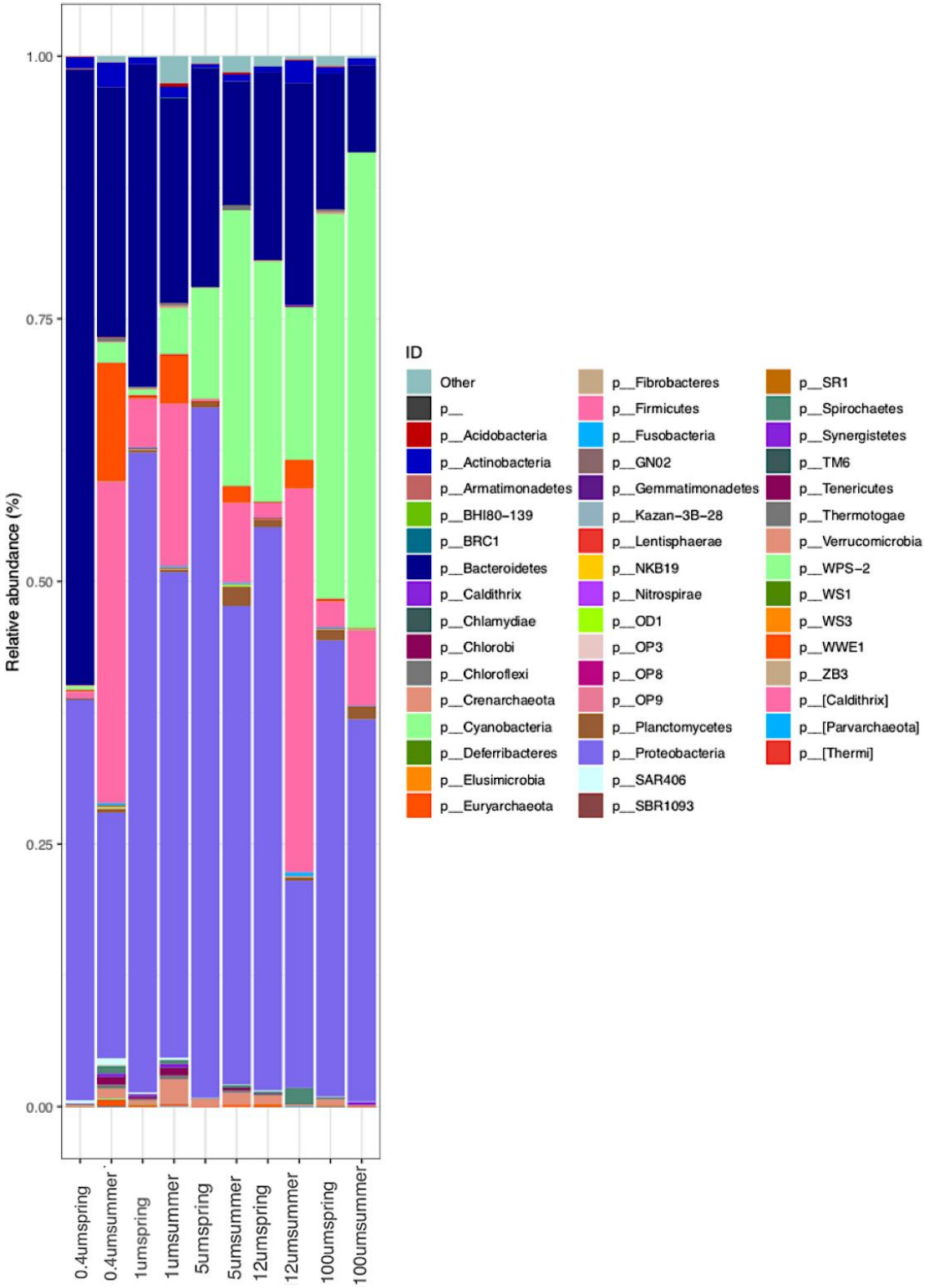

Figure 3.7 The relative abundance of the bacterial taxa associated with different MGP size fractions; 0.4 μm, 1 μm, 5 μm, 12 μm and 100 μm from spring and summer, classified at phylum level.

To obtain a better resolution for bacterial community composition, a family level was selected as this appears to be better identified in comparison with genera and species levels, providing a reasonable outlook to represent taxa. There are significant differences in the abundance of the bacterial communities associated with MGP in spring and summer. The families Alteromonadaceae ($P_{t\text{-}test}$=0.002<0.05), Flavobacteriaceae ($P_{t\text{-}test}$= 0.003<0.05), Erythrobacteraceae ($P_{t\text{-}test}$= 0.008 <0.05), Rhizobiaceae ($P_{t\text{-}test}$ = 0.036 <0.05) and unclassified families from the order Alteromonadales ($P_{t\text{-}test}$= 0.044 <0.05) all have significantly higher abundance in spring. Furthermore, the families Prevotellaceae ($P_{t\text{-}test}$= 0.003< 0.05), Lactobacillaceae ($P_{t\text{-}test}$= 0.018<0.05), Paraprevotellaceae ($P_{t\text{-}test}$=0.023<0.05), Porphyromonadaceae ($P_{t\text{-}test}$= 0.024 < 0.05), Bacteroidaceae ($P_{t\text{-}test}$= 0.026 <0.05), unclassified families of Clostridiales ($P_{t\text{-}test}$= 0.031<0.05), Fusobacteriaceae ($P_{t\text{-}test}$= 0.034<0.05), Lachnospiraceae ($P_{t\text{-}test}$= 0.037 < 0.05) and Enterobacteriaceae ($P_{t\text{-}test}$= 0.39 <0.05) all showed noticeably higher abundance in summer samples (Figure 3.8).

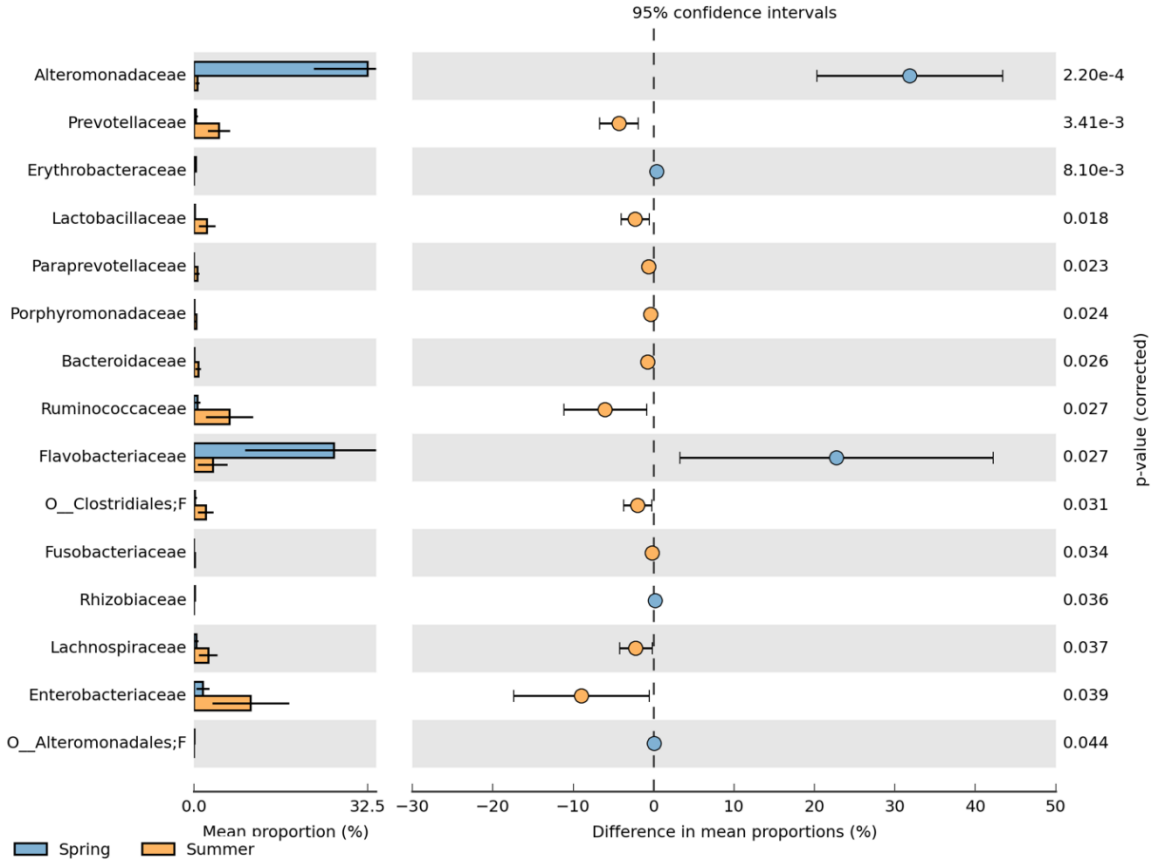

Figure 3.8 Comparasion of taxonomic profiles of MGP and differences among family abundances of spring and summer (P<0.05). P-values corrected using Benjamini-Hochberg false discovery rate. Horizontal bar plots display the mean (± S.D.) proportional contribution (%) of each family to the total community.

Family abundances showed variations among different MGP size fractions. The families OCS155 (P_{ANOVA}<0.001), Pelagibacteraceae (P_{ANOVA}<0.001), Methylophilaceae (P_{ANOVA}<0.05), A714017 (P_{ANOVA}<0.05), Pelagibacteraeae (P_{ANOVA}<0.001) and Halomonadaceae (P_{ANOVA}<0.001) have noticeably higher abundances in the 0.4 μm MGP size fraction, as shown in Figure 3.9. Pelagibacteraceae is abundant in 1 μm size fractions (P_{ANOVA}<0.05) (Figure 3.9), indicating that these families are linked to smaller MGP sizes.

On the other hand, different families are considerably abundant in association with larger MGP size fractions; the family Micrococcaceae (P_{ANOVA}<0.02) and an unclassified family of the order Stramenopiles (P_{ANOVA}<0.05) showed higher abundance in 100 μm MGP size fractions.

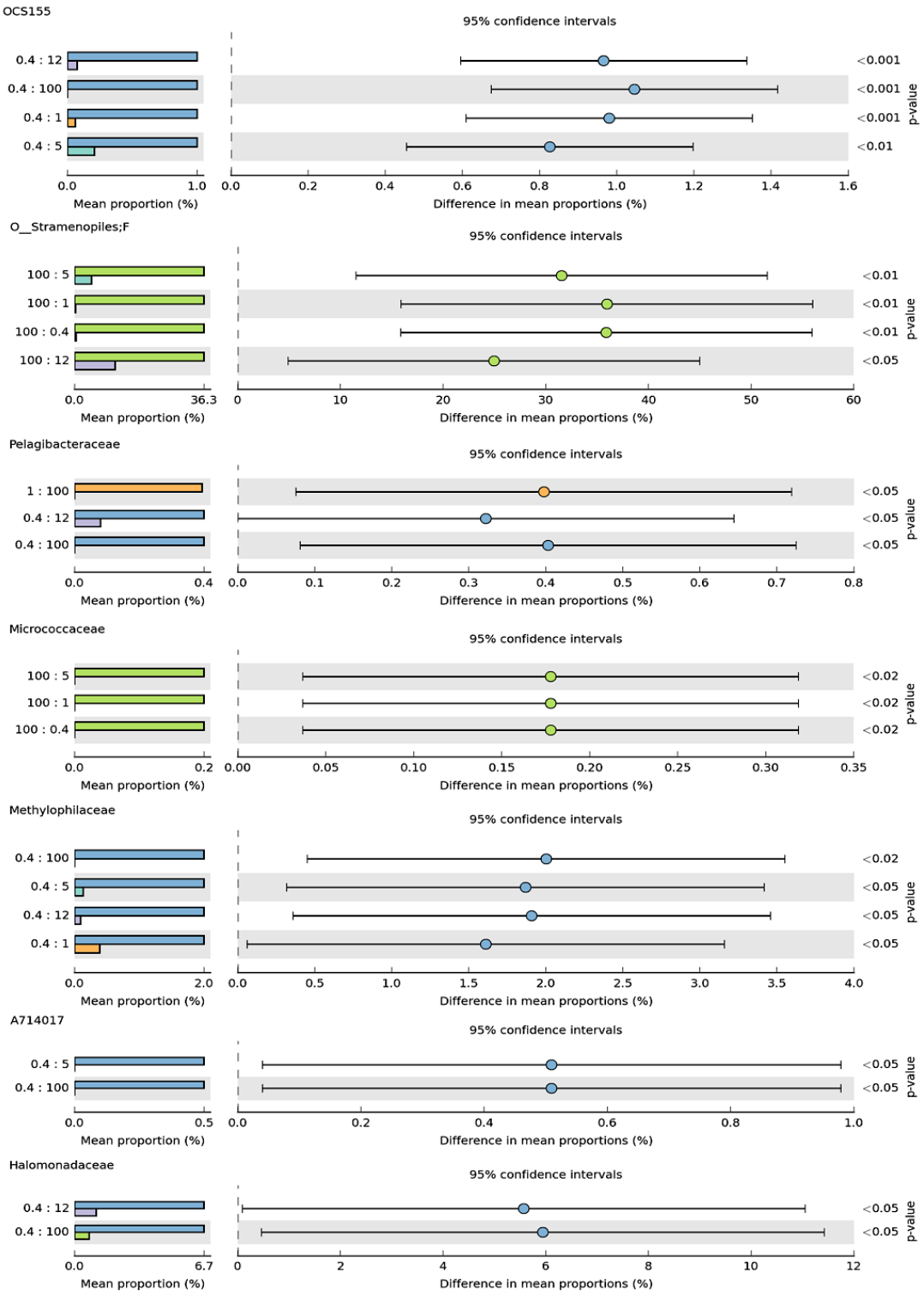

Figure 3.9 The mean proportion and differences in mean proportions (%) of the taxonomic profile of MGP, differences between the abundance of families (OCS155, unclassified family of the order Stramenopiles, Pelagibacteraceae, Micrococcaceae, Methylophilaceae, A714017 and Halomonadaceae) in different MGP size fractions: 0.4 μm (blue), 1 μm (orange), 5 μm (turquoise), 12 μm (purple) and 100 μm (green) ($P<0.05$). Horizontal bar plots display proportional contribution (%) of each

3.4.3 Functional capability profile of the MGP-associated bacterial communities

To get an insight into the putative functional capability profiles of the MGP-associated bacterial community of different size groups of spring and summer, the filtered 16S rRNA bacterial sequences were annotated in KEGG. The KO profiles were generated from OTU tables created in SILVA. There is a total of 286 identified pathways within the bacterial community associated to MGP. The relative abundances of the predicted metabolic pathways are presented in Figure 3.10. Despite the various metabolic pathways present within the genes of the bacterial community associated with MGP, there is a similar pattern of pathway abundances within the community, irrespective of size fractions or seasons (Figure 3.10).

Generally, the relative abundance results of the pathways indicate a functional redundancy when compared to the relative abundance of the bacterial community of MGP size fractions that differs by size and season.

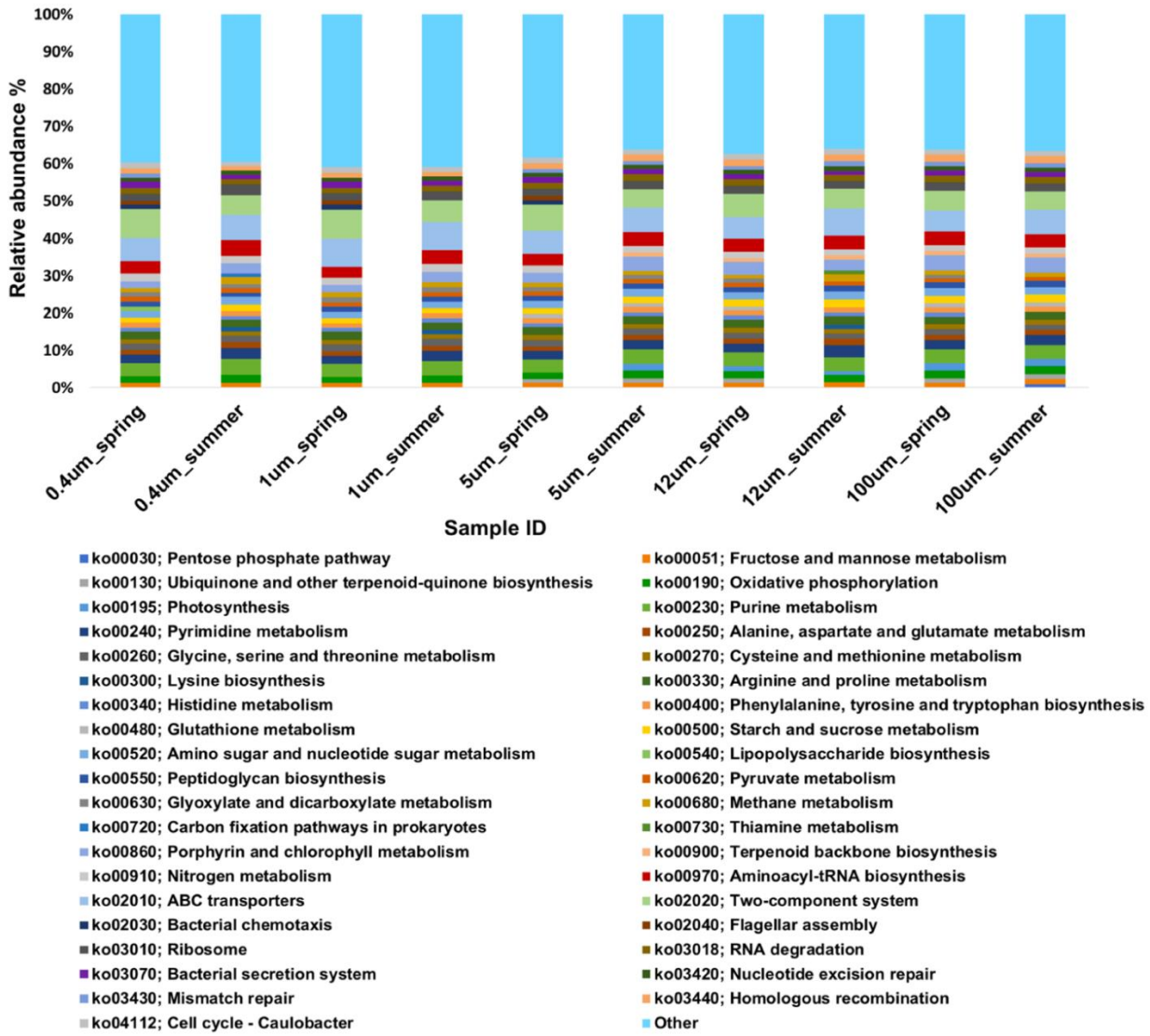

Figure 3.10 The relative abundance of predicted metabolic pathways (pathways that count for <1 percent is considered as other).

There are significant differences (P<0.05) in some of the genes involved in the metabolic pathways present within the MGP-associated bacteria between spring and summer. Five metabolic pathways, the cell cycle-caulobacter (P= 0.004), bacterial secretion system (P= 0.005), two-component system (P= 0.013), bacterial chemotaxis (P= 0.041) and flagellar assembly (P= 0.043) were significantly enriched in the spring. Six other metabolic pathways, pyrimidine metabolism (P= 0.009), purine metabolism (P= 0.011), aminoacyl-tRNA biosynthesis (P= 0.017), ribosome (P= 0.004), fructose and mannose metabolism (P= 0.036) and lysine biosynthesis (P= 0.040) were significantly higher in the summer community, as shown in Figure 3.11. Moreover, relating the significantly different functional profiles in Figure 3.11 of the bacterial community in spring and summer to the significantly different family

abundance in spring and summer in Figure 3.8 suggests that pathway enrichment may perhaps be driven by the corresponding abundant families.

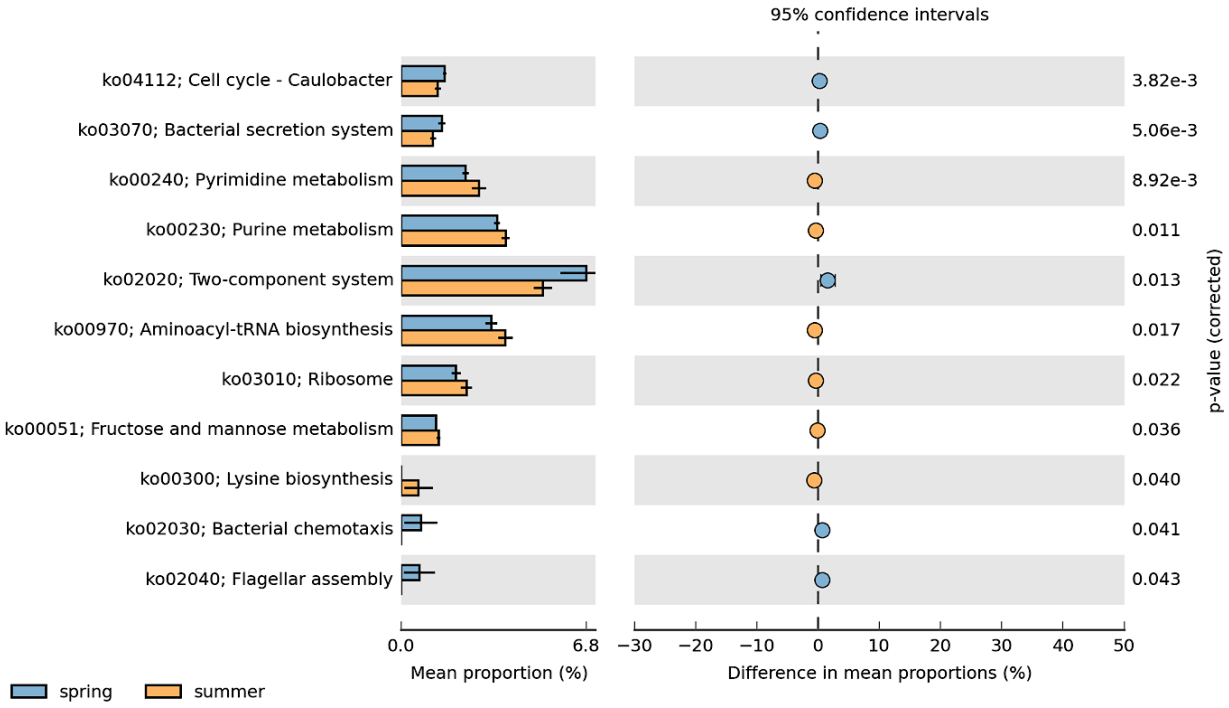

Figure 3.11 The mean proportion and difference in mean proportion (%) showing a proportion of sequences with significant differences ($P < 0.05$) of the functional capabilities among different size fractions of MGP between spring and summer.

Furthermore, the metabolic pathways show variation between the different size fractions of MGP as illustrated in Figure 3.12. The heat map reveals that the most predominant functional genes “homologous recombination”, “starch and sucrose metabolism”, “glycine, serine and threonine metabolism”, and nitrogen metabolism are found in all size fractions. The results point to what functional genes the taxa groups are encoding, while the photosynthesis functional genes are enriched in the size groups of 5, 12 and 100 µm with a higher abundance in 100 µm.

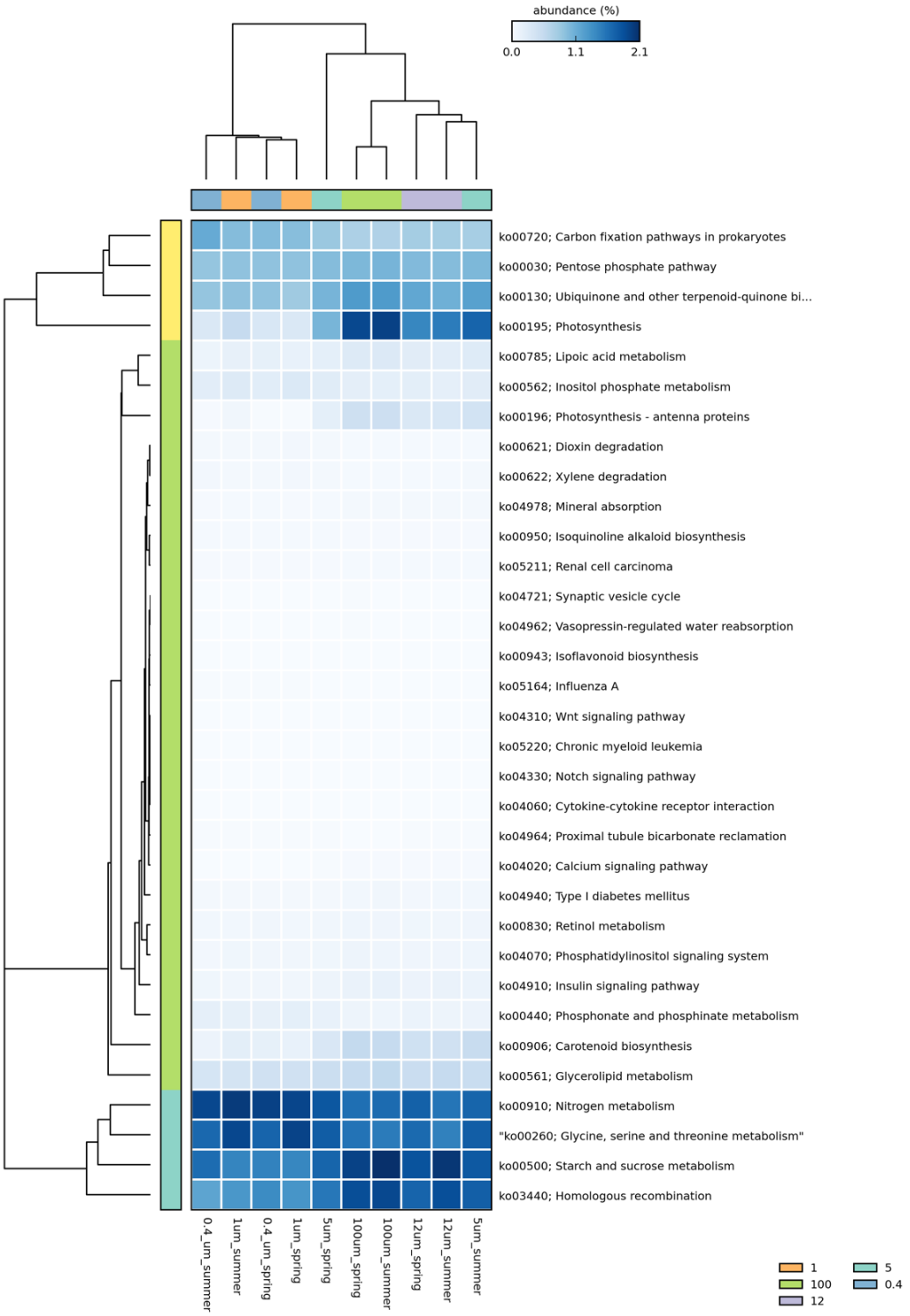

Figure 3.12 Heat map dendrogram showing the distribution of the functional categories illustrated by metabolic pathways between different MGP size groups.

3.4.4 Abundance of nuclease enzymes

There are total of 6605 enzymes predicted within the bacterial community associated with different MGP size fractions in this study. All nuclease-related enzyme genes were mined from the Tax4Fun output. A total of 75 enzymes were found related to nucleases, accounting for about 1.7 percent of the total putative predicted enzymes. There are significant differences ($P_{t\text{-}test} < 0.05$) in the presence of nuclease genes with MGP-associated bacterial communities between spring and summer, as presented in Figure 3.13A, where regulation of ribonuclease activity A ($P = 0.003$), ribonuclease PH ($P = 0.017$), deoxyribonuclease I ($P = 0.021$), ribonuclease R ($P = 0.027$), ribonuclease T ($P = 0.027$), exodeoxyribonuclease I ($P = 0.030$) and putative endonuclease ($P = 0.045$) all have a higher abundance in spring. Ribonuclease HIII ($P = 0.044$) is the only one that showed higher abundance in summer.

Moreover, Figure 3.13B provides an overview of the differences between nuclease enzyme gene abundance between different MGP size fractions. It is illustrated that overall MGP size fractions are enriched with exonuclease ABC subunit A. The 0.4 and 1 µm size fractions, which are considered smaller, display similar enrichment patterns. On the other hand, the larger size fractions (5, 12 and 100 µm) showed less comparable patterns. The enrichment of exodeoxyribonuclease V gamma subunit and exodeoxyribonuclease V beta subunit were found to be higher in size fractions of 100 µm in spring and summer.

By comparing the two results, it is thought that the functional variability could be related to the significantly different abundant taxa because taxonomic diversity would alter the distribution of nuclease genes among season and size fractions.

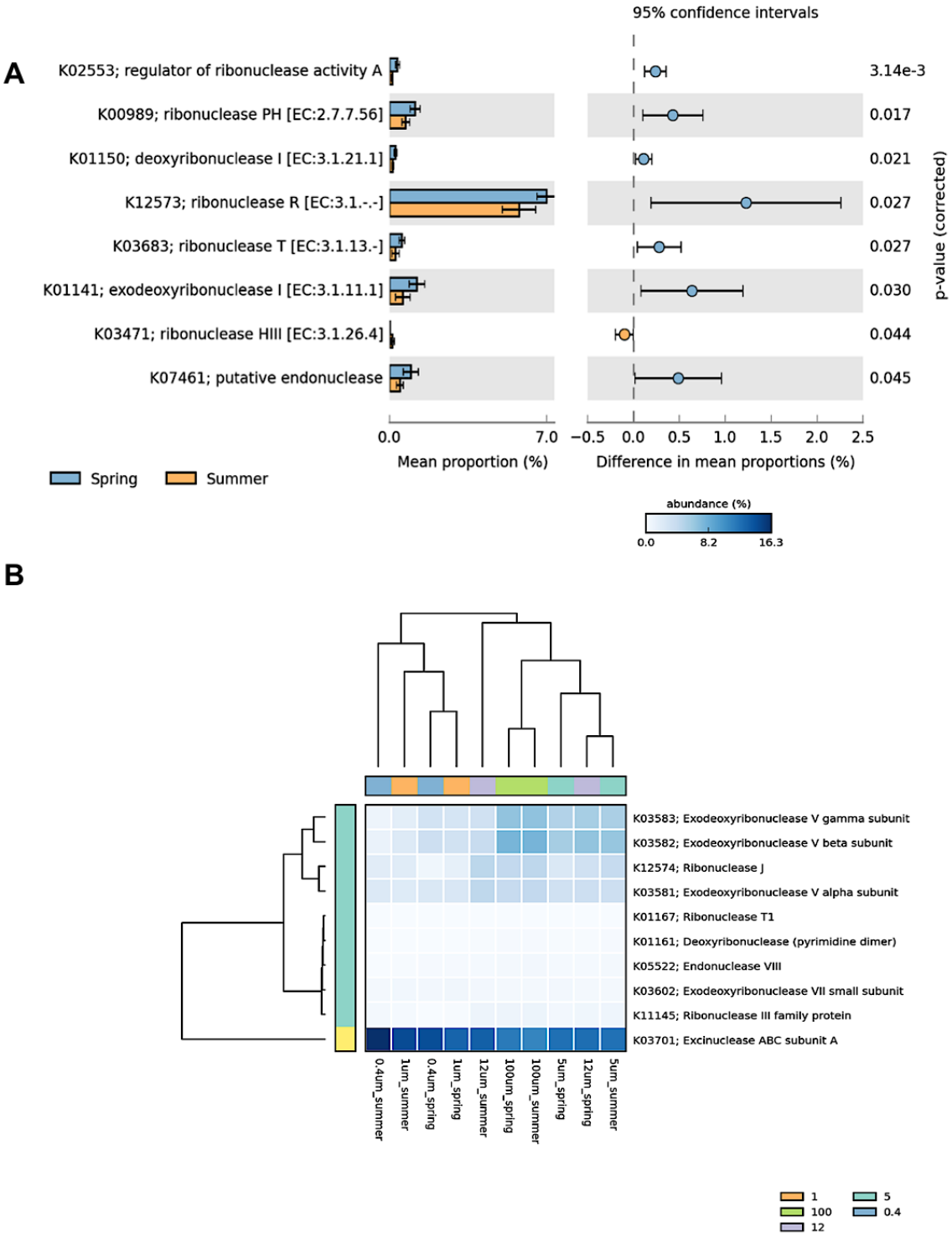

Figure 3.13 The group of nuclease enzymes present within the bacterial community associated with different size fractions of MGP. A: Extended error plots with 95 percent confidence intervals using Benjamin-Hochberg FDR procedure. B: Heatmap presenting the distribution (in relative abundances) of predicted nuclease enzymes. The dendrogram clustering threshold is 95 percent.

Overall, these results indicate that bacterial communities associated with different MGP size groups vary with season and sizes, as well as the functional profiles. Together these results provide important insights into the compositional structure and functional profile. KEGG pathways and genes associated with bacterial community associated with MGPs were mapped to functionally profile spring and summer of different MGP size groups.

3.5 Discussion

MGPs are ubiquitous in the ocean playing a key role in the carbon cycle and sedimentation of organic matter through the microbial communities they harbour. This study characterised the structure of the bacterial community associated with MGPs through amplicon-based sequencing (Illumina MiSeq) of the 16S rRNA gene. Although the best technique to provide detailed metabolic and functional profiles is to perform a whole metagenome shotgun, the 16S rRNA sequencing method is a cost-effective technique that provides a good understanding of microbial ecology (Langille *et al.*, 2013; Anahtar *et al.*, 2016; Ranjan *et al.*, 2016).

Initially, a pilot study to assess the bacterial community associated with MGP used a comprehensive size range of over 0.4 µm and 0.4-100 µm. This study was designed to help understand the overall bacterial community associated with MGP. It highlighted the general overview of bacterial community structure associated with a wider range of MGP sizes obtained from one filter size. However, the choice of the wide-range community in one filter size hindered the ability to get a clear insight into the bacterial communities associated with different sizes of MGP. The 0.4 µm filter size results were expected to overlap with the bacterial communities of the 0.4-100 µm filter. The beta diversity results of PCoA showed dissimilar communities in each size group.

Therefore, a size fractionation study was conducted to understand the bacterial communities attached to individual size groups as it provided a differentiated and comprehensive depiction of the bacterial community structure compared to the one size filter (Mestre et al., 2017). This enabled studying the functional capabilities of the taxa present in each different size group of MGPs. The fractionation of the MGP size range (0.2-100µm) resulted in five discrete MGP size groups for spring and summer, (the detailed corresponding sample IDs are in section 3.3.2, Table 3.1). In addition, functional profiling of the bacterial community of the five MGP size groups was explored to describe the potential functions associated with the bacterial community of each size of the MGP.

Previous studies that depended on size fractionation of water samples reported distinct groups of bacterial communities between free living (FL) and particle attached (PA) bacteria (DeLong *et al.*, 1993; Acinas *et al.*, 1999; Moeseneder *et al.*, 2001; Zhang *et al.*, 2016; Mestre *et al.*, 2017). Conversely, other studies have shown high resemblance between the FL and PA bacterial communities (Hollibaugh *et al.*, 2000; Ortega-Retuerta *et al.*, 2013). Thus far, there is an argument in the literature on the overlap size range of FL and PA bacteria as well as their similarity and dissimilarity within these size groups. These could possibly suggest a spatial

and/or temporal variation or an error in the serial filtration in the fractionation procedure to separate the FL and PA fractions of the bacterial communities as some bacteria may stick to the filters of the bigger size fractions (Mohit *et al.*, 2014; Rieck *et al.*, 2015).

3.5.1 Bacterial communities associated with different MGP size fractions

Pilot study

The pilot study results revealed that the richness and diversity of the one filter size 0.4-100 µm and 0.4 µm are distinct. Despite the overlap in gel particle sizes that were collected on each of them, interestingly, the 0.4-100 µm fraction showed higher richness and diversity compared to < 0.4 µm. Although the filter size >0.4 µm which was expected to be richer and more diverse because it contains particles larger than 100 µm. This would suggest that the upper size cut-off has affected in some way the abundance and composition of the bacterial community. Studying the MGP bacterial community in a larger range of sizes, 0.-100 µm and >0.4 µm, has provided general insight into the taxonomic groups within the particles without distinguishing between different sizes on the filters.

The diversity of bacterial community of MGPs size groups

The choice of these seasons and size fractionation was based on gel particles in the ocean being higher in abundance during spring and summer (Engel *et al.*, 2004; Verdugo *et al.*, 2004). Despite this there are few studies available on the abundance of MGP in the current study area of the North Sea (Sintes *et al.*, 2010), these two seasons were chosen because MGP numbers are high during spring-summer periods (Mestre *et al.*, 2017).

The rarefaction analysis of the sequencing data indicates that the achieved sequencing depth of 8500 per sample was sufficient to reveal the low abundance species as the rarefaction plots of the reads against OTUs levelled off (Schloss *et al.*, 2009; Daley and Smith, 2013). More samples and taxonomic descriptions of the MGPs are needed to decrease the proportion of squandered sequences reads and to fully describe the nature of the bacterial community composition of different MGP size fractions. Despite these limitations, the results revealed valuable phylogenetic information on the MGP bacterial diversity.

The two large size fractions of 12 µm and 100 µm during summer had fewer OTUs in comparison to other filters size, which would indicate that the bacterial abundance on MGP is not size dependent. Interestingly, the species richness did not increase with increasing MGP

size. This suggests that there is no correlation between the richness of bacterial taxa and the associated particle size. This would be inferred by the abundance of dominant bacteria that facilitate attachment and prevent others from attachment to the same particles. In addition, variations in environmental conditions and substrate availability may also be important (Yawata *et al.*, 2014; Cordero and Datta, 2016; Datta *et al.*, 2016). In contrast, a similar study did find an increase in community species richness with particle size (Mestre et al., 2017).

The results showed that there was significant variation between the five discrete size fractions of MGP in spring and summer, harbouring distinct bacterial taxa. As the results show, the major bacterial communities associated to MGP belong to Proteobacteria. These findings are consistent with some previous studies that the major phylum associated with gel particles like TEP are Proteobacteria (Levi et al., 2016). This may suggest that the composition of the outer membrane of Proteobacteria (Gram negative) that is made up from lipopolysaccharides may facilitate the attachment of Proteobacteria to marine gel aggregates which are mainly made up from polysaccharides. Lipopolysaccharides are an amphipathic molecule and have been reported in pathogenic bacteria as self-aggregating, forming larger molecules in the blood stream (Santos et al., 2003). Moreover, taxa within the Bacteroidetes, including Cytophaga and Flavobacteria, are often found associated with marine snow and phytoplankton blooms in marine environments (DeLong *et al.*, 1993; Teeling *et al.*, 2012a; Fontanez *et al.*, 2015).

The present data show that different MGP size groups harboured distinct bacterial community compositions, although the differences was not consistent. It was hypothesised that bacterial communities differ greatly between particle fractions and this may result in a connection of microbial properties of different size groups. However, the current study reveals a significant difference in the bacterial community abundances within different MGP size fractions and between seasons. The Alteromonadaceae and Flavobacteriaceae families that differ significantly in abundance, favouring spring. Alteromonadaceae are a marine family reported to utilise a variety of organic molecules and to produce extracellular enzymes like amylase, lipase, gelatinase and chitinases (López-Pérez and Rodriguez-Valera, 2014). Flavobacteriaceae are cytophages belonging to Bacteroides and commonly found attached to marine particles (Tully *et al.*, 2014). The variation of bacterial community associated with MGP in spring and summer is common and it is in agreement with previous studies (Glöckner et al., 1999; Kirchman, 2002; Amaral-Zettler et al., 2010).

At this point, by describing the MGP diverse bacterial communities associated with various size fractions, insights into the size and seasonal differences of the bacterial community structure of the North Sea MGPs were gained. Size fractionation e allowed a further overview

of the microorganisms in the FL and PA modes. Generally, the taxa of particle-associated communities contributed to a significantly higher phylogenetic and metabolic diversity of the MGPs, that is in agreement with studies of particulate material in other aquatic ecosystems (Dang and Lovell, 2016; Yung *et al.*, 2016).

3.5.2 The functional profile of MGP-associated bacterial communities

The potential functional profile of the bacterial community associated with MGP has been investigated here by using 16S rRNA bacterial community analysis functional predictions were carried out by sequence annotation in KEGG. The results of the predicted bacterial functional profiles of the MGP size fractions displayed diverse putative functions represented by various pathways and enzymes present within the bacterial communities associated to different MGP size fractions. Putative functions predicted that there is significant difference in the metabolic pathways encoded by the bacterial communities between spring and summer. The results showed noteworthy variation in the genes encoding the pathways of purine and pyrimidine metabolism, where both pathways showed higher appearance in summer MGPs. This is indicating a higher demand of the enzymes related to these two pathways in the summer. This observation is in agreement with a prior study that reported higher uptake rates of dissolved eDNA in the coastal Northwest Mediterranean in the summer using a radioactive bioassay method to quantify the dissolved eDNA (Salter, 2018). This investigation reported a turnover rate of the eDNA in less than five hours during extreme limited phosphate period.

Moreover, to map the findings of high purine and pyrimidine putative genes present in summer with the significantly abundant families found in association with the MGPs in summer: Prevotellaceae Lactobacillaceae, Paraprevotellaceae, Porphyromonadaceae, Bacteroidaceae, Fusobacteriaceae, Lachnospiraceae and Enterobacteriaceae. This would suggest that these bacterial groups might be responsible for the high pathway's abundance. Presumably, these families might be consuming eDNA and thus encoding purine and pyrimidine. A recent study has investigated the intake of eDNA by marine bacteria in the sea bottom (Wasmund *et al.*, 2019). This study revealed that diverse bacteria of the sea floor like *Candidatus* Izemoplasma, *Lutibacter*, *Shewanella*, *Fusibacteraceae* and *Nitrincolaceae* are able to utilise eDNA derived carbon. Where the genomic study of *Candidatus* showed several genes of extracellular nucleases and the Fusibacteraceae genome haven't encode any extracellular nuclease genes. Other earlier studies have also reported the consumption of DNA by marine bacteria like some

species of α-Proteobacteria, *Vibrio, Alteromonas*, *Pseudoalteromonas and Shewanella oneidensis*, yet the last is reported to use the DNA by secreting extracellular nucleases (Lennon, 2007; Gödeke *et al.*, 2011).

A variety of pathways were significantly enriched in spring MGPs, such as those associated with bacterial chemotaxis and flagella assembly pathways. This points to the fact that observed pathways may be highly involved in the motility of bacteria present within the community toward freshly-produced particles that thrive and are abundant in spring following algal blooms (Teeling et al., 2016). Notably, the photosynthesis pathway was higher in MGP size fractions 5, 12 and 100 μm in spring and summer, in agreement with the taxonomic profile of these groups that was dominated by Cyanobacteria *Synechococcus*. This suggests that these MGP bacterial communities are playing a role in the fixation of carbon by photosynthesis during the spring-summer. This finding is in agreement with a prior study that reported a correlation between bigger particle sizes and cyanobacterial abundance in Lake Taihu, China (Shi *et al.*, 2018).

The results showed that distinct bacterial taxonomic structural composition in the MGP may drives distinct functional profiles. This work has shown that shifts in the taxonomic composition of MGPs in different size fractions did not correspond to the metabolic potential profile that remained relatively the same across size fractions and seasons. This indicates a partial functional redundancy, consistent with recent studies (Allison and Martiny, 2008; Sunagawa *et al.*, 2015; Louca *et al.*, 2016; Louca *et al.*, 2018). However, another study reported a strong correlation between microbial taxa and their functions (Galand *et al.*, 2018).

MGPs undergo continuous aggregation and dispersion, where the dispersed particles have low buoyancy fragments tend to travel upward to the ocean surface (Cunliffe *et al.*, 2011; Wurl *et al.*, 2011). This natural dynamic of the MGP is thought to be controlled by bacterial extracellular enzymes (Baltar *et al.*, 2010; Baltar *et al.*, 2017). Therefore, it is important to understand the bacterial community composition of MGPs and their functional profiles in order to increase the knowledge of bacterial-particles interactions and their potential ecological dynamics in the ocean.

Despite its exploratory nature, this study for the first time offers some insight into the functional diversity of the bacterial community composition associated with MGPs size groups of 0.2-100 μm. However, major contributors to bacterial community diversity and corresponding functional gene abundances in MGP remains largely uncharacterised. Marine microorganisms

attach to particles as a preferred lifestyle (Smith et al., 1992; Simon et al., 2002; Grossart et al., 2007). Gene expression of enzymes in bacteria is a feature distinct for the planktonic mode of life or biofilm mode where bacteria live in consortia (Chua et al., 2014). Therefore, extracellular enzymes may be secreted by expression, depending on bacterial demand and mode of life. Notwithstanding that this is a functional predictions study of the MGP bacterial community, the encoded genes presents are not necessarily expressed. Hence, it is important to assess the functions by either transcriptome or culture dependent studies. The next chapter, therefore, moves on to discuss the capability of secretion of DNases by cultured FL and PA marine bacteria.

3.6 Conclusions

This work examined bacterial community structure and profiled the functional capabilities of different size groups of MGP from Northumberland offshore waters collected in spring and summer. The size fractionation of MGPs enabled a better understanding of the bacterial communities associated with each MGP size range. The several findings of this study are:

1. The taxonomic structures of microbial communities attached to MGP's vary between seasons (spring and summer) and size groups.
2. The MGP microbial community is dominated by diverse families, mainly Proteobacteria, Bacteroidetes, Firmicutes and Cyanobacteria. The variation was size and season dependent.
3. Seventy-five putative nuclease genes are present within the MGP attached bacterial communities.
4. Some taxonomic groups may influence the functional capabilities of the MGP bacterial communities.
5. The data showed evidence of partial functional redundancy of the microbial functional profiles of the bacterial communities.
6. This work can contribute to a further consideration of the role of MGP bacterial metabolites in the gel particle dynamics in the ocean.

Chapter 4. Isolation and identification of marine bacteria producing extracellular nucleases

4.1 Abstract

The vast majority of bacteria in the natural environment are present in the form of aggregates and/or biofilms. Microbial aggregates are ubiquitous in the marine environment and are inhabited by diverse microbial communities that often express intense extracellular enzymatic activities. This has implications for marine biogeochemical processes, such as nutrient cycling for example. Despite this, the secretion of DNases, an important group of enzymes released by marine aggregate associated bacteria, has not been studied. Therefore, in this work a culture-based approach was used to examine the diversity of bacterial nucleases in particle-attached and free-living marine bacteria. Bacterial isolates were identified by 16S rRNA and MALDI-TOF mass spectrometry and investigated for DNase production on methyl green DNase test agar. The results showed that of the 115 isolates identified, most of them belonged to Gammaproteobacteria (58%), with smaller contributions from by Actinobacteria (16%) and Firmicutes (14%). Further, 36% of the isolates showed positive DNase activity, dominated by the *Vibrio* and *Shewanella* genera. Further characterisation was carried out for the most active isolates, and these showed DNA hydrolysis comparable to high concentrations of micrococcal nuclease (MNase) and Nuclease B (NucB), the positive controls. Indicating a potent DNase producer from marine sediments is classified as *Serratia marcescens*. This study highlighted the prevalent production of DNases among marine bacteria and the secretion of nucleases by bacteria isolated from marine gel particle (MGPs) for the first time in marine aggregates. This has important implications for understanding the dynamics and fate of marine aggregates in relation to extracellular DNase in the oceans.

4.2 Introduction

The marine environment contains enormous microbial diversity (Salazar and Sunagawa, 2017) and density; on average, bacteria are present in seawater at concentrations of 10^6 cells ml^{-1} (Azam, 1998; Azam and Malfatti, 2007). Bacteria can live in one of two modes: either as free living (planktonic) or aggregated, either attached to surfaces (biofilm) or in the form of particle aggregates (flocs). In the aggregated forms, bacteria live embedded in a hydrated slimy matrix made up of extracellular polymeric substances (EPS) in the form of biofilms and protobiofilms (Verdugo, 2007; Verdugo and Santschi, 2010; Bar-Zeev *et al.*, 2012; Elias and Banin, 2012). "Marine gel particles" is a term used to describe suspended bacterial aggregates that initiate biofilm formation when they become attached to surfaces, and which are found ubiquitously in the oceans (Bar-Zeev *et al.*, 2012; Neukermans *et al.*, 2016; Busch *et al.*, 2017).

Marine bacteria can impact nutrient cycling by secreting extracellular enzymes (ECE) (Azam, 1998; Azam and Malfatti, 2007; Arnosti, 2011; Luo *et al.*, 2017; Ivančić *et al.*, 2018). These play a fundamental role in the hydrolysis of high-molecular-weight organics, forming low-molecular-weight compounds that can be readily taken up by bacteria, and they can also affect the stability of MGP aggregates (Vetter and Deming, 1999; Hoppe *et al.*, 2002; Lechtenfeld *et al.*, 2015; Balmonte *et al.*, 2016; Liu and Liu, 2018). As a result of their implicit role in the sequestration of carbon and its transfer to the deep ocean on sinking aggregates, MGPs and their associated bacterial ECEs have recently attracted a lot of attention (Karner and Herndl, 1992; Grossart *et al.*, 2007; Arnosti *et al.*, 2012; Kellogg and Deming, 2014).

Although many hydrolytic extracellular enzymes are produced by free-living bacteria and bacteria attached to marine aggregates, for instance chitinases and proteases (Smith *et al.*, 1992; Traving *et al.*, 2015; Baltar *et al.*, 2017), information on the presence and diversity of deoxyribonucleases (DNases) in marine bacteria, especially those associated with aggregates, is absent.

DNases catalyse the hydrolysis of deoxyribonucleic acid (DNA) by breaking down phosphodiester bonds (Nishino and Morikawa, 2002). Consequently, DNase is considered to have a pivotal role in DNA utilisation, horizontal gene transfer and nutrient cycling in the environment (Mulcahy *et al.*, 2010a). In addition, extracellular DNA (eDNA) plays an important role in the formation and structure of biofilms (Whitchurch *et al.*, 2002). Indeed, it is now considered a key structural component of the biofilm matrix (Tetz *et al.*, 2009). eDNA plays a critical role in the attachment and stability of the biofilm matrix and DNases are now well recognised as agents that can effectively break up biofilms (Nijland *et al.*, 2010;

Jakubovics *et al.*, 2013; Shields *et al.*, 2013; Okshevsky *et al.*, 2015). It is of considerable interest whether eDNA is also an important component of marine flocs, and if so, whether the secretion of DNases by floc-associated bacteria can affect the structural integrity (and sinking rates) of marine particles. The secretion of extracellular DNases has been reported in several species of marine bacteria such as *Bacillus licheniformis* (Nijland *et al.*, 2010), *Vibrio* sp. (Maeda and Taga, 1976), *Myroides*, *Planococcus*, *Sporosarcina* and *Halomonas* (Dang *et al.*, 2009), although their precise role remains obscure.

The well recognised availability of eDNA to serve as a source of nutrients in the oceans (Dell'Anno and Danovaro, 2005) might also explain the production of DNases by marine bacteria. However, little is known about the diversity of DNases produced by marine bacteria generally and in marine aggregates in particular. This study therefore examined the hypothesis that the production of extracellular DNases among marine bacteria is commonplace. The diversity of extracellular DNase production by free-living marine bacteria and by bacteria attached to aggregates was therefore examined.

4.2.1 Aim and objectives

The aim of this chapter is to investigate the diversity of marine bacteria producing DNase. This is accomplished by pursuing the following objectives:

1. To isolate free living and aggregate-associated marine bacteria.
2. To investigate the ability of the isolated marine bacteria to produce DNase.
3. To identify the DNase-producing bacteria using 16S rRNA gene marker sequencing.
4. To understand the phylogeny of marine DNase-producing bacteria.
5. To investigate the nuclease gene distribution in the genomic DNA of the best isolate producing DNase.

4.3 Materials and Methods

4.3.1 Sampling sites

Marine bacteria from seawater, sediments, MGPs and biofilm were isolated and investigated for nuclease production. Seawater and sediments were collected on 25/03/2015 and MGP was collected on 31/10/2016, from the North Sea, one-mile off the Northumberland coast (55^{o}06.972 N, 1^{o}25.600 W), using the Research Vessel Princess Royal. Surface seawater was collected from sea surface top 30 cm and at 5 m depth using polyethylene clean buckets and 5 L Niskin bottles. Surface sediment was collected by a Van Veen sediment grabber in 50 m of water. Samples were transported in clean 5 litre Nalgene bottles, and stored at 4^{o}C in a cold room until been processed within 24 hours. Marine biofilm bacteria were collected on 25/08/2015 from intertidal zone rocks on Ras Alhamra beach in Muscat, Oman (23^{o}38’20.8 N 58^{o}29’28.5 E). Samples were collected using sterile swabs (Sterilin, UK), and transported in a cool box for less than 2 hours until reaching the laboratory. Sampling details are shown in Table 4.1.

A batch of previously isolated and identified marine bacterial strains preserved in 50% glycerol and stored at -80^{o}C (Burgess Group Culture Collection (BGCC); Newcastle University) were also used.

Table 4.1 Sampling dates, location, and source of isolates.

Sampling date	**Location**	**Source of isolates**
25/03/2015	The North Sea	Seawater Sediment
10/08/2015	Ras Alhamra beach, Muscat, Oman	Biofilm from on rocks intertidal zone
31/10/2016	The North Sea	MGP

4.3.2 Isolation of bacterial strains and growth conditions

Isolation and purification of the marine isolates were carried out using marine agar 2216 (Difco™ Marine Agar, BD, UK) and marine broth 2216 (Difco™, Marine broth BD, UK) under aseptic conditions. Bacteria were isolated from seawater by direct spreading onto marine agar plates. Sterile swabs were used to rub the sediments and the polycarbonate filters (PC) filters 0.4-100 µm (MGP filtration process described in Chapter 2) and then streaked onto marine agar plates. Biofilm isolates were streaked as well directly from the swabs onto the marine agar plates. The preserved BGCC bacterial collection was propagated from glycerol stocks by streaking about 5 µl onto the marine agar. All isolates were incubated at room temperature (22^{o}C). Growth of the isolates was examined after 24-72 hours of incubation. Isolate colonies with a distinct morphology and colour were cultivated onto fresh marine agar plates to obtain pure single colonies. The colonies were further purified up to the fourth and fifth generations. All pure bacterial isolates were cultivated in marine broth at room temperature with agitation at 150 rpm using an orbital shaker. The overnight liquid marine broth cultures of isolates were preserved in 50% glycerol and stored at -80^{o}C for further use.

4.3.3 16S rRNA gene identification of DNase-producing bacteria

Bacterial isolates were cultured overnight in 5 mL marine broth (Difco™ Marine Broth 2216, BD) with agitation at 150 rpm. Bacteria cell cultures (OD 1.8-1.9) were centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and the bacterial pellets were extracted using a Genomic DNA mini kit (Invitrogen PureLink^{TM}, USA), following the manufacturer's instructions. DNA extracts were checked by Nanodrop 2000 spectroscopy (Thermo-Fisher Scientific, USA). The 16S rRNA gene universal primers 27F (5'-AGA-GTT-TGA-TCC TGG CTC-AG-3') and 1492R (5'-ACG-GCT-ACC-TTG-TTA-CGA-CTT-3') (Eurogentec, Belgium) were used to bind with the target gene. The amplification was carried out with a thermocycler PCR (Bio-Rad). Prior to the PCR, a 50 µL reaction was prepared with 1µL of DNA and 1µL of each forward and reverse primer, mixed with 22 µL MyTaq^{TM} Red Mix 2X (Bioline, Meridian Bioscience Inc., USA, www.bioline.com). The PCR reaction was made up to 50 µL with sterile MilliQ water. The PCR thermocycles were performed under the following cycling conditions: an initial denaturation step at 95°C for 1 minute**,** followed by 35 cycles of 15 seconds at 95°C**,** 15 seconds at 55°C**,** and 10 seconds' extension at 72°C. Gene sequences of isolates were sequenced using the Geneius laboratories service (Newcastle, upon Tyne**,** UK,

http://www.geneiuslabs.co.uk), except for samples obtained from Oman, which were sequenced at the Central Analytical and Applied Research Unit (CAARU), Sultan Qaboos University, Oman. The 16S rRNA gene sequences were obtained in the ABI file format, edited in MEGA7, and converted to the FASTA format file. The edited sequences were aligned with the nearest sequences in the Basic Local Alignment Search Tool (BLAST, NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi). The matched sequences were compared to the 20 nearest similar matches. Alignment and phylogenetic trees of positive nuclease-producing isolates were constructed following procedures described at: http://www.phylogeny.fr.

4.3.4 MALDI TOF-MS identification

Identification of non-DNase-producing marine biofilm bacteria obtained from the Omani coast of the Sea of Oman was by Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS, BRUKER, https://www.bruker.com) at CAARU. Pure single colonies at 12-24 hours were picked up from agar plates and placed directly onto the MALDI target plate to dry and be read.

4.3.5 Examination of DNase production by the bacterial isolates

Nuclease test for cultivable bacteria

All purified isolates were investigated for DNase secretion by an initial test and the positive isolates were then further tested following the procedure described here. All purified bacterial isolates were examined for the production of DNase by streaking onto methyl green DNase test agar (Difco 263220, BD, UK) incubated for 24-72 hours at room temperature. This is an efficient and low-cost method for detecting the production of DNase that is based on a medium enriched with 2 g of DNA that binds to methyl green (positive charged dye binds to DNA). A positive reaction is indicated by colourless clear zones around the colonies of the tested isolates. Hydrolysis of the DNA occurs as a result of DNase secretion that breaks down the DNA-methyl green (Jeffries *et al.*, 1957; Smith *et al.*, 1969). Positive bacterial isolates producing nuclease, showing a halo around the bacterial colonies, were selected for further investigation with the bacterial supernatant test.

Well-diffusion method and bacterial filtered supernatant

The agar well-diffusion method (Jatt *et al.*, 2015) was employed for the supernatant deriving from DNase-positive bacterial isolates. Broth bacterial cultures were centrifuged at 7000 rpm for 5 minutes. The supernatant was filtered through 0.2 µm Millipore syringe filters (Sigma-Alrdich, UK) to investigate extracellular nuclease production on the wells (Tredwell *et al.*, 2011). Wells of 5 mm were punched in the solid DNase agar plates by sterile 1000 µl tips, four wells per plate. Each well was filled with 100 µl of the DNase-positive isolate filtered supernatant. A negative control of marine broth and positive controls of Micrococcal Nuclease (MNase, stock concentration $2x10^6$ gel U/ml) (New England Biolabs, Life Technologies) and NucB (0.65 µg/ml), a marine deoxyribonuclease from *Bacillus licheniformis* EL-34-6 (Nijland *et al.*, 2010) provided by the Institute for Cell and Molecular Biosciences (Newcastle University, UK), were used in this analysis. MNase concentrations of 20, 200 and 2000 gel Unit/ml, and NucB concentrations of 0, 10, 100 and 1000 ng/ml were studied. The extracellular DNase activity is measured by the diameter of the halos surrounding the bacterial culture supernatants. The active bacterial supernatant with active nuclease production was tested for DNA digestion.

Digestion of DNA by DNase active supernatant

To characterise the activity of the positive bacterial supernatant against the DNA, a digestion test reaction was prepared in a total volume of 250 µL, containing 25 µL Calf-thymus DNA (CT-DNA of 1 mg/mL, Sigma Aldarich, UK), 12.5 µL $MnSO_4$ (5 mM final concentration of 100 mM stock), Mn^{2+} divalent cations to enhance nuclease activity (Philip *et al.*, 2017) and 125 µl Tris buffer (100 mM stock, pH 8). Ten µl of 0.2 µm filtered bacterial supernatant and controls were added separately, plus sterile MilliQ, to bring the mix to the final volume. After incubation at 37^{o}C for 24 hours, 5 µl of each sample mix was mixed with 1 µl of blue 6x DNA loading dye (Thermo-Fisher scientific, USA). The digestion of DNA was monitored by 0.8% agarose (Sigma-Aldrich) gel electrophoresis and run for 45 minutes at 100V using a gel casting system (Vari-Gel Midi-System, Fisher Brand, UK). The gel was visualised with UV imaging (Bio-Rad, Gel Doc EZ system, UK).

4.3.6 Genomic DNA sequencing for bacterial isolates with highest DNase production

The marine isolate that showed the highest DNase production, AW2 was selected for the genomic analysis. The genomic DNA extraction was carried out using a DNA extraction kit

following the manufacturer's instructions (Genomic DNA mini kit, Invitrogen PureLink™, USA). The genomic DNA purity was checked with a Nanodrop™ to maintain A260/280 > 1.8. The sequencing was performed at NUOMICS (Northumbria University, Newcastle upon Tyne) using the Illumina Nextera XT platform (Lahra *et al.*, 2018), V2 chemistry and sequencing depth of 15Mb reads. The sequence files were received in FASTQ format. The sequence quality control (QC) checking was processed in GALAXY a bioinformatics web-based platform https://usegalaxy.org/. The assembly of the sequences was carried out in SPAdes (http://spades.bioinf.spbau.ru/release3.10.1/manual.html) with the help of Dr. Darren Smith, NUOMICS. Classification of the isolate was carried out with Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/). Sequence annotation was performed in PROKKA (Cuccuru *et al.*, 2014) via GALAXY.

4.4 Results

4.4.1 Classification of isolated marine bacteria

In the present study, a total of 115 bacterial isolates were obtained from two different marine origins, the North Sea off the Northumberland coast and the coast of the Sea of Oman. A culture-based study was performed on free-living as well as aggregate-attached bacteria forms, from different marine niches, seawater, sediments, marine biofilm, and marine gel particles (MGP), in addition to the previously collected and identified isolates of the Burgess Culture Collection (BGCC) (Table 4.2). Twenty different isolates recovered from MGP aggregates from the North Sea and 23 marine biofilm isolates from the coast of Oman were investigated for DNase production (Table 4.2).

Table 4.2 The number of isolated marine bacteria and their origin.

Isolates origin	**Number**
Seawater (the North Sea)	14
Sediments (the North Sea)	2
Burgess group culture collection (BGCC)	56
MGP (the North Sea)	20
Marine biofilm (the Sea of Oman)	23
Total	115

The results showed that most of the bacterial isolates belong to Gammaproteobacteria (58%), followed by Actinobacteria (16%), Firmicutes (14%), Alphaproteobacteria (7%), Bacteroidetes (4%), and Betaproteobacteria (1%), as shown in Figure 4.1. Most of the bacterial isolates were classified into 20 different families dominated by Vibrionaceae (26.7%), followed by Bacillaceae (13.3%), Pseudoalteromonadaceae (8.6%), Alteromonadales (7.6%), and Micrococcaceae (6.7%) (Figure 4.2).

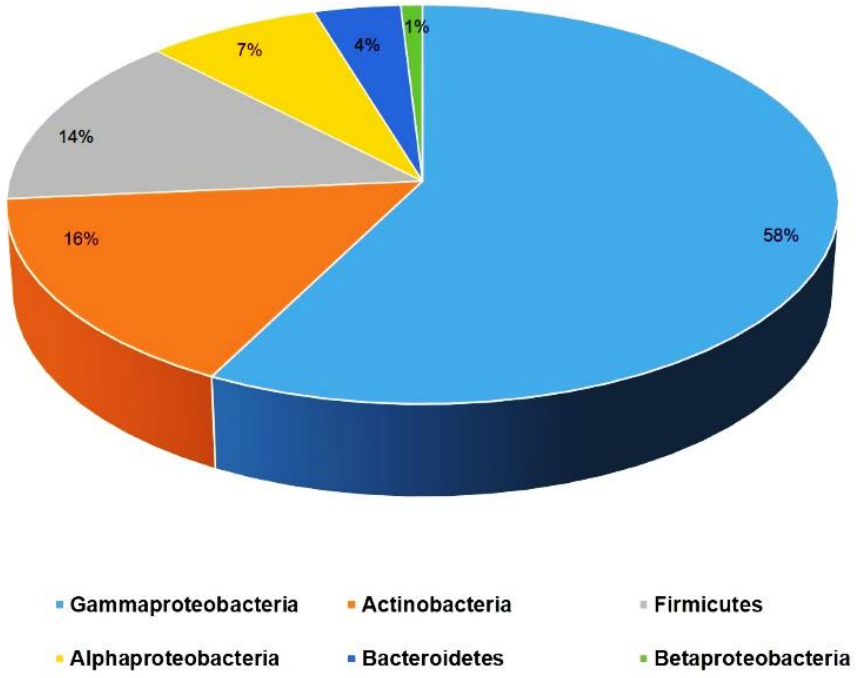

Figure 4.1 The classification of isolated bacteria at phylum level. Gammaproteobacteria is dominant, accounting for 58%, followed by Actinobacteria (16%).

Moreover, the results illustrated that most bacteria that secrete DNase are affiliated to the family Vibrionaceae (26.7%), followed by Bacillaceae (13%) and Pseudoalteromonadaceae (8%) (Figure 4.2).

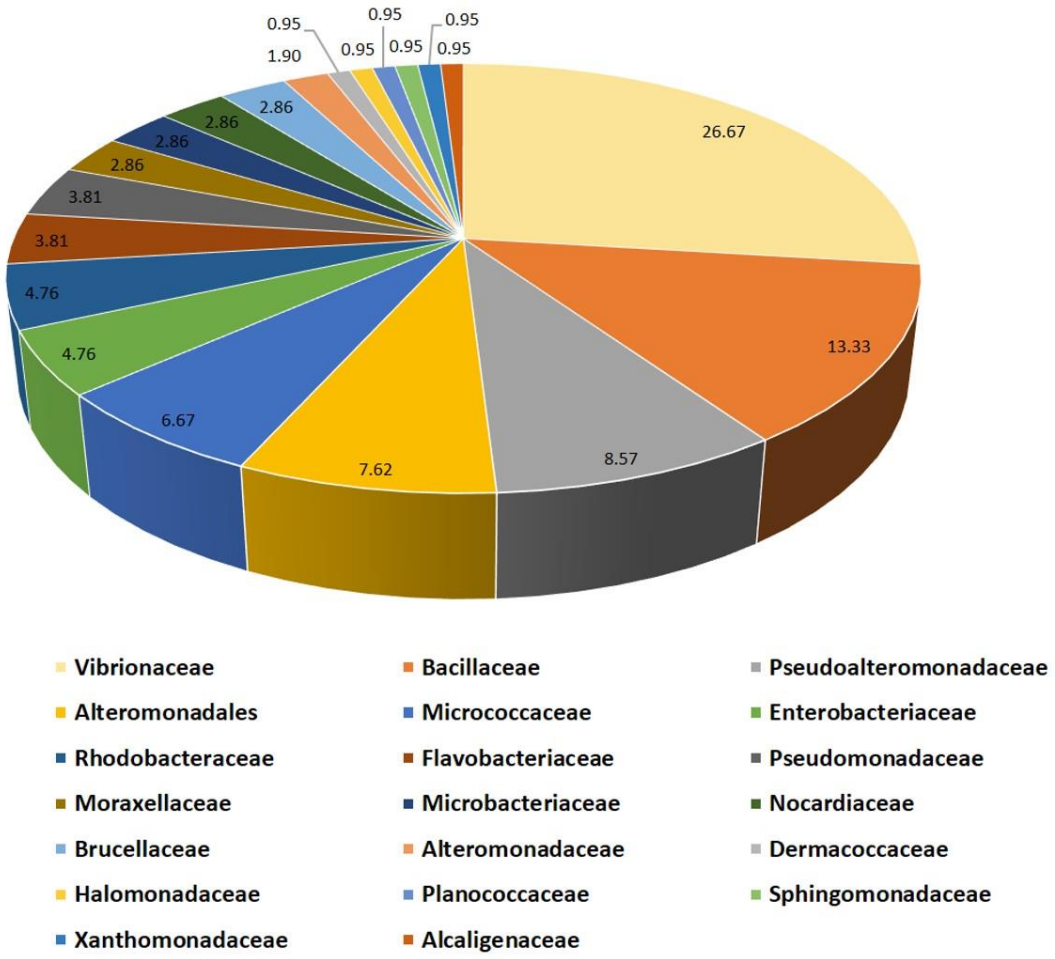

Figure 4.2 The classification of the isolated bacteria at family level. Vibrionaceae dominates, followed by Bacillaceae.

As shown in Table 4.3, the identification of the isolates in this study was completed primarily by sequencing the16S rRNA gene. Sequences of the identified strains were available, deposited in GenBank NCBI (https://www.ncbi.nlm.nih.gov/genbank/) accession numbers MK599164-MK599196. However, the non-DNase-producing bacteria from Oman were identified using MALDI-TOF as a cost cutting procedure. The non-DNase-producing isolates from the North Sea have not been identified (all preserved in 50% glycerol and stored at -80 in a freezer in Ridley Building 2, Newcastle University).

Table 4.3 The isolated bacteria tested for DNase activity on methyl green DNase test agar, DNase active (+), DNase inactive (-), the source of each strain (BGCC-CB = Cullercoats Bay, BGCC-AS = Arctic Sediment, BGCC-DSS = Deep Sea Sediment, BGCC-BB = Blyth Beach, BGCC-TR = Tyne Riverbank, BGCC-SSH = Seaton Sluice Harbour and BGCC = Burgess Group Culture Collection). Identification is based on 16S rRNA sequence similarities using online BLAST-NCBI. MALDI-TOF identification of non-DNase-producing isolates obtained from Oman.

No.	Code	Source	DNase activity	16S rRNA nearest match	Sequence match %
1	AW1	N. Sea Seawater	+	*Vibrio hemicentroti* strain AlyHP32	99
2	AW2	N. Sea Sediments	+	*Serratia marcescens* strain NBRC 102204	95
3	AW3	N. Sea Sediments	+	*Serratia marcescens* strain JCM	96
4	AW4	N. Sea Seawater	+	*Shewanella algicola* strain ST-6	92
5	AW5	N. Sea Seawater	+	*Shewanella basaltis* strain J83	99
6	AW8	N. Sea Seawater	+	*Vibrio lentus strain* CIP 107166	99
7	AW101	N. Sea Seawater	+	*Croceibacter atlanticus* strain HTCC2559	99
8	AW104	N. Sea Seawater	+	*Sulfitobacter dubius* strain LMG20555	94
9	A31	N. Sea MGP	-	*Pseudoalteromonas citrea* strain CERBOM CH10	99
10	D2	N. Sea MGP	-	*Planktotalea lamellibrachiae* strain JAM 119	99
11	E2	N. Sea MGP	+	*Bacillus proteolyticus* strain MCCC 1A00365	99
12	F3	N. Sea MGP	+	*Pseudoalteromonas espejiana* strain NBRC 102222	99
13	G22	N. Sea MGP	+	*Vibrio atlanticus* strain VB 11.11	99
14	H2	N. Sea MGP	-	*Shewanella fidelis* strain KMM 3582	99
15	I41	N. Sea MGP	-	*Pseudovibrio ascidiaceicola* strain F423	99
16	L3	N. Sea MGP	+	*Pseudoalteromonas citrea* strain CERBOM CH10	99
17	M2	N. Sea MGP	-	*Microbulbifer hydrolyticus* strain DSM 11525	99
18	O2	N. Sea MGP	-	*Polaribacter atrinae* strain WP25	98
19	P21	N. Sea MGP	-	*Dermacoccus nishinomiyaensis* strain DSM	99
20	P22	N. Sea MGP	-	*Micrococcus yunnanensis* strain YIM 65004	99
21	Q2	N. Sea MGP	-	*Psychrobacter cryohalolentis* strain K5	99

22	S2	N. Sea MGP	-	*Psychrobacter aquaticus* strain CMS 56	86
23	T2	N. Sea MGP	-	*Psychrobacter maritimus* strain Pi2-20	98
24	U2	N. Sea MGP	+	*Vibrio atlanticus* VB 11.11	99
25	V2	N. Sea MGP	-	*Cobetia amphilecti* strain 46-2	99
26	W2	N. Sea MGP	-	*Shewanella surugensis* strain c959	98
27	X2	N. Sea MGP	-	*Pseudoalteromonas issachenkonii* strain KMM 3549	99
28	Y	N. Sea MGP	-	*Polaribacter atrinae* WP25	98
29	1A202	BGCC	-	*Maribacter dokdonensis*	87
30	1B002	BGCC	-	*Bacillus simplex*	99
31	1B201	BGCC	-	*Paenisporosarcina macmurdoensis*	95
32	2A205	BGCC	-	*Kocuria carniphila*	93
33	2A601	BGCC	-	*Microbacterium maritypicum*	96
34	2A603	BGCC	-	*Sphingopyxis baekryungensis*	99
35	10A	BGCC -CB	-	*Ruegeria atlantica*	94.14
36	1A	BGCC -CB	-	*Vibrio sp. EJY3*	98
37	2A	BGCC -CB	-	*Pseudoalteromonas undina*	99.77
38	2B	BGCC-CB	+	*Pseudomonas fluorescens*	95.24
39	2D	BGCC-CB	+	*Polaribacter sp.*	96
40	5A	BGCC-CB	-	*Pseudoalteromonas carrageenovora*	98.53
41	AS1	BGCC-AS	-	*Bacillus pumilus*	88.34
42	AS2	BGCC-AS	-	*Arthrobacter pascens*	95.34

43	AS4	BGCC-AS	-	*Arthrobacter phenanthrenivorans*	99
44	AS5	BGCC-AS	-	*Stenotrophomonas maltophilia*	98
45	BB2	BGCC-BB	-	*Alcaligenes faecalis*	98.97
46	BB7	BGCC-BB	+	*Rahnella aquatilis*	99.78
47	BB9	BGCC-BB	+	*Terrabacter tumescens*	95.54
48	BBf	BGCC-BB	-	*Roseobacter denitrificans*	98
49	BO7	BGCC-TR	-	*Bacillus pumilus*	99.41
50	BOa	BGCC-TR	-	*Kluyvera georgiana*	96.86
51	BOb	BGCC-TR	-	*Roseobacter denitrificans*	97
52	BR6	BGCC-BB	+	*Rhodococcus erythropolis*	98.74
53	DS1a	BGCC-DSS	-	*Bacillus pumilus*	99.86
54	DS2a	BGCC-DSS	-	*Bacillus pumilus*	99.55
55	DS5	BGCC-DSS	+	*Bacillus simplex*	99.89
56	DS6	BGCC-DSS	-	*Bacillus licheniformis*	99.6
57	DS8	BGCC-DSS	+	*Bacillus pumilus*	99.22
58	G1Y	BGCC-CB	-	*Kocuria varians*	99
59	IP3	BGCC-TR	+	*Arthobacter protophormiae*	94.35
60	IP4	BGCC-TR	-	*Bacillus pumilus*	99.48
61	IPa	BGCC-TR	-	*Bacillus subtilis*	99.94
62	IPoI	BGCC-TR	-	*Bacillus sp.*	92
63	LM1/SH	BGCC-SSH	-	*Kocuria varians*	98.52

64	SB2C	BGCC	+	*Shewanella putrefaciens*	98.65
65	SH10	BGCC-SSH	+	*Bacillus weihenstephanensis*	99
66	SH13	BGCC-SHH	-	*Rhodococcus pyridinivorans*	98
67	SH14	BGCC-SHH	-	*Ochrobactrum anthropi*	98.71
68	SH15	BGCC-SHH	-	*Ochrobactrum anthropi*	98.67
69	SH2	BGCC-SHH	+	*Serratia marcescens*	99.87
70	SH4	BGCC-SHH	-	*Arthobacter uratoxydans*	99.89
71	SH5	BGCC-SHH	-	*Pseudomonas mendocina*	99
72	SH7	BGCC-SHH	+	*Psuedomonas sp.*	NA
73	SH8	BGCC-SHH	-	*Pseudomonas oleovorans*	99.93
74	SHa	BGCC-SHH	-	*Bacillus lichenformis*	99.6
75	SW1-3	BGCC	-	*Shewanella baltica*	99
76	TR10	BGCC-TR	-	*Rhodococcus erythropolis*	98..03
77	TR13	BGCC-TR	-	*Ochrobactrum anthropi*	98.89
78	TR16	BGCC-TR	-	*Pseudorhodobacter ferrugineus*	92.82
79	TR4A	BGCC-TR	+	*Shewanella putrefaciens*	98.36
80	TR4B	BGCC-TR	-	*Shewanella putrefaciens*	99.64
81	TR7	BGCC-TR	-	*Pseudomonas fluorescens*	99.52
82	TR7A	BGCC-TR	-	*Pseudoalteromonas tetraodonis*	99.04
83	TR8	BGCC-TR	+	*Microbacterium liquefaciens*	99.61
84	TR9	BGCC-TR	-	*Microbacterium liquefaciens*	NA

85	LB1	Marine biofilm	+	*Vibrio parahaemolyticus* ATCC 17802	99
86	LB3	Marine biofilm	-	*Vibrio fortis* strain CAIM 629	97
87	NB11	Marine biofilm	+	*Vibrio parahaemolyticus* ATCC 17802	99
88	MB11	Marine biofilm	+	*Vibrio inhibens* BFLP-10	99
89	MB12	Marine biofilm	+	*Vibrio parahaemolyticus* RIMD 2210633 O3:K6	99
90	MB12Y	Marine biofilm	+	*Pseudoalteromonas piscicida* NBRC 103038	99
91	MB51	Marine biofilm	+	*Vibrio alginolyticus* NBRC 15630	99
92	MB33	Marine biofilm	+	*Vibrio alginolyticus* NBRC 15630	98
93	MB44	Marine biofilm	+	*Vibrio alginolyticus* ATCC 17749	98
				MALDITOF-MS identification	**Score**
94	LB4	Marine biofilm	-	*Vibrio alginolyticus*	1.905
95	NB21	Marine Biofilm	+	*Vibrio alginolyticus*	2.018
96	NB31	Marine Biofilm	-	*Vibrio pelagius*	2.267
97	NB41	Marine Biofilm	-	*Vibrio pelagius*	1.871
99	MB13	Marine Biofilm	-	*Vibrio pelagius*	2.194
100	MB21	Marine Biofilm	-	*Vibrio alginolyticus*	2.078
101	MB22	Marine Biofilm	-	*Vibrio alginolyticus*	2.161
102	MB23	Marine Biofilm	-	*Vibrio alginolyticus*	2.064
103	MB31	Marine Biofilm	-	*Vibrio chagasii*	1.993
104	MB32	Marine Biofilm	-	*Vibrio alginolyticus*	2.074

105	MB41	Marine Biofilm	-	*Vibrio pelagius*	2.275
106	MB42	Marine Biofilm	-	*Vibrio alginolyticus*	1.988
107	MB43	Marine Biofilm	+	*Vibrio alginolyticus*	2.069
Unidentified					
108	AW8	N. Sea Seawater	-		
109	AW101	N. Sea Seawater	-		
110	AW102	N. Sea Seawater	-		
111	AW103	N. Sea Seawater	-		
112	AW105	N. Sea Seawater	-		
113	AW106	N. Sea Seawater	-		
114	AW107	N. Sea Seawater	-		
115	AW108	N. Sea Seawater	-		

4.4.2 Deoxyribonuclease (DNase) production by marine bacterial isolates

The results show that nearly 33% (38 out of 115) of the investigated isolates displayed positive DNase activity, revealed by clear halos around the colonies as a result of DNA hydrolysis (Figure 4.4). The DNase-producing bacteria are dominated by the genus *Vibrio* (12 isolates) followed by *Shewanella* (6 isolates), then *Bacillus* and *Pseudoalteromonas*, which each account for 4 isolates, *Serratia* (3 isolates), *Pseudomonas* (2 isolates), *Croceibacter* (1), *Sulfitobacter* (1), *Polaribacter* (1), *Rahnella* (1), *Terrabacter* (1), *Rhodococcus* (1) and *Microbacterium* (1). Of the DNase-producing isolates belong to the genera *Vibrio*, eight were obtained from the bacterial biofilm from the coast of the Sea of Oman and 4 were obtained from the Omani coast (Table 4.3).

The results of the MGP isolates showed that five isolates obtained from MGP were reported here for the first time to secrete DNase. These isolates belong to the families Pseudoalteromonadacea (2), Vibrionaceae (2) and Bacillaceae (1).

A phylogenetic tree was constructed for bacterial isolates producing DNase based on partial 16S rRNA gene sequences (sequence length about 800-1200 bp). The DNase-producing isolates of the BGCC (previously identified) are not included in the phylogenetic tree here as their sequences were not accessible.

The phylogenetic tree showed the evolutionary relationships between 20 DNase-producing isolates (AW1, AW2, AW3, AW5, AW8, AW101, AW104, LB1, LB3, NB11, MB11, MB12, MB12Y, MB33, MB44, MB51, E3, F3, G22, and L3) and their closest matched bacterial species in BLAST, NCBI as presented in Figure 4.3.

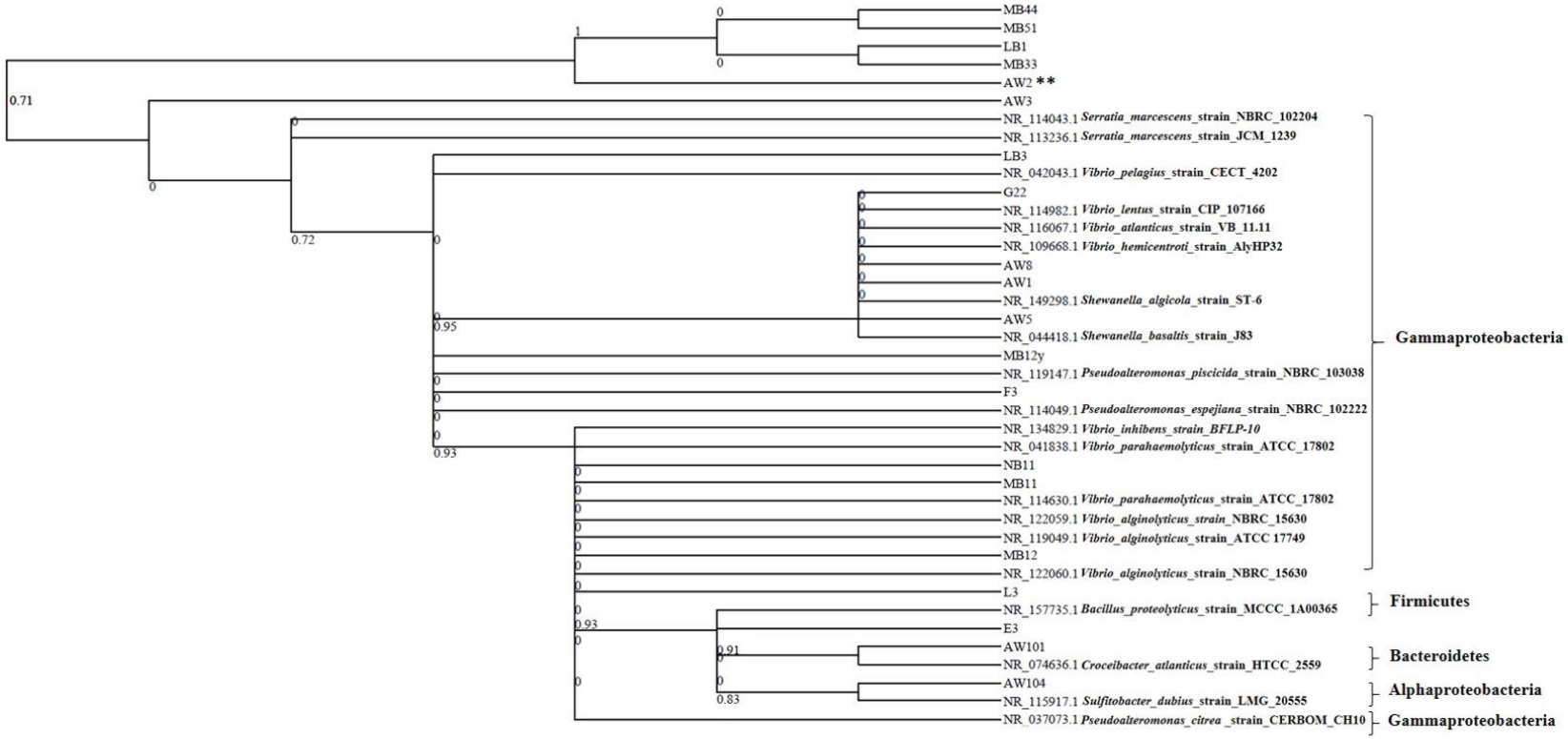

Figure 4.3 Neighbour-joining phylogenetic tree based on partial 16S rRNA gene sequences showing the relationships between marine bacterial isolates producing DNase: AW1, AW2, AW3, AW5, AW8, AW101, AW104, LB1, LB3, NB11, MB11, MB12, MB12Y, MB33, MB44, , MB51, E3, F3, G22, L3 and their phylogenetic nearest neighbours (related genera or species). Asterisks ** indicate potential new strains with high DNase activity. Scale bar represents 0.5 sequence similarity.

The DNase-producing bacteria were further characterised by testing the filtered supernatant from the bacterial overnight culture in DNase agar using a well agar diffusion technique. The zone of hydrolysis of DNA degradation was measured in millimetres to compare the DNase production capacity of the marine bacteria with standard nuclease enzymes (Figure 4.4). The data in Figure 4.5 show that the filtered supernatants of AW2 and AW3 had relatively higher DNase activity than the supernatants of G22, L3, LB1, NB21, and MB12. Meanwhile, the levels of DNase activity showed by the AW2 and AW3 filtered supernatants are comparable to high concentrations of MNase (2000 gel U/ml) and NucB (1000 ng/ml). This is indicative of high concentrations of DNase in the supernatants of AW2 and AW3.

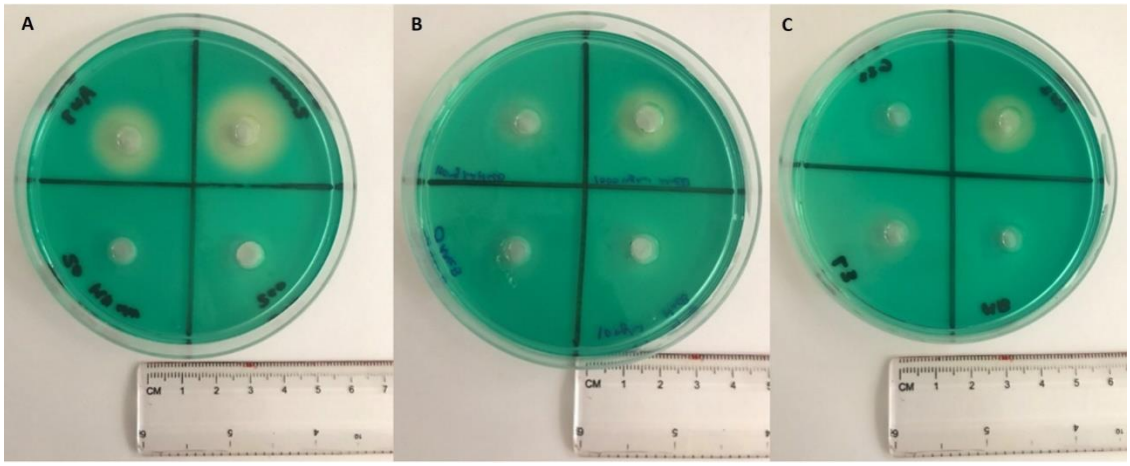

Figure 4.4 Zone of hydrolysis indicates DNase activity in the filtered supernatant of the bacterial isolates using a well diffusion method: (clockwise starting from top left corner) (A) AW3, 2000 gel U/ml, 200 gel/ml, 20 gel/mml and (C) AW2, L3, G22 compared to (B) different concentrations of micrococcal nuclease MNase (0, 20,200, 2000 gel U/ml) and NucB (0, 10, 100 1nd 1000 ng/ml).

As presented in Figure 4.5 the diameter of the clear halos around the wells indicates DNase hydrolysis of the DNA substrate in the methyl green DNase agar. AW2 and AW3 have the largest diameters: 22.7 mm and 22 mm respectively.

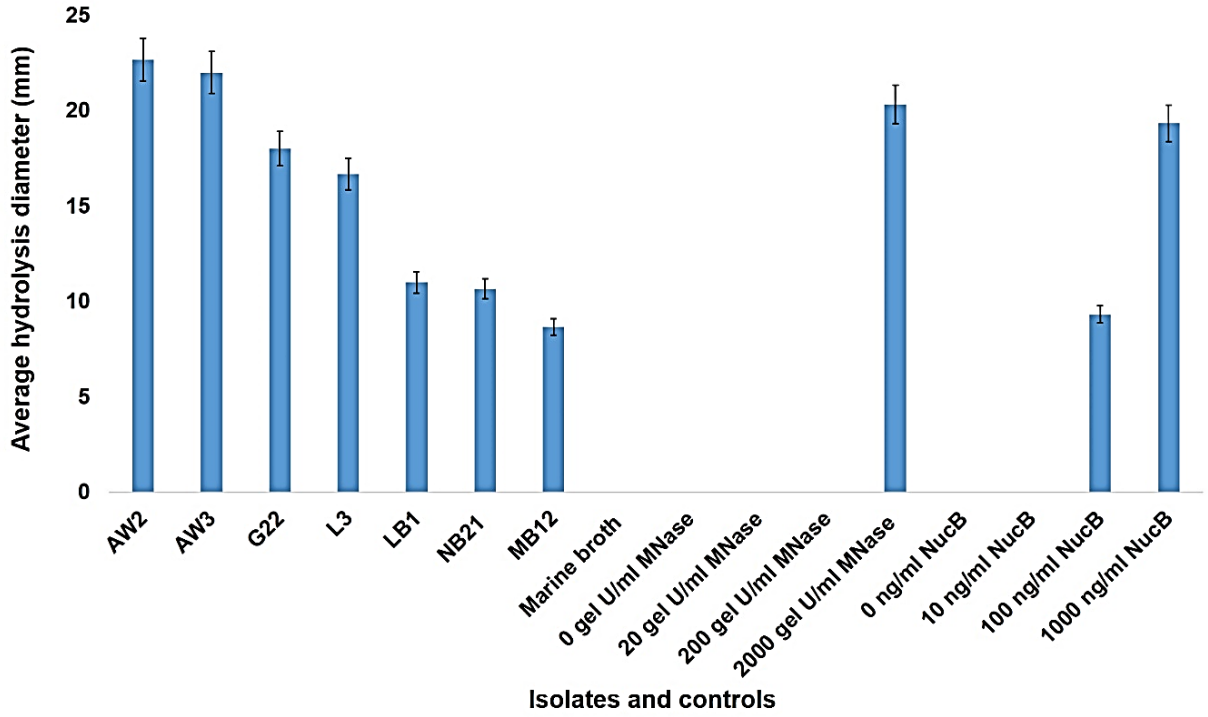

Figure 4.5. The diameters of the zone of hydrolysis of filtered supernatants of the DNase-producing bacterial isolates. AW2 and AW3 show comparable diameters to the positive control 2000 gel U/ ml MNase and 1000 ng/ml NucB. Error bar indicated ± standard deviation.

4.4.3 DNA digestion by bacterial supernatant

The potent DNase producers in the well assay (AW2, AW3, G22 and L3) were selected for confirmation of extracellular DNase production by the DNA digestion method, monitored by gel electrophoresis. This tested the ability of the filtered supernatants to digest high-molecular-weight DNA. The results revealed that the filtered supernatants of AW2 and AW3 displayed noticeable digestion on high-molecular-weight DNA (Calf thymus, 1 mg/ml) after 1 hour of incubation and complete digestion after 24 hours at 37^{o}C, as shown in Figure 4.6. The filtered supernatant of G22 and L3 showed complete digestion of the DNA after 24 hours of incubation at 37^{o}C.

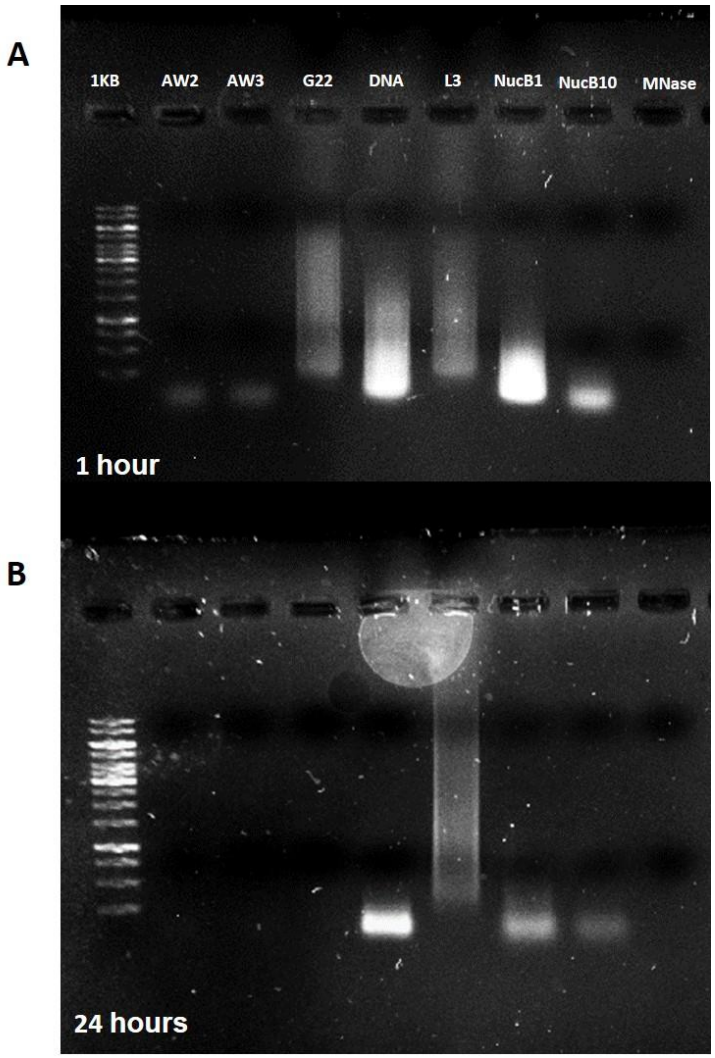

Figure 4.6. The activity of the bacterial supernatant against high-molecular-weight DNA (1 mg/ml Calf thymus). Lane 1, 1KB ladder as marker. Lanes 2, 3, 4 and 6 are filtered supernatants of bacterial isolates, AW2, AW3, G22, and L3 respectively. Lane 5, DNA, remained unaffected. Lane 7 and 8, NucB1 of 1 ng/ml and NucB10 of 10 ng/ml. Lane 9, 2000 gel U/ml of MNase after incubation of (A) 1 hour and (B) 24 hours at 37^{o}C.

The supernatant of biofilm bacteria has not shown any DNA digestion as shown by gel electrophoresis (Figure 4.7).

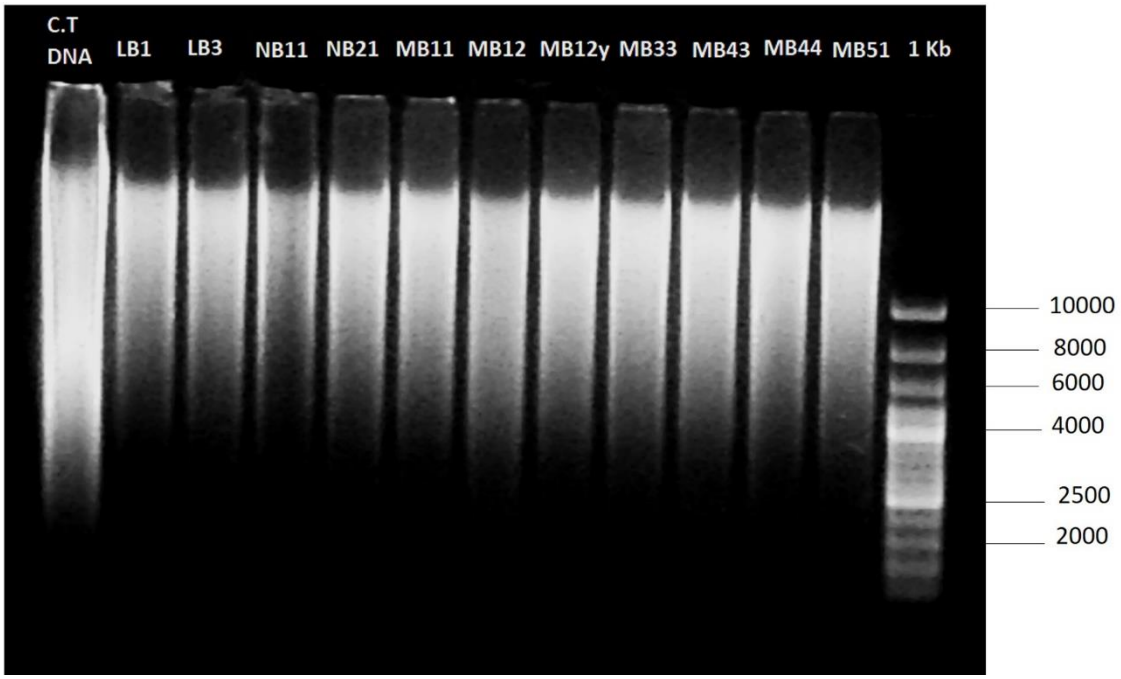

Figure 4.7 The activity of the bacterial supernatant of biofilm isolates from the coast of Oman against high-molecular-weight DNA (1 mg/ml Calf thymus). Filtered supernatants from the isolates did not show any digestion reaction against high-molecular-weight DNA (1 mg/ml).

4.4.4 Genomic DNA of AW2 a prolific DNase producer

The AW2 strain was the best DNase producers based on the diameter of the zone of hydrolysis on the methyl green DNase agar plates and from the DNA digestion assays. The genomic DNA extracted showed a bright band on the agarose gel. The sequencing resulted in a total of 323,751 sequences, with sequence lengths of 35 to 251bp. GALAXY bioinformatics https://usegalaxy.org/ Quality control of the sequences resulted in < 30 PHRED, which means the probability of an incorrect base is 1 in 1000 and the base call accuracy is 99.9%. The sequence assembly of the genome contained 115 contigs of about 7 Mb in total. The classification of the isolate AW2 in BLAST has showed a 99.4 % similarity to *Serratia marcescens*.

4.4.5 Genes for nuclease enzymes in the genome of AW2

The genome of bacterial strain *S. marcescens* (AW2) is about 5Mb in size (annotation statistics in Table 4.4), resulted in 43 annotated nuclease related enzymes including a variety of ribonucleases and deoxyribonucleases (the list of annotated nuclease genes and their functions are presented in Table 4.5). Furthermore, there are seven extracellular deoxyribonuclease genes present within the genome of AW2 including; Exodeoxyribonuclease III, Crossover junction endodeoxyribonuclease RuvC, Exodeoxyribonuclease 7 small subunit, Exodeoxyribonuclease 7 large subunit, Exodeoxyribonuclease 8, Exodeoxyribonuclease 10, and Exodeoxyribonuclease I. These results indicating a widespread distribution of nuclease genes within the genome of AW2.

Table 4.4 Prokka annotation statistics of AW2 bacteria.

Contigs		115
Genome size (bp)		5225681
Features predicted	tRNA	86
	rRNA	11
	tmRNA	1
	CDS	4853

Table 4.5 Annotated nuclease genes of the AW2 genome and their functions based on UniProt databse https://www.uniprot.org/.

No.	Locus tag	Feature type	Length bp	Gene	EC number	COG	Product	Function
1	DHIAJNHD_00169	CDS	1935	rnb	3.1.13.1	COG4776	Exoribonuclease 2	Transfer RNA biogenesis.
2	DHIAJNHD_00199	CDS	882	yciV	3.1.13.-	COG0613	5'-3' exoribonuclease	Ribosome biogenesis in eukaryotes and RNA degradation.
3	DHIAJNHD_00250	CDS	807	xthA	3.1.11.2	COG0708	Exodeoxyribonuclease III	Base excision repair.
4	DHIAJNHD_00292	CDS	1122	rnd	3.1.13.5	COG0349	Ribonuclease D	Exonuclease involved in the 3' processing of various precursor tRNAs. Initiates hydrolysis at the 3'-terminus of an RNA molecule and releases 5'-mononucleotides.
5	DHIAJNHD_00312	CDS	522	ruvC	3.1.22.4	COG0817	Crossover junction endodeoxyribonuclease RuvC	Homologous recombination.
6	DHIAJNHD_00618	CDS	468	NA	3.1.27.-	NA	Ribonuclease	Catalyse the cleavage of RNA.
7	DHIAJNHD_00631	CDS	1470	rng	3.1.26.-	COG1530	Ribonuclease G	Cytoplasmic axial filament protein.
8	DHIAJNHD_00743	CDS	2475	rnr	3.1.13.1	COG0557	Ribonuclease R	RNA degradation.
9	DHIAJNHD_00760	CDS	546	orn	3.1.-.-	COG1949	Oligoribonuclease	Ribosome biogenesis in eukaryotes.
10	DHIAJNHD_00786	CDS	1449	addA	3.6.4.12	NA	ATP-dependent helicase/nuclease subunit A	Acts as both an ATP-dependent DNA helicase and an ATP-dependent, dual-direction single-stranded exonuclease.
11	DHIAJNHD_00962	CDS	801	nucA	3.1.30.2	NA	Nuclease	Catalyse the hydrolysis of both double- or single-stranded DNA and RNA, at the 3'position of the phosphodiester bond.
12	DHIAJNHD_01149	CDS	792	nei	3.2.2.-	COG266	Endonuclease 8	Involved in base excision repair of DNA damaged by oxidation or by mutagenic agents.
13	DHIAJNHD_01195	CDS	474	ybeY	3.1.-.-	COG0319	Endoribonuclease YbeY	Single strand-specific metallo-endoribonuclease involved in late-stage 70S ribosome quality control and in maturation of the 3' terminus of the 16S rRNA.
14	DHIAJNHD_01578	CDS	327	rnpA	3.1.26.5	COG0594	Ribonuclease P protein component	Generates mature tRNA molecules by cleaving their 5'-ends.
15	DHIAJNHD_01960	CDS	531	mutS2	3.1.-.-	NA	Endonuclease MutS2	Suppression of homologous recombination.
16	DHIAJNHD_02082	CDS	555	rnt_1	3.1.13.-	COG0847	Ribonuclease T	Maturation of transfer RNA and ribosomal RNA in bacteria.

17	DHIAJNHD_02165	CDS	3252	sbcC	NA	COG0419	Nuclease SbcCD subunit C	Inhibit DNA replication and are intermediates in certain DNA recombination reactions.
18	DHIAJNHD_02166	CDS	1233	sbcD	NA	COG0420	Nuclease SbcCD subunit D	Inhibit DNA replication and are intermediates in certain DNA recombination reactions.
19	DHIAJNHD_02173	CDS	192	higB_2	3.1.-.-	NA	Endoribonuclease HigB	Inhibits translation by sequence-specific cleavage of mRNA.
20	DHIAJNHD_02195	CDS	264	xseB	3.1.11.6	COG1722	Exodeoxyribonuclease 7 small subunit	Bidirectionally degrades single-stranded DNA into large acid-insoluble oligonucleotides.
21	DHIAJNHD_02332	CDS	783	tatD	3.1.11.-	COG0084	3'-5' ssDNA/RNA exonuclease TatD	3'-5' exonuclease that prefers single-stranded DNA and RNA.
22	DHIAJNHD_02487	CDS	594	rnhB	3.1.26.4	COG0164	Ribonuclease HII	Endonuclease that specifically degrades the RNA of RNA-DNA hybrids.
23	DHIAJNHD_02515	CDS	759	ygdG	3.1.-.-	COG0258	Flap endonuclease Xni	Cleave the 5'-overhanging flap structure.
24	DHIAJNHD_02603	CDS	717	rph	2.7.7.56	NA	Ribonuclease PH	An expressed but non-active exoribonuclease allele.
25	DHIAJNHD_02655	CDS	486	rraA	NA	COG0684	Regulator of ribonuclease activity A	Modulates RNA abundance by binding to RNase E (Rne) and regulating its endonucleolytic activity.
26	DHIAJNHD_02820	CDS	681	rnc	3.1.26.3	COG0571	Ribonuclease 3	Digests double-stranded RNA. Involved in the processing of primary rRNA transcript to yield the immediate precursors to the large and small rRNAs (23S and 16S). Processes some mRNAs, and tRNAs when they are encoded in the rRNA operon.
27	DHIAJNHD_02911	CDS	537	smrA	3.1.-.-	COG2840	Putative DNA endonuclease SmrA	Catalysis of the hydrolysis of ester linkages within nucleic acids by creating internal breaks.
28	DHIAJNHD_02947	CDS	1734	recJ	3.1.-.-	COG0608	Single-stranded-DNA-specific exonuclease RecJ	Single-stranded-DNA-specific exonuclease acting in a 5' to 3' direction.
29	DHIAJNHD_03233	CDS	1299	xseA	3.1.11.6	COG1570	Exodeoxyribonuclease 7 large subunit	Bidirectionally degrades single-stranded DNA into large acid-insoluble oligonucleotides, which are then degraded further into small acid-soluble oligonucleotides.
30	DHIAJNHD_03242	CDS	822	recE	3.1.11.-	NA	Exodeoxyribonuclease 8	Catalyse the degradation of double-stranded DNA.
31	DHIAJNHD_03379	CDS	4035	rhsA	3.1.-.-	COG3209	putative deoxyribonuclease RhsA	Catalysis of the hydrolysis of various phosphoric anhydride bonds.

32	DHIAJNHD_03658	CDS	696	nucM	3.1.21.-	COG2356	Nuclease NucM	Catalysis of the hydrolysis of ester linkages within nucleic acids.
33	DHIAJNHD_03828	CDS	351	chpB	3.1.-.-	COG2337	Endoribonuclease toxin ChpB	ChpB is a sequence-specific mRNA and (weak) tmRNA endoribonuclease that inhibits protein synthesis and induces bacterial stasis.
34	DHIAJNHD_03883	CDS	669	exoX	3.1.11.-	COG0847	Exodeoxyribonuclease 10	Capable of degrading both single-strand and double-strand DNA with 3' to 5' polarity. Has higher affinity for ssDNA ends than for dsDNA.
35	DHIAJNHD_03971	CDS	426	rraB	NA	COG3076	Regulator of ribonuclease activity B	Modulate RNA abundance by binding to RNase E (Rne) and regulating its endonucleolytic activity.
36	DHIAJNHD_04092	CDS	840	nfo	3.1.21.2	COG0648	Endonuclease 4	DNA repair and cleaves phosphodiester bonds at apurinic or apyrimidinic sites (AP sites) to produce new 5'-ends that are base-free deoxyribose 5-phosphate residues.
37	DHIAJNHD_04108	CDS	1428	sbcB	3.1.11.1	COG2925	Exodeoxyribonuclease I	Degrades single-stranded DNA (ssDNA) in a highly processive manner.
38	DHIAJNHD_04152	CDS	390	vapC	3.1.-.-	COG1487	tRNA(fMet)-specific endonuclease VapC	Cleaves both charged and uncharged tRNA-(fMet) between positions 38 and 39 at the anticodon stem-loop boundary.
39	DHIAJNHD_04225	CDS	468	rnhA	3.1.26.4	COG0328	Ribonuclease HI	Degrades the RNA of RNA-DNA hybrids.
40	DHIAJNHD_04338	CDS	849	cho	3.1.25.-	COG0322	Excinuclease cho	Incises the DNA at the 3' side of a lesion during nucleotide excision repair.
41	DHIAJNHD_04371	CDS	642	nth	4.2.99.18	COG0177	Endonuclease III	DNA repair enzyme that has both DNA N-glycosylase activity and AP-lyase activity.
42	DHIAJNHD_04560	CDS	651	rnt_2	3.1.13.-	COG0847	Ribonuclease T	Trims short 3' overhangs of a variety of RNA species, leaving a one or two nucleotide 3' overhang.
43	DHIAJNHD_04884	CDS	555	nfi	3.1.21.7	COG1515	Endonuclease V	DNA repair enzyme involved in the repair of deaminated bases. Selectively cleaves double-stranded DNA at the second phosphodiester bond 3' to a deoxyinosine leaving behind the intact lesion on the nicked DNA.

4.5 Discussion

Extracellular DNA and bacterial extracellular enzymes are ubiquitous in the ocean and play an important role in the cycling and fate of organic matter (Luo *et al.*, 2017; Ivančić *et al.*, 2018; Liu and Liu, 2018). However, knowledge regarding DNase diversity in marine bacteria in general, and in marine aggregates more specifically, remains rudimentary. DNase production has been reported in several bacterial species, including *Serratia marcescens* (Nestle and Roberts, 1969), marine isolates of *Vibrio* sp. (Maeda and Taga, 1976), *Pseudomonas aeruginosa* (Mulcahy *et al.*, 2010a), *Myroides*, *Planococcus*, *Sporosarcina* and *Halomonas* (Dang et al., 2009) and *Bacillus licheniformis* (Nijland *et al.*, 2010). In addition, there are some studies on DNase produced by the human pathogen *Streptococcus* (Porschen and Sonntag, 1974; Palmer *et al.*, 2012). However, most studies have focused only on individual cultivable species and the recombinant gene expression of nuclease genes (Cao *et al.*, 2017). The diversity of DNase production by marine bacteria and aggregate-associated bacteria has not been addressed before. In this study, a culture-based method was used to highlight the diversity of isolated marine bacteria producing DNase.

4.5.1 Deoxyribonuclease (DNase) production by marine bacteria

This study is the first to identify DNase-producing bacteria from marine aggregates (isolated from MGP from the North Sea and marine biofilm from the coast of Oman). Overall, 33% of the isolated bacteria produced DNase, predominated by Gammaproteobacteria. This work indicates that DNase secretion is relatively common among diverse marine bacterial isolates and is greater in Gammaproteobacteria than in other classes. This is in agreement with Lennon (2007), who demonstrated the ability of marine bacteria, mostly Gammaproteobacteria (*Vibrio*, *Pseudoalteromonas*, *Alteromanas*), Alphaproteobactria (*Roseobacter*, *Koriijomonas*, *Gwanyang*) and Bacteroidetes (*Flexibacter* and *Microscilla*), to consume DNA as a nutrient by producing DNase enzymes.

The production of DNase by Gammaproteobacteria has been studied in bioinformatics investigations and may be explained by the fact that Gammproteobacteria employ a nuclease bacteriocin (NB) defence mechanism (Parret and De Mot, 2002; Sharp *et al.*, 2017). However, the results presented in this chapter confirm the common production of DNase among

Gammaproteobacteria in a culture-based study. This could be attributed to the fact that this is a dominant cultivable class of marine bacteria.

Most of the bacteria that produced DNase in this study belong to twenty families, most of which are affiliated to Vibrionacea (26%). *Vibrio* employs extracellular nucleases for natural transformation and for defence to elude neutrophil extracellular traps like in *Vibrio cholera* (Blokesch and Schoolnik, 2008; Seper *et al.*, 2013). The next highest cultivable family was Bacillaceae (13%). Bacillus are well-known enzyme producers (Lei *et al.*, 2017; Parab *et al.*, 2017; Anjum *et al.*, 2018) including extracellular DNase production, for example, NucB production by *Bacillus licheniformis* (Nijland et al., 2010). The results demonstrated that 8 % of the species produced DNase belong to *Pseudoalteromonadaceae*, which is known for its production of bioactive compounds and enzymes (Tutino *et al.*, 2002; Zeng *et al.*, 2006; de Pascale *et al.*, 2008; Bian *et al.*, 2012; Bhattacharya *et al.*, 2018). A recent study of extracellular enzymes from marine cultivable bacteria from The New Britain Trench showed that *Pseudoalteromonas* produces various extracellular enzymes, mostly proteases and chitinases (García-Fraga *et al.*, 2015; Liu *et al.*, 2018). Another study on cultivable bacteria in Laizhou Bay, China, showed that *Pseudoalteromonas* was the most common cultivable genus producing extracellular proteases (Li *et al.*, 2017). Moreover, *Pseudoalteromonas* have also been reported to produce nuclease bacteriocins (Desriac *et al.*, 2010).

In the current study bacteria associated with marine aggregates were investigated for DNase production for the first time. The results revealed that eight out of twenty isolates from MGP were able to produce DNase. These isolates belong to the families Pseudoalteromonadacea (3), Vibrionaceae (2), Bacillaceae (1), Shewanellaceae (1) and Halomonadaceae (1). Most of these families have been reported previously to produce DNase, but not when isolated from particle aggregate bacteria (Balabanova *et al.*, 2017). *Vibrio* is the highest DNase producer in this study, obtained from different environments, the North Sea and the coast of Oman, from free-living and aggregated forms. *Vibrio* are prevalent marine bacteria that are able to alternate between free living (FL) and particle attached (PA), and form biofilms (Karunasagar *et al.*, 1996; Nair *et al.*, 2007; Yildiz and Visick, 2009). Extracellular enzymes from *Vibrio* (Maeda and Taga, 1976; Bunpa *et al.*, 2016) and extracellular agents and enzymes from *Pseudoalteromonas citrea* have been reported previously (Holmström and Kjelleberg, 1999; Roca *et al.*, 2016; Yamada *et al.*, 2016; Imbs *et al.*, 2018), however extracellular DNase from *Vibrio* within MGPs have not been described previously. This can be explained by that *Vibrio* are possibly exploiting MGPs material by secreting DNase. In addition, there is evidence that *Pseudomonas* and *Vibrio* produce extracellular deoxyribonucleases to utilise DNA as a nutrient source, as well as

synthesising DNases for the horizontal gene transfer of DNA as part of natural transformation (Blokesch and Schoolnik, 2008; Mulcahy *et al.*, 2010a).

The finding that *Vibrio atlanticus* and *Pseudoalteromonas citrea* isolated from MGP produces DNase may implicate DNase in the dispersal of MGPs, based on the hypothesis that eDNA is a key component of MGP and plays a vital role in its structural integrity.

Furthermore, extracellular enzymes produced by heterotrophic bacteria are a key player in organic matter cycling in the ocean (Azam and Malfatti, 2007; Rier *et al.*, 2014; Balmonte *et al.*, 2016; Burns *et al.*, 2016) and bacterial extracellular enzyme activity in aggregates was reported to be two orders of magnitude higher than in free-living bacteria (Smith *et al.*, 1992; Grossart *et al.*, 2006; Ziervogel *et al.*, 2010; Kellogg and Deming, 2014). These extracellular enzymes are thought to be involved in the dissolution of MGP and marine snow (D'ambrosio *et al.*, 2014; Balmonte *et al.*, 2016; Luo *et al.*, 2017; Baltar, 2018; Ivančić *et al.*, 2018). However, the effect of DNase is understudied with regard to its implications for MGP and marine snow dispersal.

Additional studies are required to observe the production of DNase in situ. However, our results of cultivable isolates revealed various bacteria that secrete DNase. The versatile ability of marine isolates to produce DNase suggests that they play an important ecological role in extracellular DNA degradation in the marine environment. The present study provides data on DNase production by marine cultivable bacteria associated with MGPs for the first time and hence provides insight into the diversity of extracellular enzyme production by marine bacteria (Al-Wahaibi *et al.*, 2019). Moreover, the diverse nuclease genes present within the genome of the *sediment isolate Serratia marcescens* (AW2) may suggest ecological importance of nucleases in the seafloor niche.

4.6 Conclusions

This study has highlighted the following outcomes:

1. About 33% of the isolated bacteria obtained from free-living and MGP associated isolates from the North Sea and the Sea of Oman were identified as nuclease producers, and most of them belonged to diverse genera.
2. This is a first study to show DNase secretion by cultivable MGP associated bacteria.
3. The sediment isolate AW2 that produced high levels of secreted nucleases was identified as *Serratia marcescens*.
4. Based on the genome sequencing of *S. marcescens*, it contains 43 diverse nuclease genes including 7 extracellular deoxyribonuclease genes.
5. The findings demonstrate the ecological role that bacterial DNase may play in the bacterial community of various marine niches.
6. This study highlights the diversity of DNase-producing marine bacteria, as representative isolates from several distinct bacterial genera recovered were able to produce DNases. There is a great need to understand the diversity of marine bacteria that produce extracellular nucleases as this can increase our knowledge of the role of bacterial nucleases biogeochemical processes and MGP dynamics in the ocean.

Chapter 5. General Discussion and Future Directions

5.1 Introduction

In spite of the importance of MGPs that resides in the fact that they are a vertical vehicle of carbon flux into the deep ocean and as an important component of the global carbon cycle and in the sedimentation of marine aggregates, little is known yet about the precise biochemical composition of MGPs. This study has begun to characterise the biochemical composition of MGPs by looking at the presence of eDNA and their associated bacterial communities. As this has not been recognised previously and would be of integral implication to the MGP dynamics. However, eDNA is recognised as an important component in the coherence of bacterial aggregates, specifically biofilms (Suzuki *et al.*, 2009; Okshevsky and Meyer, 2015; Vorkapic *et al.*, 2016). Likewise, it may play a similar role in MGPs and flocs in the ocean. Moreover, the ecological aspects of the bacterial interaction within MGPs, such as foraging on the matrix constituents and expelling extracellular material into the particle milieu, have not been well explored in the context of gel organic matter and bacterial interactions. As bacterial communities live attached to the MGP, consuming the compositional materials and expelling extracellular materials including eDNA, into the MGP microenvironment, implies that there may be bacterial communities within the MGPs that might also utilise eDNA.

5.2 Main findings

5.2.1 The isolation of MGPs in water suspension

In Chapter 2, a method was developed for the first time to isolate MGPs in suspension in a small volume of water. This permitted access to concentrated particles and allowed a comprehensive insight into the three-dimensional structure of the particles and the way they behave in their natural habitats. Current studies in the literature on MGPs and the vast majority of studies on organic matter (OM) in aquatic systems carry out investigations using filters (Alldredge *et al.*, 1993; Passow and Alldredge, 1994; Passow and Alldredge, 1995; Villacorte *et al.*, 2015). Here the MGPs are filtered onto filter paper and generally studied biochemically while still attached to the filter paper. Another widely used method for the study of TEPs is staining with Alcian blue dye by using filter-transfer-freeze (FTF) and fixing them on glass slides (Hewes and Holm-Hansen, 1983). Recently, a FlowCAM technique has been used to investigate TEPs based on

online in-situ quantifications (Thuy *et al.*, 2017). Hitherto, gel particles have been studied either by using microscope imaging or by the conventional staining-based methods. Regardless of the benefits of these methods, their limitation, as evidenced by the current investigations into MGPs, is that the particles have to be fixed. Fixing the particles would hamper the fulfilment of the current study's aim, which is investigating MGPs in their fully intact three-dimensional structure for a careful spatial characterization of natural particles. Therefore, the isolation and resuspension of MGPs concentrated in a small volume has enabled a holistic visualisation of the MGPs by confocal laser scanning microscopy (CLSM) and the use of particles in different experiments.

Bar-Zeev *et al.* (2012) have studied the TEP as biofilm initiator on reverse osmosis membranes of desalination plant by using real-time microscopy to detect the particles in a flow cell experiment and employing SYTO-9, Con A and Alcian Blue. Although there is some similarity in the stains implemented and in visualising the particles in real time in-situ and in full structure of the current study. However, the results presented here have totally established a novel approach by isolating the particles and suspend it in smaller volume (5 mL) of new fresh sterile artificial seawater. Additionally, our current results have combined SYTO-9, TOTO-3 and Con A in order to differentiate between extracellular DNA (TOTO-3) and intracellular DNA (SYTO-9).

5.2.2 The co-occurrence of eDNA and DNase in the MGP

The most remarkable result to emerge from this study is that it has provided the first evidence of the presence of eDNA in natural MGPs and in the *Pseudoalteromonas atlantica* EPS a marine floc model. Labelling the eDNA with fluorescent dyes in the MGPs' has illustrated that eDNA is present within the MGP matrix, as proven by the results of this study in Chapter 2. This is an important milestone contributing to the understanding of the biochemical composition of the MGPs that haven't been found elsewhere. As previously reported the gel particles composition was limited to acidic polysaccharides and/or protein particles (Alldredge *et al.*, 1993; Long and Azam, 1996; Neukermans *et al.*, 2016; Pinu and Villas-Boas, 2017). This current result may now be elucidated by the fact that eDNA is an abundant biomaterial in the ocean with important ecological functions for the marine environment for example, in preserving genetic diversity (Paul *et al.*, 1987; Dell'Anno *et al.*, 2002; Dell'Anno and Danovaro, 2005; Corinaldesi *et al.*, 2014; Collins *et al.*, 2018; Corinaldesi *et al.*, 2018). In addition, the occurrence of eDNA in the oceans in various quantities that can reach up to 22 µg L^{-1} in anoxic

deep sea (Corinaldesi *et al.*, 2005; Corinaldesi *et al.*, 2007), 15 µg L^{-1} in coastal waters (Deflaun *et al.*, 1987) and 44 µg L^{-1} in estuarine (Paul *et al.*, 1989). Hence, the higher quantities of eDNA in the oceans are found to be in sediments in comparison to the water column (Dell'Anno and Danovaro, 2005). This can now be explained by that the eDNA is may also transported with the marine sinking aggregates. Recently, a study has reported that the microbiome of the ocean surface is linked to the deep communities as a result of sinking particles transporting the genetic material like DNA (Mestre *et al.*, 2018). Therefore, the ample eDNA in marine sediments is assumed now to be mobilising with marine snow aggregates alongside with the carbon flux and playing vital unidentified roles, that yet need to be further explored.

Furthermore, the observation of eDNA within the MGPs in this study, as well as it is pronounced availability in the ocean, as explained earlier, may support the assumption of the sticky nature of eDNA and its ability to act as adherent material (Seeman, 2007; Vilain *et al.*, 2009; Kirkpatrick and Viollier, 2010) that enable attachment or adsorption to form aggregates with marine organic matter (Lorenz and Wackernagel, 1992; Nielsen *et al.*, 2007). One possible notion is that the eDNA of the seawater may stick to the MGPs. A second likely assumption is that the PA microbiome is the source of eDNA as a result of their ecological activities in the MGP microenvironment like the intense colonisation that requires EPS production that could facilitate adhesion to gel particles (Yawata *et al.*, 2014). It is already reported that some bacterial cultures flocculation is due to EPS agglomeration properties (Sun *et al.*, 2017b). Additionally, there is reports on the presence of extracellular nucleic acids; DNA and RNA that causes aggregation in *Rhodovulum sulfidophilum* cultures (Watanabe *et al.*, 1999; Suzuki *et al.*, 2009). Further, Suzuki *et al.* (2009) have also proved that the *R. sulfidophilum* flocs stability is retained as well by nucleic acid presence. eDNA is a known cell-to-cell communicating molecule in *Pseudomonas aeruginosa* biofilms (Nemoto *et al.*, 2003; Allesen-Holm *et al.*, 2006). Thus far, all these evidences are all in association to bacterial cultures and no evidence yet reported on eDNA effects on MGPs structure stability.

Commonly, bacterial EPS is studied as part of surface-attached biofilms (Sun *et al.*, 2017b; Deng *et al.*, 2018; Nagaraj *et al.*, 2018). The common feature of marine gel flocs and biofilms is that their basic hydrogel EPS composition is similar, especially the eDNA, regardless of the possible variation in some other constituents (Deng *et al.*, 2018; Xu *et al.*, 2019). Additionally, marine gel particles have a natural ability to clump and form bigger aggregates contributing to the sedimentation sink to the ocean floor (Chin *et al.*, 1998; Engel, 2004). Further, the flocculation behavior of the bacterial aggregates in the oceans resembles the biofilm formation (Watanabe *et al.*, 1999; Suzuki *et al.*, 2009) and the soft biofloc formation in wastewater and

water desalination treatments reviewed by Tansel (2018). Moreover, marine bacteria like *Sagittula stellate* and Cyanobacteria have been reported to contribute in the aggregation processes of dissolved organic matter (DOM) (Ding *et al.*, 2008; Lama *et al.*, 2016). Whereas, *Pseudoalteromonas* spp. EPS have shown to enhance chitin particles aggregation (Yamada *et al.*, 2013; Yamada *et al.*, 2016). Therefore, the eDNA can possibly affect the strength of the MGPs as in surface biofilms (Whitchurch *et al.*, 2002; Das *et al.*, 2010). Taken together, these results may also suggest that eDNA would contribute to the characterisation of the POM form as recalcitrant or labile by affecting their resistance to decomposition and thus their residence time of organic matter in the ocean.

Furthermore, the MGPs specifically TEP are involved in biofilm initiation of marine structures like reverse osmosis membrane (RO) of desalination plants (Bar-Zeev *et al.*, 2009; Bar-Zeev *et al.*, 2012). Further, the term "protobiofilm" is introduced as planktonic version that is similar to the surface attached biofilms (Bar-Zeev *et al.*, 2012). Resemblance of the MGP and biofilms be in their composition that is made up of hydrogel, in their sticky character, in their affinity to be colonised by bacteria reviewed in Bar-Zeev *et al.* (2015).

However, there is some evidence that bacterial communities of the marine sediments are using DNA as a source of energy (Wasmund *et al.*, 2019). Marine Roseobacter Clade of coastal surface marine waters are reported to exploit dissolved DNA as a source of carbon (Taylor *et al.*, 2018). This could be attributed to the ability of diverse bacteria to exploit the available forms of DNA in the oceans (Lennon, 2007).

On the other hand, this present study has also confirmed in Chapter 2 the presence of eDNA in the EPS of *P. atlantica*, the marine bacterial floc model. The possible interference of this is that eDNA may be released with the bacterial EPS flux. This is advantageous step in further unraveling of the nature of bacterial EPS of *P. atlantica* as a suspended biomaterial in the water column. In accordance with this current result, previous studies have demonstrated that DNA is released via active release in membrane vesicles of some environmental bacteria as such marine cyanobacteria *Prochlorococcus*, *Alteromonas*, *Salinicola* and *Thalassospira* (Biller *et al.*, 2014; Biller *et al.*, 2017). *Pseudomonas aeruginosa* is also reported to consume DNA as nutrient by secreting extracellular DNase (Mulcahy *et al.*, 2010b).

The co-occurrence of eDNA and DNase as antipodes are accentuated here by the outcomes of the current study. It was hypothesized in Chapter 3 that eDNA presence in MGPs milieus necessitate bacterial communities to encode DNA metabolism. Demonstrating a functional capability of the bacterial community to meet the substrate availability. Both the bioinformatics approach in Chapter 3 and the culture-based study in Chapter 4 has displayed concurring results

of the MGP bacterial communities' potential encoding nuclease genes and isolates secreting DNase. Presumably eDNA and DNase are thus playing antagonistic roles by which affecting the MGP integrity. There are 75 putative nuclease genes encoded by the MGP associated bacterial community as the bioinformatics investigation showed. It seems that nucleases are highly present in the bacterial community. In addition, the culture-based approach of the MGP bacterial isolates illustrated that species belong to Pseudoalteromonadacea, Vibrionaceae and Bacillaceae families have revealed DNase secretion.

The interaction of the bacterial communities within gel particles involves the secretion of extracellular enzymes, as discussed in the previous chapters, suggesting that it may have a key implication for the dissolution of gel particles and marine carbon cycling in the ocean (Huston and Deming, 2002; Arnosti, 2011; Kellogg *et al.*, 2011; Arnosti, 2014; Arnosti *et al.*, 2014; Kellogg and Deming, 2014; Lechtenfeld *et al.*, 2015; Luo *et al.*, 2017). Microbial extracellular enzymes in the ocean present attached to bacterial cells or dissolved in the water are both may excrete implications on organic matter stability (D'ambrosio *et al.*, 2014). Marine snow bacteria hydrolyses the organic matter by extracellular enzymes that are regulated by quorum sensing (QS) (Hmelo *et al.*, 2011; Jatt *et al.*, 2015; Krupke *et al.*, 2016). However, the extracellular enzymes associated with marine snow aggregates are getting more attention (Balmonte *et al.*, 2016). Several microbial extracellular enzymes have been reported with marine aggregates; laminarinase (Arnosti *et al.*, 2012) and bacteria degrading phosphonate, which is associated with DOM (Sosa *et al.*, 2017). Conversely, there are no reports describing bacterial DNase in PA and their effect on particles integrity and dissolution. The results of the current study as presented in Chapter 4 highlighted the marine bacterial isolates that produced DNase that was accomplished with a culture-based technique. This is the first study to show the ability of PA bacterial isolates of MGPs to secrete DNase (Al-Wahaibi *et al.*, 2019). This early step leading discovery is a crucial finding that contextualize extracellular DNases with MGPs and ocean carbon cycling. This indicates the potentially important role that DNase might play in the gel particle and marine snow cycling dynamics in the ocean. This has not been underlined elsewhere and offers an increase in the knowledge of oceans microbial extracellular enzymes and their implications on particles cycling. Particle attached (PA) bacteria employ specific extracellular enzymes to utilise components within the particles (Smith *et al.*, 1992; Ziervogel *et al.*, 2010; Arnosti *et al.*, 2012). Furthermore, this current study investigated the production of extracellular DNase by marine free- living (FL) and PA bacteria. The presence of eDNA as a substrate might trigger the production of the nuclease enzymes for bacterial utilisation, as in hydrolytic enzyme regulation (Boetius and Lochte, 1994; Boetius and Lochte, 1996; Arnosti *et al.*, 2014).

5.2.3 The composition and potential functional capabilities of the bacterial community associated with MGPs

Bacterial communities attached to MGPs and their associated metabolites are driving alterations in the biogeochemical cycles of the ocean and they play a role in shaping the function of the sinking particles and larger accumulations designated 'marine snow' (Azam and Malfatti, 2007; Balmonte *et al.*, 2016; Luo *et al.*, 2017). Therefore, the study of bacterial communities associated with marine particles is important as it will enable a better understanding of the putative role of bacteria and through identifying encoded metabolites. This could have important ramifications in predicting the fate of the MGPs. As microbial communities are one of the main factors of particles degradation and carbon turnover in the ocean (Enke *et al.*, 2018; Enke *et al.*, 2019). Prior studies on the bacterial communities associated with MGP and marine aggregates all focused on their composition. Here in our current study we investigate the functional diversity of the bacterial community associated with MGPs. The results have pointed to the dominance by particle degrades taxa like Firmicutes, Alphaproteobacteria and Gammaproteobacteria (Dang *et al.*, 2009; Teeling *et al.*, 2012b). The presence of these bacteria presumably associated with featured metabolomics functions. However, the functional investigations are based on the match with encoded gene rather than expression and production (Ferrer *et al.*, 2018).

Irrespective to the diversity of this community, the functional capabilities remained similar across all size fractions, which illustrates a functional redundancy. This is unexpected and possible may be explained by that the same taxa are inhabiting the particles all the year round. Another possible reason may be that the phylogenetic identification of the OTUs was based on the sequencing of the 16Sr RNA marker gene that may limit the functions to this site of the genome (Sevigny *et al.*, 2019). While the search for functional capabilities have been based on broader search in databases of genomes of cultivable bacteria (Shamim *et al.*, 2019). Hence, this probably insufficient to get insight into all functional capabilities. This is supported by the study of a microbial community of seafloor aquifers (Louca *et al.*, 2018; Tully *et al.*, 2018). However, a recent study has denied the notion of functional redundancy in marine microbial communities (Galand *et al.*, 2018). This is therefore provoking a debate on the functional redundancy of microbial communities.

5.2.4 The presence of nuclease genes in the genome of marine sediment bacteria Serratia marcescens

The short genome sequence of the prolific marine bacteria DNase producer AW2 identified as *Serratia marcescens*, isolated from the North Sea, revealed a high distribution of various nuclease genes within the genome. This wide distribution may suggest a high production of DNase. However, while this production was observed on the methyl green DNase agar plates, it is not necessarily the same in situ as high concentration substrate availability and/or some environmental conditions could affect the secretion (Küchler *et al.*, 2016). Transcriptomic and transproteomic studies are therefore required to fully characterise the actual activity and secretion behaviour of the DNase in situ. The sea bottom is known to contain high levels of DNA in the sediments (Dell'Anno and Danovaro, 2005; Torti *et al.*, 2015; Torti *et al.*, 2018). This may explain why numerous nuclease genes are present in the genome of *S. marcescens* that was isolated from sediments. Although *S. marcescens* are a very well-known for nuclease production (Benedik and Strych, 1998; Vafina *et al.*, 2018), it has not been reported among bacteria that consumes DNA (Lennon, 2007; Wasmund *et al.*, 2019). In addition, it is presence with the sediments might be linked to the abundant eDNA pools in the sediments that was exported by MGP aggregates (Pietramellara *et al.*, 2009; Guardiola *et al.*, 2015; Nagler *et al.*, 2018a).

5.3 Limitations of the study

Although the current study has provided some useful insights into the composition of MGPs and developed a strategy to produce an MGP floc model from *Pseudoalteromonas atlantica* EPS, there are still more areas to explore in this realm by overcoming the following limitations.

1. There is a lack of techniques that help with the serial filtrations and separation of MGPs, mainly due to the fact that these gel pools are always studied on filters. In addition, the filtration process applied here required frequent filter changing, which may cause an undetectable loss of particles.
2. The inability to stain the gel particles simultaneously by Alcian Blue and Coomassie Brilliant Blue for the initial conventional classification of the type of particles.
3. There have been attempts to measure the number of MGPs present in 5 ml water suspension as the particles concentrated. Efforts to use a flow cytometer (core facility at Newcastle University) were hindered by the rapid growth of the gel particles, as they naturally tend to coagulate. The flow cytometry instrument was not able to take these particles as the rapidly aggregating particles could block their capillaries. Another attempt was made to use dynamic laser scattering (DLS) instrumentation, which is widely used to measure growing particles sizes (Chin *et al.*, 1998; Ding *et al.*, 2008; Shiu *et al.*, 2014). However, the unavailability (at Newcastle University) of this instrument within the size range of up to 300 µm hindered the endeavour to obtain data on MGP size measurements and aggregate size increase.

5.4 Conclusions

The studies carried out in this thesis have provided original contributions to the knowledge of the characterisation of MGPs and the *Pseudoalteromonas atlantica* marine floc model.

1. In this study, a new method has been developed to isolate MGPs in a small volume of liquid suspension, imitating their natural occurrence in the ocean, but in a concentrated state. This has been done for the first time here for MGPs of 0.4-100 µm.
2. One of the most significant findings to emerge from this study is that the structural composition of MGPs and the *P. atlantica* floc model comprises eDNA. This is a remarkable finding to be added to the global perception of MGPs' ecological role in the ocean. The presence of eDNA would answer further questions about the dynamics of MGPs aggregation in the ocean.
3. This study provided insights that facilitated a better understanding of the composition of the bacterial community attached to MGPs. Diverse bacterial taxa inhabit different MGP size groups and encode various putative metabolic pathways that vary between spring and summer.
4. Another obvious finding from the current study is the secretion of DNase by marine bacteria isolated from MGPs for the first time. This finding points to a potential implication of DNase within the MGPs' micro-niche.
5. The best DNase producer of the bacterial isolate from the North Sea sediments AW2 identified as *Serratia* marcescens. It is genome contains 43 nuclease genes including seven extracellular deoxyribonuclease genes.

5.5 Future work perspectives

Although this current study has provided a useful new insight into the presence of eDNA in the matrices of MGPs and the particle model of *P. atlantica*, applying two specific eDNA stains (YOYO-1 and TOTO-3), the mechanisms by which extracellular DNase degrades MGPs remain largely unknown. Therefore, several suggestions for future research are listed below.

1. Investigating the presence of eDNA in MGPs produced by bacteria and diatoms in large-scale studies in a microcosm, as well as understanding the occurrence of eDNA in other organic matter and marine snow from different depths of the ocean.
2. Further spatial and temporal exploration of the MGP size fractions in bacterial communities is required, as well as detailed profiling of their composition using a shotgun metagenome.
3. There is abundant room for further progress in determining the precise functional properties of MGP-associated bacteria using a multi-omics investigation that involves metatranscriptomics, metatransproteomics and metabolomics (Jansson and Baker, 2016).
4. Conducting a transcriptomic study of the DNase production of the AW2 strain marine bacteria.
5. Further research should be undertaken to investigate the potential role of bacterial DNases in the structural integrity of MGPs and their dynamics, as the degradation of the eDNA may be a key step in the turnover of marine organic matter.
6. Investigating the in-vitro role of eDNA in MGP aggregation. We have provided preliminary results (see Appendix A).
7. Investigating the biotechnological application of marine bacterial extracellular DNase, specifically for biofilms caused by MGPs on reverse osmosis (RO) membrane of desalination plants.

References

Acinas, S.G., Antón, J. and Rodríguez-Valera, F. (1999) 'Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA', *Appl. Environ. Microbiol.*, 65(2), pp. 514-522.

Al-Wahaibi, A.S., Lapinska, E., Rajarajan, N., Dobretsov, S., Upstill-Goddard, R. and Burgess, J.G. (2019) 'Secretion of DNases by marine bacteria: a culture based and bioinformatics approach', *Frontiers in Microbiology*, 10, p. 969.

Alawi, M., Schneider, B. and Kallmeyer, J. (2014) 'A procedure for separate recovery of extra- and intracellular DNA from a single marine sediment sample', *Journal of microbiological methods*, 104, pp. 36-42.

Alldredge, A.L., Cole, J.J. and Caron, D.A. (1986) 'Production of heterotrophic bacteria inhabiting macroscopic organic aggregates (marine snow) from surface waters1', *Limnology and Oceanography*, 31(1), pp. 68-78.

Alldredge, A.L., Passow, U. and Logan, B.E. (1993) 'The Abundance and Significance of a Class of Large, Transparent Organic Particles in the Ocean', *Deep-Sea Research Part I-Oceanographic Research Papers*, 40(6), pp. 1131-1140.

Alldredge, A.L. and Silver, M.W. (1988) 'Characteristics, dynamics and significance of marine snow', *Progress in Oceanography*, 20(1), pp. 41-82.

Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M. and Tolker-Nielsen, T. (2006) 'A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms', *Molecular microbiology*, 59(4), pp. 1114-1128.

Allison, S.D., Chao, Y., Farrara, J.D., Hatosy, S. and Martiny, A. (2012) 'Fine-scale temporal variation in marine extracellular enzymes of coastal southern California', *Frontiers in microbiology*, 3, p. 301.

Allison, S.D. and Martiny, J.B. (2008) 'Resistance, resilience, and redundancy in microbial communities', *Proceedings of the National Academy of Sciences*, 105(Supplement 1), pp. 11512-11519.

Amaral-Zettler, L., Artigas, L.F., Baross, J., Bharathi, L., Boetius, A., Chandramohan, D., Herndl, G., Kogure, K., Neal, P. and Pedrós-Alió, C. (2010) 'A global census of marine microbes', *Life in the world's oceans: diversity, distribution and abundance. Oxford: Blackwell Publishing Ltd*, pp. 223-245.

Anahtar, M.N., Bowman, B.A. and Kwon, D.S. (2016) 'Efficient Nucleic Acid Extraction and 16S rRNA Gene Sequencing for Bacterial Community Characterization', *Journal of visualized experiments: JoVE*, (110).

Anjum, K., Bi, H., Chai, W., Lian, X.-Y. and Zhang, Z. (2018) 'Antiglioma pseurotin A from marine Bacillus sp. FS8D regulating tumour metabolic enzymes', *Natural product research*, 32(11), pp. 1353-1356.

Arnosti, C. (2003) 'Microbial extracellular enzymes and their role in dissolved organic matter cycling', *Aquatic Ecosystems: Interactivity of Dissolved Organic Matter*, pp. 315-342.

Arnosti, C. (2011) 'Microbial extracellular enzymes and the marine carbon cycle', *Ann Rev Mar Sci*, 3, pp. 401-25.

Arnosti, C. (2014) 'Patterns of microbially driven carbon cycling in the ocean: links between extracellular enzymes and microbial communities', *Advances in Oceanography*, 2014.

Arnosti, C., Bell, C., Moorhead, D.L., Sinsabaugh, R.L., Steen, A.D., Stromberger, M., Wallenstein, M. and Weintraub, M.N. (2014) 'Extracellular enzymes in terrestrial, freshwater, and marine environments: perspectives on system variability and common research needs', *Biogeochemistry*, 117(1), pp. 5-21.

Arnosti, C., Fuchs, B., Amann, R. and Passow, U. (2012) 'Contrasting extracellular enzyme activities of particle-associated bacteria from distinct provinces of the North Atlantic Ocean', *Frontiers in Microbiology*, 3(425).

Arruda Fatibello, S.H.S., Henriques Vieira, A.A. and Fatibello-Filho, O. (2004) 'A rapid spectrophotometric method for the determination of transparent exopolymer particles (TEP) in freshwater', *Talanta*, 62(1), pp. 81-85.

Aßhauer, K.P., Wemheuer, B., Daniel, R. and Meinicke, P. (2015) 'Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data', *Bioinformatics*, 31(17), pp. 2882-2884.

Azam, F. (1998) 'Microbial control of oceanic carbon flux: The plot thickens', *Science*, 280(5364), pp. 694-696.

Azam, F. and Malfatti, F. (2007) 'Microbial structuring of marine ecosystems', *Nat Rev Micro*, 5(10), pp. 782-791.

Azetsu-Scott, K. and Passow, U. (2004) 'Ascending marine particles: significance of transparent exopolymer particles (TEP) in the upper ocean', *Limnol Oceanogr*, 49, pp. 741-748.

Bachmann, J., Heimbach, T., Hassenrück, C., Kopprio, G.A., Iversen, M.H., Grossart, H.P. and Gärdes, A. (2018) 'Environmental drivers of free-living vs. particle-attached bacterial community composition in the Mauritania upwelling system', *Frontiers in microbiology*, 9.

Balabanova, L., Podvolotskaya, A., Slepchenko, L., Eliseikina, M., Noskova, Y., Nedashkovskaya, O., Son, O., Tekutyeva, L. and Rasskazov, V. (2017) 'Nucleolytic enzymes from the marine bacterium Cobetia amphilecti KMM 296 with antibiofilm activity and biopreservative effect on meat products', *Food Control*, 78, pp. 270-278.

Balmonte, J., Teske, A. and Arnosti, C. (2016) *American Geophysical Union, Ocean Sciences Meeting 2016, abstract# MM44A-0465*.

Baltar, F. (2018) 'Watch Out for the "Living Dead": Cell-Free Enzymes and Their Fate', *Frontiers in microbiology*, 8, p. 2438.

Baltar, F., Arístegui, J., Gasol, J.M., Sintes, E., van Aken, H.M. and Herndl, G.J. (2010) 'High dissolved extracellular enzymatic activity in the deep central Atlantic Ocean', *Aquatic Microbial Ecology*, 58(3), pp. 287-302.

Baltar, F., Morán, X.A.G. and Lønborg, C. (2017) 'Warming and organic matter sources impact the proportion of dissolved to total activities in marine extracellular enzymatic rates', *Biogeochemistry*, 133(3), pp. 307-316.

Bar-Zeev, E., Berman-Frank, I., Girshevitz, O. and Berman, T. (2012) 'Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles', *Proceedings of the National Academy of Sciences*, 109(23), pp. 9119-9124.

Bar-Zeev, E., Berman-Frank, I., Liberman, B., Rahav, E., Passow, U. and Berman, T. (2009) 'Transparent exopolymer particles: Potential agents for organic fouling and biofilm formation in desalination and water treatment plants', *Desalination and Water Treatment*, 3(1-3), pp. 136-142.

Bar-Zeev, E., Passow, U., Castrillon, S.R. and Elimelech, M. (2015) 'Transparent exopolymer particles: from aquatic environments and engineered systems to membrane biofouling', *Environ Sci Technol*, 49(2), pp. 691-707.

Bartual, A., Vicente-Cera, I., Flecha, S. and Prieto, L. (2017) 'Effect of dissolved polyunsaturated aldehydes on the size distribution of transparent exopolymeric particles in an experimental diatom bloom', *Marine Biology*, 164(5), p. 120.

Bassler, B.L. (1999) 'How bacteria talk to each other: regulation of gene expression by quorum sensing', *Current opinion in microbiology*, 2(6), pp. 582-587.

Benedik, M.J. and Strych, U. (1998) 'Serratia marcescens and its extracellular nuclease', *FEMS microbiology letters*, 165(1), pp. 1-13.

Bhattacharya, S., Choudhury, J.D., Gachhui, R. and Mukherjee, J. (2018) 'A new collagenase enzyme of the marine sponge pathogen Pseudoalteromonas agarivorans NW4327 is uniquely linked with a TonB dependent receptor', *International journal of biological macromolecules*, 109, pp. 1140-1146.

Bian, F., Xie, B.B., Qin, Q.L., Shu, Y.L., Zhang, X.Y., Yu, Y., Chen, B., Chen, X.L., Zhou, B.C. and Zhang, Y.Z. (2012) 'Genome Sequences of Six Pseudoalteromonas Strains Isolated from Arctic Sea Ice', *Journal of Bacteriology*, 194(4), pp. 908-909.

Biller, S.J., McDaniel, L.D., Breitbart, M., Rogers, E., Paul, J.H. and Chisholm, S.W. (2017) 'Membrane vesicles in sea water: heterogeneous DNA content and implications for viral abundance estimates', *The ISME journal*, 11(2), p. 394.

Biller, S.J., Schubotz, F., Roggensack, S.E., Thompson, A.W., Summons, R.E. and Chisholm, S.W. (2014) 'Bacterial vesicles in marine ecosystems', *science*, 343(6167), pp. 183-186.

Bižić-Ionescu, M., Zeder, M., Ionescu, D., Orlić, S., Fuchs, B.M., Grossart, H.P. and Amann, R. (2015) 'Comparison of bacterial communities on limnic versus coastal marine particles reveals profound differences in colonization', *Environmental microbiology*, 17(10), pp. 3500-3514.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R. and Abebe, E. (2005) 'Defining operational taxonomic units using DNA barcode data', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360(1462), pp. 1935-1943.

Blokesch, M. and Schoolnik, G.K. (2008) 'The Extracellular Nuclease Dns and Its Role in Natural Transformation of Vibrio cholerae', *Journal of Bacteriology*, 190(21), pp. 7232-7240.

Bochdansky, A.B., Van Aken, H.M. and Herndl, G.J. (2010) 'Role of macroscopic particles in deep-sea oxygen consumption', *Proceedings of the National Academy of Sciences*, 107(18), pp. 8287-8291.

Böckelmann, U., Manz, W., Neu, T.R. and Szewzyk, U. (2002) 'Investigation of lotic microbial aggregates by a combined technique of fluorescent in situ hybridization and lectin-binding-analysis', *Journal of microbiological methods*, 49(1), pp. 75-87.

Boetius, A. and Lochte, K. (1994) 'Regulation of microbial enzymatic degradation of organic matter in deep-sea sediments', *Marine Ecology-Progress Series*, 104, pp. 299-299.

Boetius, A. and Lochte, K. (1996) 'Effect of organic enrichments on hydrolytic potentials and growth of bacteria in deep-sea sediments', *Marine Ecology Progress Series*, 140, pp. 239-250.

Boyd, P.W. and Trull, T.W. (2007) 'Understanding the export of biogenic particles in oceanic waters: Is there consensus?', *Progress in Oceanography*, 72(4), pp. 276-312.

Bunpa, S., Sermwittayawong, N. and Vuddhakul, V. (2016) 'Extracellular enzymes produced by Vibrio alginolyticus isolated from environments and diseased aquatic animals', *Procedia Chemistry*, 18, pp. 12-17.

Burns, W., Marchetti, A., White, B., Prairie, J. and Ziervogel, K. (2016) *American Geophysical Union, Ocean Sciences Meeting 2016, abstract# MM44B-0496*.

Busch, K., Endres, S., Iversen, M.H., Michels, J., Nöthig, E.-M. and Engel, A. (2017) 'Bacterial colonization and vertical distribution of marine gel particles (TEP and CSP) in the Arctic Fram Strait', *Frontiers in Marine Science*, 4(Art. No. 166).

Cao, Z., Casabona, M.G., Kneuper, H., Chalmers, J.D. and Palmer, T. (2017) 'The type VII secretion system of Staphylococcus aureus secretes a nuclease toxin that targets competitor bacteria', *Nature microbiology*, 2(1), p. 16183.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A. and Turnbaugh, P.J. (2011) 'Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample', *Proc Natl Acad Sci USA*, 15, pp. 4516-4522.

Chen, I. and Dubnau, D. (2004) 'DNA uptake during bacterial transformation', *Nature Reviews Microbiology*, 2(3), p. 241.

Chen, J. and Thornton, D.C.O. (2015) 'Transparent exopolymer particle production and aggregation by a marine planktonic diatom (Thalassiosira weissflogii) at different growth rates', *Journal of Phycology*, 51(2), pp. 381-393.

Chin, W.-C., Orellana, M.V. and Verdugo, P. (1998) 'Spontaneous assembly of marine dissolved organic matter into polymer gels', *Nature*, 391(6667), pp. 568-572.

Christina, L. and Passow, U. (2007) 'Factors influencing the sinking of POC and the efficiency of the biological carbon pump', *Deep Sea Research Part II: Topical Studies in Oceanography*, 54(5), pp. 639-658.

Chróst, R.J. (1990) 'Microbial ectoenzymes in aquatic environments', in *Aquatic microbial ecology*. Springer, pp. 47-78.

Chua, S.L., Liu, Y., Yam, J.K.H., Chen, Y., Vejborg, R.M., Tan, B.G.C., Kjelleberg, S., Tolker-Nielsen, T., Givskov, M. and Yang, L. (2014) 'Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles', *Nature communications*, 5.

Ciais, P., Dolman, A., Bombelli, A., Duren, R., Peregon, A., Rayner, P., Miller, C., Gobron, N., Kinderman, G. and Marland, G. (2014) 'Current systematic carbon-cycle observations and the need for implementing a policy-relevant carbon observing system', *Biogeosciences*, 11, pp. 3547-3602.

Cisternas-Novoa, C., Lee, C. and Engel, A. (2014) 'A semi-quantitative spectrophotometric, dye-binding assay for determination of Coomassie Blue stainable particles', *Limnology and Oceanography: Methods*, 12, pp. 604-616.

Cisternas-Novoa, C., Lee, C. and Engel, A. (2015) 'Transparent exopolymer particles (TEP) and Coomassie stainable particles (CSP): Differences between their origin and vertical distributions in the ocean', *Marine Chemistry*, 175, pp. 56-71.

Collins, R.A., Wangensteen, O.S., O'Gorman, E.J., Mariani, S., Sims, D.W. and Genner, M.J. (2018) 'Persistence of environmental DNA in marine systems', *Communications biology*, 1(1), p. 185.

Copeland, A., Lucas, S., Lapidus, A., Barry, K., Detter, J.C. and Glavina del Rio, T. (2006) 'Complete sequence of Pseudoalteromonas atlantica T6c', *Walnut Creek, CA: US DOE Joint Genome Institute*

Costa-Ramos, C. and Rowley, A.F. (2004) 'Effect of extracellular products of Pseudoalteromonas atlantica on the edible crab Cancer pagurus', *Applied and. Environmental Microbiology*, *70*(2), pp.729-735.

Cordero, O.X. and Datta, M.S. (2016) 'Microbial interactions and community assembly at microscales', *Current opinion in microbiology*, 31, pp. 227-234.

Corinaldesi, C., Danovaro, R. and Dell'Anno, A. (2005) 'Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments', *Applied and Environmental Microbiology*, 71(1), pp. 46-50.

Corinaldesi, C., Dell'Anno, A. and Danovaro, R. (2007) 'Early diagenesis and trophic role of extracellular DNA in different benthic ecosystems', *Limnology and oceanography*, 52(4), pp. 1710-1717.

Corinaldesi, C., Tangherlini, M., Luna, G.M. and Dell'Anno, A. (2014) 'Extracellular DNA can preserve the genetic signatures of present and past viral infection events in deep hypersaline anoxic basins', *Proceedings of the Royal Society of London B: Biological Sciences*, 281(1780), p. 20133299.

Corinaldesi, C., Tangherlini, M., Manea, E. and Dell'Anno, A. (2018) 'Extracellular DNA as a genetic recorder of microbial diversity in benthic deep-sea ecosystems', *Scientific reports*, 8(1), p. 1839.

Cowen, J. and Holloway, C. (1996) 'Structural and chemical analysis of marine aggragates: in situ macrophotography and laser confocal and electron microscopy', *Marine Biology*, 126(2), pp. 163-174.

Crump, B.C., Armbrust, E.V. and Baross, J.A. (1999) 'Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean', *Applied Environmental Microbiology*, 65(7), pp. 3192-3204.

Cuccuru, G., Orsini, M., Pinna, A., Sbardellati, A., Soranzo, N., Travaglione, A., Uva, P., Zanetti, G. and Fotia, G. (2014) 'Orione, a web-based framework for NGS analysis in microbiology', *Bioinformatics*, 30(13), pp. 1928-1929.

Cunliffe, M., Engel, A., Frka, S., Gasparovic, B., Guitart, C. Murrell, J.C., M., Stolle, C., Upstill-Goddard, R. and Wurl, O. (2013) 'Sea surface microlayers: A unified physicochemical and biological perspective of the air-ocean interface', *Progress in Oceanography*, 109, pp. 104-116.

Cunliffe, M., Upstill-Goddard, R.C. and Murrell, J.C. (2011) 'Microbiology of aquatic surface microlayers', *FEMS microbiology reviews*, 35(2), pp. 233-246.

D'ambrosio, L., Ziervogel, K., MacGregor, B., Teske, A. and Arnosti, C. (2014) 'Composition and enzymatic function of particle-associated and free-living bacteria: a coastal/offshore comparison', *The ISME journal*, 8(11), p. 2167.

Daley, T. and Smith, A.D. (2013) 'Predicting the molecular complexity of sequencing libraries', *Nature methods*, 10(4), p. 325.

Dang, H. and Lovell, C.R. (2016) 'Microbial Surface Colonization and Biofilm Development in Marine Environments', *Microbiology and Molecular Biology Reviews*, 80(1), pp. 91-138.

Dang, H., Zhu, H., Wang, J. and Li, T. (2009) 'Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough', *World Journal of Microbiology and Biotechnology*, 25(1), pp. 71-79.

Das, T., Sehar, S., Koop, L., Wong, Y.K., Ahmed, S., Siddiqui, K.S. and Manefield, M. (2014) 'Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation', *PloS one*, 9(3), p. e91935.

Das, T., Sharma, P.K., Busscher, H.J., van der Mei, H.C. and Krom, B.P. (2010) 'Role of extracellular DNA in initial bacterial adhesion and surface aggregation', *Applied Environmental Microbiology*, 76(10), pp. 3405-3408.

Datta, M.S., Sliwerska, E., Gore, J., Polz, M.F. and Cordero, O.X. (2016) 'Microbial interactions lead to rapid micro-scale successions on model marine particles', *Nature Communications*, 7, p. 11965.

de Pascale, D., Cusano, A.M., Autore, F., Parrilli, E., Di Prisco, G., Marino, G. and Tutino, M.L. (2008) 'The cold-active Lip1 lipase from the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125 is a member of a new bacterial lipolytic enzyme family', *Extremophiles*, 12(3), pp. 311-323.

Decho, A.W. (2010) 'Overview of biopolymer-induced mineralization: what goes on in biofilms?', *Ecological Engineering*, 36(2), pp. 137-144.

Decho, A.W. and Gutierrez, T. (2017) 'Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems', *Frontiers in microbiology*, 8.

Decho, A.W. and Moriarty, D.J.W. (1990) 'Bacterial exopolymer utilization by a harpacticoid copepod: a methodology and results', *Limnology and Oceanography*, 35(5), pp. 1039-1049.

Deflaun, M.F., Paul, J.H. and Jeffrey, W.H. (1987) 'Distribution and Molecular-Weight of Dissolved DNA in Subtropical Estuarine and Oceanic Environments', *Marine Ecology Progress Series*, 38(1), pp. 65-73.

Dell'Anno, A. and Danovaro, R. (2005) 'Extracellular DNA plays a key role in deep-sea ecosystem functioning', *Science*, 309(5744), p. 2179.

Dell'Anno, A., Stefano, B. and Danovaro, R. (2002) 'Quantification, base composition, and fate of extracellular DNA in marine sediments', *Limnology and Oceanography*, 47(3), pp. 899-905.

DeLong, E.F., Franks, D.G. and Alldredge, A.L. (1993) 'Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages', *Limnology and Oceanography*, 38(5), pp. 924-934.

DeMott, P.J., Hill, T.C., McCluskey, C.S., Prather, K.A., Collins, D.B., Sullivan, R.C., Ruppel, M.J., Mason, R.H., Irish, V.E. and Lee, T. (2016) 'Sea spray aerosol as a unique source of ice nucleating particles', *Proceedings of the National Academy of Sciences*, 113(21), pp. 5797-5803.

Deng, B., Ghatak, S., Sarkar, S., Ghatak, P., McComb, D.W. and Sen, C.K. (2018) 'STEM Observation of eDNA as a Dominant Component of EPS in Pseudomonas aeruginosa Biofilm', *Microscopy and Microanalysis*, 24(S1), pp. 1334-1335.

Deng, W., Cruz, B.N. and Neuer, S. (2016) 'Effects of nutrient limitation on cell growth, TEP production and aggregate formation of marine Synechococcus', *Aquatic Microbial Ecology*, 78(1), pp. 39-49.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G.L. (2006) 'Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB', *Applied and environmental microbiology*, 72(7), pp. 5069-5072.

Desriac, F., Defer, D., Bourgougnon, N., Brillet, B., Le Chevalier, P. and Fleury, Y. (2010) 'Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic', *Marine drugs*, 8(4), pp. 1153-1177.

Ding, Y.-X., Chin, W.-C., Rodriguez, A., Hung, C.-C., Santschi, P.H. and Verdugo, P. (2008) 'Amphiphilic exopolymers from Sagittula stellata induce DOM self-assembly and formation of marine microgels', *Marine Chemistry*, 112(1), pp. 11-19.

Discart, V. (2015) *Transparent exopolymer particles: detection and role in membrane based systems*.

Dominiak, D.M., Nielsen, J.L. and Nielsen, P.H. (2011) 'Extracellular DNA is abundant and important for microcolony strength in mixed microbial biofilms', *Environmental microbiology*, 13(3), pp. 710-721.

Dubnau, D. (1999) 'DNA uptake in bacteria', *Annual Reviews in Microbiology*, 53(1), pp. 217-244.

Elias, S. and Banin, E. (2012) 'Multi-species biofilms: living with friendly neighbors', *FEMS Microbiology Reviews*, 36(5), pp. 990-1004.

Engel, A. (2004) 'Distribution of transparent exopolymer particles (TEP) in the northeast Atlantic Ocean and their potential significance for aggregation processes', *Deep Sea Research Part I: Oceanographic Research Papers*, 51(1), pp. 83-92.

Engel, A. (2009) 'Determination of marine gel particles', *Practical Guidelines for the Analysis of Seawater*, pp. 125-142.

Engel, A. and Galgani, L. (2016) 'The organic sea-surface microlayer in the upwelling region off the coast of Peru and potential implications for air–sea exchange processes', *Biogeosciences (BG)*, 13, pp. 989-1007.

Engel, A., Thoms, S., Riebesell, U., Rochelle-Newall, E. and Zondervan, I. (2004) 'Polysaccharide aggregation as a potential sink of marine dissolved organic carbon', *Nature*, 428(6986), pp. 929-932.

Enke, T.N., Datta, M.S., Schwartzman, J., Cermak, N., Schmitz, D., Barrere, J., Pascual-García, A. and Cordero, O.X. (2019) 'Modular assembly of polysaccharide-degrading marine microbial communities', *Current Biology*, 29(9), pp. 1528-1535. e6.

Enke, T.N., Leventhal, G.E., Metzger, M., Saavedra, J.T. and Cordero, O.X. (2018) 'Micro-scale ecology regulates particulate organic matter turnover in model marine microbial communities', *bioRxiv*, p. 241620.

Ferrer, M., Méndez-García, C., Bargiela, R., Chow, J., Alonso, S., García-Moyano, A., Bjerga, G.E., Steen, I.H., Schwabe, T. and Blom, C. (2018) 'Decoding the ocean's microbiological secrets for marine enzyme biodiscovery', *FEMS microbiology letters*, 366(1), p. fny285.

Flemming, H.-C. (2016) 'EPS—Then and Now', *Microorganisms*, 4(4), p. 41.

Flintrop, C.M., Rogge, A., Miksch, S., Thiele, S., Waite, A.M. and Iversen, M.H. (2018) 'Embedding and slicing of intact in situ collected marine snow', *Limnology and Oceanography: Methods*, 16(6), pp. 339-355.

Fontanez, K.M., Eppley, J.M., Samo, T.J., Karl, D.M. and DeLong, E.F. (2015) 'Microbial community structure and function on sinking particles in the North Pacific Subtropical Gyre', *Frontiers in microbiology*, 6.

Fowler, S.W. and Knauer, G.A. (1986) 'Role of Large Particles in the Transport of Elements and Organic-Compounds through the Oceanic Water Column', *Progress in Oceanography*, 16(3), pp. 147-194.

Galand, P.E., Pereira, O., Hochart, C., Auguet, J.C. and Debroas, D. (2018) 'A strong link between marine microbial community composition and function challenges the idea of functional redundancy', *The ISME journal*, p. 1.

Galgani, L. and Engel, A. (2013) 'Accumulation of gel particles in the sea-surface microlayer during an experimental study with the diatom Thalassiosira weissflogii'.

Gallo, P.M., Rapsinski, G.J., Wilson, R.P., Oppong, G.O., Sriram, U., Goulian, M., Buttaro, B., Caricchio, R., Gallucci, S. and Tükel, Ç. (2015) 'Amyloid-DNA composites of bacterial biofilms stimulate autoimmunity', *Immunity*, 42(6), pp. 1171-1184.

Galloway, T.S., Cole, M. and Lewis, C. (2017) 'Interactions of microplastic debris throughout the marine ecosystem', *Nature ecology & evolution*, 1(5), p. 0116.

Ganesh, S., Parris, D.J., DeLong, E.F. and Stewart, F.J. (2014) 'Metagenomic analysis of size-fractionated picoplankton in a marine oxygen minimum zone', *The ISME journal*, 8(1), pp. 187-211.

García-Fraga, B., da Silva, A.F., López-Seijas, J. and Sieiro, C. (2015) 'A novel family 19 chitinase from the marine-derived Pseudoalteromonas tunicata CCUG 44952T: Heterologous expression, characterization and antifungal activity', *Biochemical engineering journal*, 93, pp. 84-93.

Glöckner, F.O., Fuchs, B.M. and Amann, R. (1999) 'Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization', *Applied and environmental microbiology*, 65(8), pp. 3721-3726.

Gödeke, J., Heun, M., Bubendorfer, S., Paul, K. and Thormann, K.M. (2011) 'Roles of two Shewanella oneidensis MR-1 extracellular endonucleases', *Appl. Environ. Microbiol.*, 77(15), pp. 5342-5351.

Gram, L., Grossart, H.-P., Schlingloff, A. and Kiørboe, T. (2002) 'Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by Roseobacter strains isolated from marine snow', *Applied and Environmental Microbiology*, 68(8), pp. 4111-4116.

Grossart, H.-P., Tang, K.W., Kiørboe, T. and Ploug, H. (2006) 'Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages', *FEMS Microbiology Letters*, 266(2), pp. 194-200.

Grossart, H.-P., Tang, K.W., Kiørboe, T. and Ploug, H. (2007) 'Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages', *FEMS microbiology letters*, 266(2), pp. 194-200.

Guardiola, M., Uriz, M.J., Taberlet, P., Coissac, E., Wangensteen, O.S. and Turon, X. (2015) 'Deep-sea, deep-sequencing: metabarcoding extracellular DNA from sediments of marine canyons', *PLoS One*, 10(10), p. e0139633.

Guidi, L., Chaffron, S., Bittner, L., Eveillard, D., Larhlimi, A., Roux, S., Darzi, Y., Audic, S., Berline, L. and Brum, J.R. (2016) 'Plankton networks driving carbon export in the oligotrophic ocean', *Nature*.

Hansell, D.A., Carlson, C.A. and Schlitzer, R. (2012) 'Net removal of major marine dissolved organic carbon fractions in the subsurface ocean', *Global Biogeochemical Cycles*, 26(1).

Hatcher, A., Hill, P. and Grant, J. (2001) 'Optical backscatter of marine flocs', *Journal of Sea Research*, 46(1), pp. 1-12.

Hedges, J.I. (2002) 'Why dissolved organics matter', *Biogeochemistry of marine dissolved organic matter*, pp. 1-33.

Herndl, G.J. and Peduzzi, P. (1988) 'The ecology of amorphous aggregations (Marine Snow) in the Northern Adriatic Sea', *Marine Ecology*, 9(1), pp. 79-90.

Hewes, C.D. and Holm-Hansen, O. (1983) 'A method for recovering nanoplankton from filters for identification with the microscope: The filter-transfer-freeze (FTF) technique 1', *Limnology and Oceanography*, 28(2), pp. 389-394.

Hmelo, L.R., Mincer, T.J. and Van Mooy, B.A. (2011) 'Possible influence of bacterial quorum sensing on the hydrolysis of sinking particulate organic carbon in marine environments', *Environmental microbiology reports*, 3(6), pp. 682-688.

Hoffman, M. and Decho, A.W. (2000) 'Proteolytic enzymes in the marine bacterium Pseudoalteromonas atlantica: post-secretional activation and effects of environmental conditions', *Aquatic Microbial Ecology*, *23*(1), pp.29-39.

Hollibaugh, J.T., Wong, P.S. and Murrell, M.C. (2000) 'Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California', *Aquatic Microbial Ecology*, 21(2), pp. 103-114.

Holloway, C.F. and Cowen, J.P. (1997a) 'Development of a scanning confocal laser microscopic technique to examine the structure and composition of marine snow', *Limnology and Oceanography*, 42(6), pp. 1340-1352.

Holloway, C.F. and Cowen, J.P. (1997b) 'Development of a scanning confocal laser microscopic technique to examine the structure and composition of marine snow', *Limnology and Oceanography*, 42, pp. 1340-1352.

Holmström, C. and Kjelleberg, S. (1999) 'Marine Pseudoalteromonas species are associated with higher organisms and produce biologically active extracellular agents', *FEMS Microbiology Ecology*, 30(4), pp. 285-293.

Hoppe, H.-G., Arnosti, C. and Herndl, G.J. (2002) 'Ecological significance of bacterial enzymes in the marine environment', *Enzymes in the Environment: Activity, Ecology, and Applications*, pp. 73-107.

Hu, W., Li, L., Sharma, S., Wang, J., McHardy, I., Lux, R., Yang, Z., He, X., Gimzewski, J.K. and Li, Y. (2012) 'DNA builds and strengthens the extracellular matrix in Myxococcus xanthus biofilms by interacting with exopolysaccharides', *PloS one*, 7(12), p. e51905.

Huston, A.L. and Deming, J.W. (2002) 'Relationships between microbial extracellular enzymatic activity and suspended and sinking particulate organic matter: seasonal transformations in the North Water', *Deep Sea Research Part II: Topical Studies in Oceanography*, 49(22), pp. 5211-5225.

Ibáñez de Aldecoa, A.L., Zafra, O. and González-Pastor, J.E. (2017) 'Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities', *Frontiers in microbiology*, 8, p. 1390.

Imbs, T.I., Silchenko, A.S., Fedoreev, S.A., Isakov, V.V., Ermakova, S.P. and Zvyagintseva, T.N. (2018) 'Fucoidanase inhibitory activity of phlorotannins from brown algae', *Algal research*, 32, pp. 54-59.

Iuculano, F., Duarte, C.M., Marbà, N. and Agustí, S. (2017) 'Seagrass as major source of transparent exopolymer particles in the oligotrophic Mediterranean coast', *Biogeosciences*, 14(22), pp. 5069-5075.

Ivančić, I., Paliaga, P., Pfannkuchen, M., Djakovac, T., Najdek, M., Steiner, P., Korlević, M., Markovski, M., Baričević, A. and Tanković, M.S. (2018) 'Seasonal variation of extracellular enzymatic activity in marine snow-associated microbial communities and their impact on the surrounding water', *FEMS Microbiology Ecology*.

Jakubovics, N.S., Shields, R.C., Rajarajan, N. and Burgess, J.G. (2013) 'Life after death: the critical role of extracellular DNA in microbial biofilms', *Lett Appl Microbiol*, 57(6), pp. 467-75.

Jansson, J.K. and Baker, E.S. (2016) 'A multi-omic future for microbiome studies', *Nat Microbiol*, 1(16049), p. 645.

Jatt, A.N., Tang, K., Liu, J., Zhang, Z. and Zhang, X.-H. (2015) 'Quorum sensing in marine snow and its possible influence on production of extracellular hydrolytic enzymes in marine snow bacterium Pantoea ananatis B9', *FEMS Microbiol. Ecol*, 91, pp. 1-13.

Jeffries, C.D., Holtman, D.F. and Guse, D.G. (1957) 'RAPID METHOD FOR DETERMINING THE ACTIVITY OF MICROORGAN-ISMS ON NUCLEIC ACIDS', *Journal of Bacteriology*, 73(4), p. 590.

Jiao, N. and Azam, F. (2011) 'Microbial carbon pump and its significance for carbon sequestration in the ocean', *Microbial Carbon Pump in the Ocean*, 10, pp. 43-45.

Jiao, N., Herndl, G.J., Hansell, D.A., Benner, R., Kattner, G., Wilhelm, S.W., Kirchman, D.L., Weinbauer, M.G., Luo, T. and Chen, F. (2010) 'Microbial production of recalcitrant dissolved organic matter: long-term carbon storage in the global ocean', *Nature Reviews Microbiology*, 8(8), pp. 593-599.

Jiao, N., Herndl, G.J., Hansell, D.A., Benner, R., Kattner, G., Wilhelm, S.W., Kirchman, D.L., Weinbauer, M.G., Luo, T. and Chen, F. (2011) 'The microbial carbon pump and the oceanic recalcitrant dissolved organic matter pool', *Nature Reviews Microbiology*, 9(7), p. 555.

Jiao, N., Robinson, C., Azam, F., Thomas, H., Baltar, F., Dang, H., Hardman-Mountford, N.J., Johnson, M., Kirchman, D.L. and Koch, B.P. (2014) 'Mechanisms of microbial carbon sequestration in the ocean–future research directions', *Biogeosciences*, 11(19), pp. 5285-5306.

Jiao, N. and Zheng, Q. (2011) 'The Microbial Carbon Pump----From genes to ecosystems', *Applied and environmental microbiology*, pp. AEM. 05640-11.

Kamer, M. and Rassoulzadegan, F. (1995) 'Extracellular enzyme activity: indications for high short-term variability in a coastal marine ecosystem', *Microbial ecology*, 30(2), pp. 143-156.

Kanehisa, M. and Goto, S. (2012) 'KEGG: Kyoto encyclopedia of genes and genomes. Kanehisa laboratories'.

Karl, D.M. and Knauer, G.A. (1984) 'Detritus-microbe interactions in the marine pelagic environment: selected results from the VERTEX experiment', *Bulletin of Marine Science*, 35(3), pp. 550-565.

Karner, M. and Herndl, G.J. (1992) 'Extracellular enzymatic activity and secondary production in free-living and marine-snow-associated bacteria', *Marine Biology*, 113(2), pp. 341-347.

Karunasagar, I., Otta, S. and Karunasagar, I. (1996) 'Biofilm formation by Vibrio harveyi on surfaces', *Aquaculture*, 140(3), pp. 241-245.

Kawato, Y., Ito, T., Kamaishi, T., Fujiwara, A., Ototake, M., Nakai, T. and Nakajima, K. (2016) 'Development of red sea bream iridovirus concentration method in seawater by iron flocculation', *Aquaculture*, 450, pp. 308-312.

Kellogg, C.T., Carpenter, S.D., Renfro, A.A., Sallon, A., Michel, C., Cochran, J.K. and Deming, J.W. (2011) 'Evidence for microbial attenuation of particle flux in the Amundsen Gulf and Beaufort Sea: elevated hydrolytic enzyme activity on sinking aggregates', *Polar biology*, 34(12), pp. 2007-2023.

Kellogg, C.T. and Deming, J.W. (2014) 'Particle-associated extracellular enzyme activity and bacterial community composition across the Canadian Arctic Ocean', *FEMS microbiology ecology*, 89(2), pp. 360-375.

Kiørboe, T., Grossart, H.-P., Ploug, H. and Tang, K. (2002) 'Mechanisms and rates of bacterial colonization of sinking aggregates', *Applied and Environmental Microbiology*, 68(8), pp. 3996-4006.

Kiørboe, T. and Hansen, J.L.S. (1993) 'Phytoplankton aggregate formation: observations of patterns and mechanisms of cell sticking and the significance of exopolymeric material', *Journal of Plankton Research*, 15(9), pp. 993-1018.

Kirchman, D.L. (2002) 'The ecology of Cytophaga–Flavobacteria in aquatic environments', *FEMS Microbiology Ecology*, 39(2), pp. 91-100.

Kirkpatrick, C.L. and Viollier, P.H. (2010) 'Cell dispersal in biofilms: an extracellular DNA masks nature's strongest glue', *Molecular microbiology*, 77(4), pp. 801-804.

Krupke, A., Hmelo, L.R., Ossolinski, J.E., Mincer, T.J. and Van Mooy, B.A. (2016) 'Quorum sensing plays a complex role in regulating the enzyme hydrolysis activity of microbes associated with sinking particles in the ocean', *Frontiers in Marine Science*, 3, p. 55.

Küchler, A., Yoshimoto, M., Luginbühl, S., Mavelli, F. and Walde, P. (2016) 'Enzymatic reactions in confined environments', *Nature Nanotechnology*, 11(5), p. 409.

Lahra, M.M., Martin, I., Demczuk, W., Jennison, A.V., Lee, K.-I., Nakayama, S.-I., Lefebvre, B., Longtin, J., Ward, A. and Mulvey, M.R. (2018) 'Cooperative recognition of internationally disseminated ceftriaxone-resistant Neisseria gonorrhoeae strain', *Emerging infectious diseases*, 24(4), p. 735.

Lama, S., Muylaert, K., Karki, T.B., Foubert, I., Henderson, R.K. and Vandamme, D. (2016) 'Flocculation properties of several microalgae and a cyanobacterium species during ferric chloride, chitosan and alkaline flocculation', *Bioresour Technol*, 220, pp. 464-70.

Landa, M., Blain, S., Christaki, U., Monchy, S. and Obernosterer, I. (2016) 'Shifts in bacterial community composition associated with increased carbon cycling in a mosaic of phytoplankton blooms', *The ISME journal*, 10(1), p. 39.

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V. and Knight, R. (2013) 'Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences', *Nature biotechnology*, 31(9), p. 814.

Lechtenfeld, O.J., Hertkorn, N., Shen, Y., Witt, M. and Benner, R. (2015) 'Marine sequestration of carbon in bacterial metabolites', *Nat Commun*, 6, p. 6711.

Lee, H., Park, C., Kim, H., Park, H. and Hong, S. (2015) 'Role of transparent exopolymer particles (TEP) in initial bacterial deposition and biofilm formation on reverse osmosis (RO) membrane', *Journal of Membrane Science*, 494, pp. 25-31.

Legendre, L., Rivkin, R.B., Weinbauer, M.G., Guidi, L. and Uitz, J. (2015) 'The microbial carbon pump concept: potential biogeochemical significance in the globally changing ocean', *Progress in Oceanography*, 134, pp. 432-450.

Lei, F., Zhao, Q., Sun-Waterhouse, D. and Zhao, M. (2017) 'Characterization of a salt-tolerant aminopeptidase from marine Bacillus licheniformis SWJS33 that improves hydrolysis and debittering efficiency for soy protein isolate', *Food chemistry*, 214, pp. 347-353.

Lennon, J.T. (2007) 'Diversity and metabolism of marine bacteria cultivated on dissolved DNA', *Applied and environmental microbiology*, 73(9), pp. 2799-2805.

Levi, A., Bar-Zeev, E., Elifantz, H., Berman, T. and Berman-Frank, I. (2016) 'Characterization of microbial communities in water and biofilms along a large scale SWRO desalination facility: Site-specific prerequisite for biofouling treatments', *Desalination*, 378, pp. 44-52.

Li, S., Winters, H., Jeong, S., Emwas, A.H., Vigneswaran, S. and Amy, G.L. (2016) 'Marine bacterial transparent exopolymer particles (TEP) and TEP precursors: Characterization and RO fouling potential', *Desalination*, 379, pp. 68-74.

Li, S., Winters, H., Villacorte, L.O., Ekowati, Y., Emwas, A.-H., Kennedy, M.D. and Amy, G.L. (2015) 'Compositional similarities and differences between transparent exopolymer particles (TEPs) from two marine bacteria and two marine algae: Significance to surface biofouling', *Marine Chemistry*, 174, pp. 131-140.

Li, Y., Wu, C., Zhou, M., Wang, E.T., Zhang, Z., Liu, W., Ning, J. and Xie, Z. (2017) 'Diversity of cultivable protease-producing bacteria in laizhou bay sediments, Bohai Sea, China', *Frontiers in microbiology*, 8, p. 405.

Liu, Q., Fang, J., Li, J., Zhang, L., Xie, B.-B., Chen, X.-L. and Zhang, Y.-Z. (2018) 'Depth-Resolved Variations of Cultivable Bacteria and Their Extracellular Enzymes in the Water Column of the New Britain Trench', *Frontiers in microbiology*, 9, p. 135.

Liu, S. and Liu, Z. (2018) 'Free extracellular enzymes dominate initial peptide hydrolysis in coastal seawater', *Marine Chemistry*, 199, pp. 37-43.

Logue, J.B., Stedmon, C.A., Kellerman, A.M., Nielsen, N.J., Andersson, A.F., Laudon, H., Lindström, E.S. and Kritzberg, E.S. (2016) 'Experimental insights into the importance

of aquatic bacterial community composition to the degradation of dissolved organic matter', *The ISME journal*, 10(3), p. 533.

Long, R.A. and Azam, F. (1996) 'Abundant protein-containing particles in the sea', *Aquatic Microbial Ecology*, 10, p. 213.

Long, R.A. and Azam, F. (2001) 'Microscale patchiness of bacterioplankton assemblage richness in seawater', *Aquatic Microbial Ecology*, 26(2), pp. 103-113.

López-Pérez, M. and Rodriguez-Valera, F. (2014) 'The family Alteromonadaceae', *The Prokaryotes: Gammaproteobacteria*, pp. 69-92.

Lorenz, M. and Wackernagel, W. (1992) 'DNA binding to various clay minerals and retarded enzymatic degradation of DNA in a sand/clay microcosm', in *Gene transfers and environment*. Springer, pp. 103-113.

Louca, S., Parfrey, L.W. and Doebeli, M. (2016) 'Decoupling function and taxonomy in the global ocean microbiome', *Science*, 353(6305), pp. 1272-1277.

Louca, S., Polz, M.F., Mazel, F., Albright, M.B.N., Huber, J.A., O'Connor, M.I., Ackermann, M., Hahn, A.S., Srivastava, D.S., Crowe, S.A., Doebeli, M. and Parfrey, L.W. (2018) 'Function and functional redundancy in microbial systems', *Nat Ecol Evol*, 2(6), pp. 936-943.

Luo, L., Meng, H. and Gu, J.-D. (2017) 'Microbial extracellular enzymes in biogeochemical cycling of ecosystems', *Journal of environmental management*, 197, pp. 539-549.

Lyons, M. and Dobbs, F. (2012) 'Differential utilization of carbon substrates by aggregate-associated and water-associated heterotrophic bacterial communities', *Hydrobiologia*, 686(1), pp. 181-193.

Maeda, M. and Taga, N. (1976) 'Extracellular nuclease produced by a marine bacterium. II. Purification and properties of extracellular nuclease from a marine Vibrio sp', *Canadian journal of microbiology*, 22(10), pp. 1443-1452.

Mari, X., Kerros, M.-E. and Weinbauer, M.G. (2007) 'Virus attachment to transparent exopolymeric particles along trophic gradients in the southwestern lagoon of New Caledonia', *Applied and environmental microbiology*, 73(16), pp. 5245-5252.

Mari, X., Passow, U., Migon, C., Burd, A.B. and Legendre, L. (2017) 'Transparent exopolymer particles: Effects on carbon cycling in the ocean', *Progress in Oceanography*, 151, pp. 13-37.

Matsushima, R., Danno, H., Uchida, M., Ishihara, K., Suzuki, T., Kaneniwa, M., Ohtsubo, Y., Nagata, Y. and Tsuda, M. (2010) 'Analysis of extracellular alginate lyase and its gene from a marine bacterial strain, Pseudoalteromonas atlantica AR06', *Applied microbiology and biotechnology*, *86*(2), pp.567-576.

Mendes, L.W., Braga, L., Navarrete, A.A., Souza, D., Silva, G.G. and Tsai, S.M. (2017) 'Using metagenomics to connect microbial community biodiversity and functions', *Curr Issues Mol Biol*, 24, pp. 103-118.

Meng, S. and Liu, Y. (2016) 'New insights into transparent exopolymer particles (TEP) formation from precursor materials at various Na+/Ca 2+ ratios', *Scientific reports*, 6, p. 19747.

Meng, S., Rzechowicz, M., Winters, H., Fane, A.G. and Liu, Y. (2013) 'Transparent exopolymer particles (TEP) and their potential effect on membrane biofouling', *Applied microbiology and biotechnology*, 97(13), pp. 5705-5710.

Mestre, M., Borrull, E., Sala, M. and Gasol, J.M. (2017) 'Patterns of bacterial diversity in the marine planktonic particulate matter continuum', *The ISME journal*, 11(4), pp. 999-1010.

Mestre, M., Ruiz-González, C., Logares, R., Duarte, C.M., Gasol, J.M. and Sala, M.M. (2018) 'Sinking particles promote vertical connectivity in the ocean microbiome', *Proceedings of the National Academy of Sciences*, 115(29), pp. E6799-E6807.

Mével, G., Vernet, M., Goutx, M. and Ghiglione, J.F. (2008) 'Seasonal to hour variation scales in abundance and production of total and particle-attached bacteria in the open NW Mediterranean Sea (0–1000 m)', *Biogeosciences*, 5(6), pp. 1573-1586.

Michels, J., Stippkugel, A., Lenz, M., Wirtz, K. and Engel, A. (2018) 'Rapid aggregation of biofilm-covered microplastics with marine biogenic particles', *Proceedings of the Royal Society B*, 285(1885), p. 20181203.

Moeseneder, M.M., Winter, C. and Herndl, G.J. (2001) 'Horizontal and vertical complexity of attached and free-living bacteria of the eastern Mediterranean Sea, determined by 16S rDNA and 16S rRNA fingerprints', *Limnology and Oceanography*, 46(1), pp. 95-107.

Mohit, V., Archambault, P., Toupoint, N. and Lovejoy, C. (2014) 'Phylogenetic differences in attached and free-living bacterial communities in a temperate coastal lagoon during summer, revealed via high-throughput 16S rRNA gene sequencing', *Appl Environ Microbiol*, 80(7), pp. 2071-83.

More, T., Yadav, J.S.S., Yan, S., Tyagi, R.D. and Surampalli, R.Y. (2014) 'Extracellular polymeric substances of bacteria and their potential environmental applications', *Journal of environmental management*, 144, pp. 1-25.

Mori, J.F., Ueberschaar, N., Lu, S.P., Cooper, R.E., Pohnert, G. and Kusel, K. (2017) 'Sticking together: inter-species aggregation of bacteria isolated from iron snow is controlled by chemical signaling', *Isme Journal*, 11(5), pp. 1075-1086.

Mühlenbruch, M., Grossart, H.P., Eigemann, F. and Voss, M. (2018) 'Mini-review: Phytoplankton-derived polysaccharides in the marine environment and their interactions with heterotrophic bacteria', *Environmental microbiology*, 20(8), pp. 2671-2685.

Mulcahy, H., Charron-Mazenod, L. and Lewenza, S. (2010a) 'Pseudomonas aeruginosa produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source', *Environmental Microbiology*, 12(6), pp. 1621-1629.

Mulcahy, H., Charron-Mazenod, L. and Lewenza, S. (2010b) 'Pseudomonas aeruginosa produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source', *Environmental microbiology*, 12(6), pp. 1621-1629.

Nagaraj, V., Skillman, L., Li, D. and Ho, G. (2018) 'Review–Bacteria and their extracellular polymeric substances causing biofouling on seawater reverse osmosis desalination membranes', *Journal of environmental management*, 223, pp. 586-599.

Nagler, M., Insam, H., Pietramellara, G. and Ascher-Jenull, J. (2018a) 'Extracellular DNA in natural environments: features, relevance and applications', *Applied microbiology and biotechnology*, pp. 1-14.

Nagler, M., Podmirseg, S.M., Griffith, G.W., Insam, H. and Ascher-Jenull, J. (2018b) 'The use of extracellular DNA as a proxy for specific microbial activity', *Applied Microbiology and Biotechnology*, 102(6), pp. 2885-2898.

Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y. and Sack, D.A. (2007) 'Global dissemination of Vibrio parahaemolyticus serotype O3: K6 and its serovariants', *Clinical microbiology reviews*, 20(1), pp. 39-48.

Nemoto, K., Hirota, K., Murakami, K., Taniguti, K., Murata, H., Viducic, D. and Miyake, Y. (2003) 'Effect of Varidase (streptodornase) on biofilm formed by Pseudomonas aeruginosa', *Chemotherapy*, 49(3), pp. 121-125.

Nestle, M. and Roberts, W. (1969) 'An extracellular nuclease from Serratia marcescens II. Specificity of the enzyme', *Journal of Biological Chemistry*, 244(19), pp. 5219-5225.

Neu, T.R. (2000) 'In situcell and glycoconjugate distribution in river snow studied by confocal laser scanning microscopy', *Aquatic Microbial Ecology*, 21(1), pp. 85-95.

Neukermans, G., Reynolds, R.A. and Stramski, D. (2016) 'Optical classification and characterization of marine particle assemblages within the western Arctic Ocean', *Limnology and Oceanography*, 61(4), pp. 1472-1494.

Nielsen, K.M., Johnsen, P.J., Bensasson, D. and Daffonchio, D. (2007) 'Release and persistence of extracellular DNA in the environment', *Environmental biosafety research*, 6(1-2), pp. 37-53.

Nijland, R., Hall, M.J. and Burgess, J.G. (2010) 'Dispersal of biofilms by secreted, matrix degrading, bacterial DNase', *PLoS One*, 5(12), p. e15668.

Nikaido, H. (2003) 'Molecular basis of bacterial outer membrane permeability revisited', *Microbiology and molecular biology reviews*, 67(4), pp. 593-656.

Nishino, T. and Morikawa, K. (2002) 'Structure and function of nucleases in DNA repair: shape, grip and blade of the DNA scissors', *Oncogene*, 21(58), pp. 9022-9032.

Okshevsky, M. and Meyer, R.L. (2014) 'Evaluation of fluorescent stains for visualizing extracellular DNA in biofilms', *J Microbiol Methods*, 105, pp. 102-4.

Okshevsky, M. and Meyer, R.L. (2015) 'The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms', *Crit Rev Microbiol*, 41(3), pp. 341-52.

Okshevsky, M., Regina, V.R. and Meyer, R.L. (2015) 'Extracellular DNA as a target for biofilm control', *Current Opinion in Biotechnology*, 33, pp. 73-80.

Orellana, M.V., Matrai, P.A., Leck, C., Rauschenberg, C.D., Lee, A.M. and Coz, E. (2011) 'Marine microgels as a source of cloud condensation nuclei in the high Arctic', *Proc Natl Acad Sci USA*, 108, pp. 13612-13617.

Ortega-Retuerta, E., Duarte, C.M. and Reche, I. (2010) 'Significance of bacterial activity for the distribution and dynamics of transparent exopolymer particles in the Mediterranean Sea', *Microbial ecology*, 59(4), pp. 808-818.

Ortega-Retuerta, E., Joux, F., Jeffrey, W.H. and Ghiglione, J.-F. (2013) 'Spatial variability of particle-attached and free-living bacterial diversity in surface waters from the Mackenzie River to the Beaufort Sea (Canadian Arctic)', *Biogeosciences*, 10(4), pp. 2747-2759.

Padilla, C.C., Ganesh, S., Gantt, S., Huhman, A., Parris, D.J., Sarode, N. and Stewart, F.J. (2015) 'Standard filtration practices may significantly distort planktonic microbial diversity estimates', *Frontiers in microbiology*, 6, p. 547.

Pakkulnan, R., Anutrakunchai, C., Kanthawong, S., Taweechaisupapong, S., Chareonsudjai, P. and Chareonsudjai, S. (2019) 'Extracellular DNA facilitates bacterial adhesion during Burkholderia pseudomallei biofilm formation', *PloS one*, 14(3), p. e0213288.

Palmer, L., Chapple, I., Wright, H., Roberts, A. and Cooper, P. (2012) 'Extracellular deoxyribonuclease production by periodontal bacteria', *Journal of periodontal research*, 47(4), pp. 439-445.

Paramor, O., Allen, K.A., Aanesen, M, Armstrong, C., Hegland, T., Le Quesne, W., Piet, G., Raakjær, J., Rogers, S., Hal, R., Van Hoof, L., Oversee, H.M.J. Frid, C (2009) 'North Sea Atlas'.

Parab, P., Khandeparker, R., Amberkar, U. and Khodse, V. (2017) 'Enzymatic saccharification of seaweeds into fermentable sugars by xylanase from marine Bacillus sp. strain BT21', *3 Biotech*, 7(5), p. 296.

Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. (2014) 'STAMP: statistical analysis of taxonomic and functional profiles', *Bioinformatics*, 30(21), pp. 3123-3124.

Parret, A.H. and De Mot, R. (2002) 'Bacteria killing their own kind: novel bacteriocins of Pseudomonas and other γ-proteobacteria', *Trends in microbiology*, 10(3), pp. 107-112.

Passow, U. (2000) 'Formation of transparent exopolymer particles, TEP, from dissolved precursor material', *Marine ecology-progress series*, 192, pp. 1-11.

Passow, U. (2002a) 'Production of transparent exopolymer particles (TEP) by phyto-and bacterioplankton', *Marine ecology-progress series*, 236, pp. 1-12.

Passow, U. (2002b) 'Transparent exopolymer particles (TEP) in aquatic environments', *Progress in oceanography*, 55(3), pp. 287-333.

Passow, U. and Alldredge, A. (1994) 'Distribution, size and bacterial colonization of transparent exopolymer particles (TEP) in the ocean', *Marine Ecology Progress Series*, pp. 185-198.

Passow, U. and Alldredge, A.L. (1995) 'A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP)', *Limnol Oceanogr*, 40, pp. 1326-1335.

Passow, U. and Carlson, C.A. (2012) 'The biological pump in a high CO_2 world', *Marine Ecology Progress Series*, 470, pp. 249-271.

Paul, J.H., Jeffrey, W.H., David, A.W., DeFlaun, M.F. and Cazares, L.H. (1989) 'Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida', *Appl. Environ. Microbiol.*, 55(7), pp. 1823-1828.

Paul, J.H., Jeffrey, W.H. and DeFlaun, M.F. (1987) 'Dynamics of extracellular DNA in the marine environment', *Applied and Environmental Microbiology*, 53(1), pp. 170-179.

Pelve, E.A., Fontanez, K.M. and DeLong, E.F. (2017) 'Bacterial Succession on Sinking Particles in the Ocean's Interior', *Frontiers in microbiology*, 8, p. 2269.

Perepelov, A.V., Shashkov, A.S., Torgov, V.I., Nazarenko, E.L., Gorshkova, R.P., Ivanova, E.P., Gorshkova, N.M. and Widmalm, G. (2005) 'Structure of an acidic polysaccharide from the agar-decomposing marine bacterium Pseudoalteromonas atlantica strain IAM 14165 containing 5, 7-diacetamido-3, 5, 7, 9-tetradeoxy-l-glycero-l-manno-non-2-ulosonic acid', *Carbohydrate research*, *340*(1), pp.69-74.

Philip, F., Ha, E.E., Seeliger, M.A. and Frohman, M.A. (2017) 'Chapter Thirteen - Measuring Phospholipase D Enzymatic Activity Through Biochemical and Imaging Methods', in Gelb, M.H. (ed.) *Methods in Enzymology*. Academic Press, pp. 309-325.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M.T., Guerri, G. and Nannipieri, P. (2009) 'Extracellular DNA in soil and sediment: fate and ecological relevance', *Biology and Fertility of Soils*, 45(3), pp. 219-235.

Pinu, F. and Villas-Boas, S. (2017) 'Extracellular microbial metabolomics: The state of the art', *Metabolites*, 7(3), p. 43.

Ploeger, L., Dullens, H., Huisman, A. and van Diest, P. (2008) 'Fluorescent stains for quantification of DNA by confocal laser scanning microscopy in 3-D', *Biotechnic & histochemistry*, 83(2), pp. 63-69.

Ploug, H. and Grossart, H.P. (2000) 'Bacterial growth and grazing on diatom aggregates: Respiratory carbon turnover as a function of aggregate size and sinking velocity', *Limnology and Oceanography*, 45(7), pp. 1467-1475.

Porschen, R.K. and Sonntag, S. (1974) 'Extracellular deoxyribonuclease production by anaerobic bacteria', *Applied microbiology*, 27(6), pp. 1031-1033.

Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S. and Qu, D. (2007) 'Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis', *Microbiology*, 153(7), pp. 2083-2092.

Quinn, P., Coffman, D., Johnson, J., Upchurch, L. and Bates, T. (2017) 'Small fraction of marine cloud condensation nuclei made up of sea spray aerosol', *Nature Geoscience*, 10(9), p. 674.

Ranjan, R., Rani, A., Metwally, A., McGee, H.S. and Perkins, D.L. (2016) 'Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing', *Biochemical and biophysical research communications*, 469(4), pp. 967-977.

Rastelli, E., Corinaldesi, C., Dell'Anno, A., Martire, M.L., Greco, S., Facchini, M.C., Rinaldi, M., O'dowd, C., Ceburnis, D. and Danovaro, R. (2017) 'Transfer of labile organic matter and microbes from the ocean surface to the marine aerosol: an experimental approach', *Scientific reports*, 7(1), p. 11475.

Rath, J., Wu, K.Y., Herndl, G.J. and DeLong, E.F. (1998) 'High phylogenetic diversity in a marine-snow-associated bacterial assemblage', *Aquatic Microbial Ecology*, 14(3), pp. 261-269.

Reichhardt, C. and Parsek, M. (2019) 'Confocal laser scanning microscopy for analysis of Pseudomonas aeruginosa biofilm architecture and matrix localization', *Frontiers in microbiology*, 10, p. 677.

Rieck, A., Herlemann, D.P., Jürgens, K. and Grossart, H.-P. (2015) 'Particle-associated differ from free-living bacteria in surface waters of the Baltic Sea', *Frontiers in microbiology*, 6, p. 1297.

Rier, S.T., Shirvinski, J.M. and Kinek, K.C. (2014) 'In situ light and phosphorus manipulations reveal potential role of biofilm algae in enhancing enzyme-mediated decomposition of organic matter in streams', *Freshwater biology*, 59(5), pp. 1039-1051.

Robinson, C., Wallace, D., Hyun, J.-H., Polimene, L., Benner, R., Zhang, Y., Cai, R., Zhang, R. and Jiao, N. (2018) 'An implementation strategy to quantify the marine microbial carbon pump and its sensitivity to global change', *National Science Review*, 5(4), pp. 474-480.

Roca, C., Lehmann, M., Torres, C.A., Baptista, S., Gaudêncio, S.P., Freitas, F. and Reis, M.A. (2016) 'Exopolysaccharide production by a marine Pseudoalteromonas sp. strain isolated from Madeira Archipelago ocean sediments', *New biotechnology*, 33(4), pp. 460-466.

Rostami, N., Shields, R.C., Yassin, S.A., Hawkins, A.R., Bowen, L., Luo, T.L., Rickard, A.H., Holliday, R., Preshaw, P.M. and Jakubovics, N.S. (2016) 'A Critical Role for Extracellular DNA in Dental Plaque Formation', *Journal of Dental Research*, p. 0022034516675849.

Salazar, G., Cornejo-Castillo, F.M., Benítez-Barrios, V., Fraile-Nuez, E., Álvarez-Salgado, X.A., Duarte, C.M., Gasol, J.M. and Acinas, S.G. (2016) 'Global diversity and biogeography of deep-sea pelagic prokaryotes', *The ISME journal*, 10(3), p. 596.

Salazar, G. and Sunagawa, S. (2017) 'Marine microbial diversity', *Current Biology*, 27(11), pp. R489-R494.

Salter, I. (2018) 'Seasonal variability in the persistence of dissolved environmental DNA (eDNA) in a marine system: The role of microbial nutrient limitation', *PloS one*, 13(2), p. e0192409.

Samo, T.J., Malfatti, F. and Azam, F. (2008) 'A new class of transparent organic particles in seawater visualized by a novel fluorescence approach', *Aquatic Microbial Ecology*, 53(3), pp. 307-321.

Santos, N.C., Silva, A.C., Castanho, M.A.R.B., Martins-Silva, J. and Saldanha, C. (2003) 'Evaluation of lipopolysaccharide aggregation by light scattering spectroscopy', *ChemBioChem*, 4(1), pp. 96-100.

Santschi, P.H. (2018) 'Marine colloids, agents of the self-cleansing capacity of aquatic systems: Historical perspective and new discoveries', *Marine Chemistry*, 207, pp. 124-135.

Schatz, D. and Vardi, A. (2018) 'Extracellular vesicles—new players in cell–cell communication in aquatic environments', *Current opinion in microbiology*, 43, pp. 148-154.

Schlafer, S. and Meyer, R.L. (2017) 'Confocal microscopy imaging of the biofilm matrix', *Journal of microbiological methods*, 138, pp. 50-59.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H. and Robinson, C.J. (2009) 'Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities', *Appl. Environ. Microbiol.*, 75(23), pp. 7537-7541.

Seeman, N.C. (2007) 'An overview of structural DNA nanotechnology', *Molecular biotechnology*, 37(3), p. 246.

Seper, A., Hosseinzadeh, A., Gorkiewicz, G., Lichtenegger, S., Roier, S., Leitner, D.R., Röhm, M., Grutsch, A., Reidl, J. and Urban, C.F. (2013) 'Vibrio cholerae evades neutrophil extracellular traps by the activity of two extracellular nucleases', *PLoS pathogens*, 9(9), p. e1003614.

Sevigny, J.L., Rothenheber, D., Diaz, K.S., Zhang, Y., Agustsson, K., Bergeron, R.D. and Thomas, W.K. (2019) 'Marker genes as predictors of shared genomic function', *BMC genomics*, 20(1), p. 268.

Shahadat, M., Teng, T.T., Rafatullah, M., Shaikh, Z.A., Sreekrishnan, T.R. and Ali, S.W. (2017) 'Bacterial bioflocculants: A review of recent advances and perspectives', *Chemical Engineering Journal*, 328(Supplement C), pp. 1139-1152.

Shakir, A., ElBadawey, M.R., Shields, R.C., Jakubovics, N.S. and Burgess, J.G. (2012) 'Removal of Biofilms from Tracheoesophageal Speech Valves Using a Novel Marine Microbial Deoxyribonuclease', *Otolaryngology-Head and Neck Surgery*, 147(3), pp. 509-514.

Shamim, K., Mujawar, S.Y. and Mutnale, M. (2019) 'Metagenomics a modern approach to reveal the secrets of unculturable microbes', in *Advances in Biological Science Research*. Elsevier, pp. 177-195.

Shammi, M., Pan, X., Mostofa, K.M., Zhang, D. and Liu, C.-Q. (2017) 'Photo-flocculation of microbial mat extracellular polymeric substances and their transformation into transparent exopolymer particles: Chemical and spectroscopic evidences', *Scientific Reports*, 7(1), p. 9074.

Shanks, A.L. (2002) 'The abundance, vertical flux, and still-water and apparent sinking rates of marine snow in a shallow coastal water column', *Continental Shelf Research*, 22(14), pp. 2045-2064.

Sharp, C., Bray, J., Housden, N.G., Maiden, M.C. and Kleanthous, C. (2017) 'Diversity and distribution of nuclease bacteriocins in bacterial genomes revealed using Hidden Markov Models', *PLoS computational biology*, 13(7), p. e1005652.

Sheng, G.-P., Yu, H.-Q. and Li, X.-Y. (2010) 'Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review', *Biotechnology advances*, 28(6), pp. 882-894.

Shi, L., Huang, Y., Zhang, M., Shi, X., Cai, Y., Gao, S., Tang, X., Chen, F., Lu, Y. and Kong, F. (2018) 'Large buoyant particles dominated by cyanobacterial colonies harbor distinct bacterial communities from small suspended particles and free-living bacteria in the water column', *MicrobiologyOpen*, 7(6), p. e00608.

Shields, R.C., Mokhtar, N., Ford, M., Hall, M.J., Burgess, J.G., ElBadawey, M.R. and Jakubovics, N.S. (2013) 'Efficacy of a marine bacterial nuclease against biofilm forming microorganisms isolated from chronic rhinosinusitis', *PloS one*, 8(2).

Shiu, R.F., Chin, W.C. and Lee, C.L. (2014) 'Carbonaceous particles reduce marine microgel formation', *Sci Rep*, 4, p. 5856.

Simon, M., Grossart, H.-P., Schweitzer, B. and Ploug, H. (2002) 'Microbial ecology of organic aggregates in aquatic ecosystems', *Aquatic Microbial Ecology*, 28(2), pp. 175-211.

Sintes, E., Stoderegger, K., Parada, V. and Herndl, G.J. (2010) 'Seasonal dynamics of dissolved organic matter and microbial activity in the coastal North Sea', *Aquatic Microbial Ecology*, 60(1), pp. 85-95.

Smith, D.C., Simon, M., Alldredge, A.L. and Azam, F. (1992) 'Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution', *Nature*, 359(6391), pp. 139-142.

Smith, D.C., Steward, G.F., Long, R.A. and Azam, F. (1995) 'Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm', *Deep Sea Res Part 2 Top Stud Oceanogr*, 42, pp. 75-97.

Smith, M.W., Zeigler Allen, L., Allen, A.E., Herfort, L. and Simon, H.M. (2013) 'Contrasting genomic properties of free-living and particle-attached microbial assemblages within a coastal ecosystem', *Frontiers in microbiology*, 4, p. 120.

Smith, P., Hancock, G. and Rhoden, D. (1969) 'Improved medium for detecting deoxyribonuclease-producing bacteria', *Applied microbiology*, 18(6), pp. 991-993.

Sosa, O.A., Repeta, D.J., Ferrón, S., Bryant, J.A., Mende, D.R., Karl, D. and DeLong, E.F. (2017) 'Isolation and characterization of bacteria that degrade phosphonates in marine dissolved organic matter', *Frontiers in microbiology*, 8, p. 1786.

Stoderegger, K.E. and Herndl, G.J. (1999) 'Production of exopolymer particles by marine bacterioplankton under contrasting turbulence conditions', *Marine Ecology Progress Series*, pp. 9-16.

Stone, R. (2010) 'The invisible hand behind a vast carbon reservoir'. American Association for the Advancement of Science.

Summers, S., Henry, T. and Gutierrez, T. (2018) 'Agglomeration of nano- and microplastic particles in seawater by autochthonous and de novo-produced sources of exopolymeric substances', *Marine Pollution Bulletin*, 130, pp. 258-267.

Sun, L., Chin, W.-C., Chiu, M.-H., Xu, C., Lin, P., Schwehr, K.A., Quigg, A. and Santschi, P.H. (2019) 'Sunlight induced aggregation of dissolved organic matter: Role of proteins in linking organic carbon and nitrogen cycling in seawater', *Science of The Total Environment*, 654, pp. 872-877.

Sun, L., Xu, C., Zhang, S., Lin, P., Schwehr, K.A., Quigg, A., Chiu, M.-H., Chin, W.-C. and Santschi, P.H. (2017a) 'Light-induced aggregation of microbial exopolymeric substances', *Chemosphere*, 181, pp. 675-681.

Sun, P., Zhang, J., Esquivel-Elizondo, S., Ma, L. and Wu, Y. (2017b) 'Uncovering the flocculating potential of extracellular polymeric substances produced by periphytic biofilms', *Bioresource Technology*.

Sunagawa, S., Coelho, L.P., Chaffron, S., Kultima, J.R., Labadie, K., Salazar, G., Djahanschiri, B., Zeller, G., Mende, D.R. and Alberti, A. (2015) 'Structure and function of the global ocean microbiome', *Science*, 348(6237), p. 1261359.

Suzuki, H., Daimon, M., Awano, T., Umekage, S., Tanaka, T. and Kikuchi, Y. (2009) 'Characterization of extracellular DNA production and flocculation of the marine photosynthetic bacterium Rhodovulum sulfidophilum', *Applied microbiology and biotechnology*, 84(2), pp. 349-356.

Tani, K. and Nasu, M. (2010) 'Roles of extracellular DNA in bacterial ecosystem', in *Extracellular nucleic acids*. Springer, pp. 25-37.

Tansel, B. (2018) 'Morphology, composition and aggregation mechanisms of soft bioflocs in marine snow and activated sludge: A comparative review', *Journal of Environmental Management*, 205(Supplement C), pp. 231-243.

Taylor, J.D., Bird, K.E., Widdicome, C.E. and Cunliffe, M. (2018) 'Active bacterioplankton community response to dissolved ‘free’deoxyribonucleic acid (dDNA) in surface coastal marine waters', *FEMS microbiology ecology*, 94(10), p. fiy132.

Teeling, H., Fuchs, B.M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C.M., Kassabgy, M., Huang, S., Mann, A.J. and Waldmann, J. (2012a) 'Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom', *Science*, 336(6081), pp. 608-611.

Teeling, H., Fuchs, B.M., Bennke, C.M., Krueger, K., Chafee, M., Kappelmann, L., Reintjes, G., Waldmann, J., Quast, C. and Gloeckner, F.O. (2016) 'Recurring patterns in bacterioplankton dynamics during coastal spring algae blooms', *Elife*, 5, p. e11888.

Tetz, G.V., Artemenko, N.K. and Tetz, V.V. (2009) 'Effect of DNase and Antibiotics on Biofilm Characteristics', *Antimicrobial Agents and Chemotherapy*, 53(3), pp. 1204-1209.

Thiele, S., Fuchs, B.M., Amann, R. and Iversen, M.H. (2015) 'Colonization in the photic zone and subsequent changes during sinking determine bacterial community composition in marine snow', *Appl. Environ. Microbiol.*, 81(4), pp. 1463-1471.

Thill, A., Veerapaneni, S., Simon, B., Wiesner, M., Bottero, J. and Snidaro, D. (1998) 'Determination of structure of aggregates by confocal scanning laser microscopy', *Journal of colloid and interface science*, 204(2), pp. 357-362.

Thornton, D.C. (2004) 'Formation of transparent exopolymeric particles (TEP) from macroalgal detritus', *Marine Ecology Progress Series*, 282, pp. 1-12.

Thornton, D.C.O. (2018) 'Coomassie Stainable Particles (CSP): Protein Containing Exopolymer Particles in the Ocean', *Frontiers in Marine Science*, 5(206).

Thuy, N.T., Huang, C.-P. and Lin, J.-L. (2017) 'Visualization and quantification of transparent exopolymer particles (TEP) in freshwater using an auto-imaging approach', *Environmental Science and Pollution Research*, 24(21), pp. 17358-17372.

Torti, A., Jorgensen, B.B. and Lever, M.A. (2018) 'Preservation of microbial DNA in marine sediments: insights from extracellular DNA pools', *Environmental Microbiology*, 20(12), pp. 4526-4542.

Torti, A., Lever, M.A. and Jørgensen, B.B. (2015) 'Origin, dynamics, and implications of extracellular DNA pools in marine sediments', *Marine Genomics*, 24, Part 3, pp. 185-196.

Traving, S.J., Thygesen, U.H., Riemann, L. and Stedmon, C.A. (2015) 'A model of extracellular enzymes in free-living microbes: which strategy pays off?', *Applied and environmental microbiology*, pp. AEM. 02070-15.

Tredwell, G.D., Edwards-Jones, B., Leak, D.J. and Bundy, J.G. (2011) 'The development of metabolomic sampling procedures for Pichia pastoris, and baseline metabolome data', *PloS one*, 6(1), p. e16286.

Tully, B.J., Sachdeva, R., Heidelberg, K.B. and Heidelberg, J.F. (2014) 'Comparative genomics of planktonic Flavobacteriaceae from the Gulf of Maine using metagenomic data', *Microbiome*, 2(1), p. 34.

Tully, B.J., Wheat, C.G., Glazer, B.T. and Huber, J.A. (2018) 'A dynamic microbial community with high functional redundancy inhabits the cold, oxic subseafloor aquifer', *The ISME journal*, 12(1), p. 1.

Tutino, M.L., Parrilli, E., Giaquinto, L., Duilio, A., Sannia, G., Feller, G. and Marino, G. (2002) 'Secretion of alpha-amylase from Pseudoalteromonas haloplanktis TAB23: Two different pathways in different hosts', *Journal of Bacteriology*, 184(20), pp. 5814-5817.

Vafina, G., Zainutdinova, E., Bulatov, E. and Filimonova, M.N. (2018) 'Endonuclease from Gram-Negative Bacteria Serratia marcescens Is as Effective as Pulmozyme in the Hydrolysis of DNA in Sputum', *Frontiers in pharmacology*, 9, p. 114.

Veening, J.-W. and Blokesch, M. (2017) 'Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria', *Nature Reviews Microbiology*, 15(10), p. 621.

Verdugo, P. (2007) 'Dynamics of marine biopolymer networks', *Polymer Bulletin*, 58(1), pp. 139-143.

Verdugo, P. (2012) 'Marine microgels', *Annual Review of Marine Science*, 4, pp. 375-400.

Verdugo, P., Alldredge, A.L., Azam, F., Kirchman, D.L., Passow, U. and Santschi, P.H. (2004) 'The oceanic gel phase: A bridge in the DOM-POM continuum', *Marine Chemistry*, 92(1-4 SPEC. ISS.), pp. 67-85.

Verdugo, P. and Santschi, P.H. (2010) 'Polymer dynamics of DOC networks and gel formation in seawater', *Deep Sea Research Part II: Topical Studies in Oceanography*, 57(16), pp. 1486-1493.

Vetter, Y.A. and Deming, J.W. (1999) 'Growth rates of marine bacterial isolates on particulate organic substrates solubilized by freely released extracellular enzymes', *Microbial ecology*, 37(2), pp. 86-94.

Vilain, S., Pretorius, J.M., Theron, J. and Brözel, V.S. (2009) 'DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms', *Appl. Environ. Microbiol.*, 75(9), pp. 2861-2868.

Villacorte, L.O., Ekowati, Y., Calix-Ponce, H.N., Schippers, J.C., Amy, G.L. and Kennedy, M.D. (2015) 'Improved method for measuring transparent exopolymer particles (TEP) and their precursors in fresh and saline water', *water research*, 70, pp. 300-312.

Vlassov, V.V., Laktionov, P.P. and Rykova, E.Y. (2007) 'Extracellular nucleic acids', *Bioessays*, 29(7), pp. 654-67.

Vorkapic, D., Pressler, K. and Schild, S. (2016) 'Multifaceted roles of extracellular DNA in bacterial physiology', *Current genetics*, 62(1), pp. 71-79.

Waite, A.M., Safi, K.A., Hall, J.A. and Nodder, S.D. (2000) 'Mass sedimentation of picoplankton embedded in organic aggregates', *Limnology and Oceanography*, 45(1), pp. 87-97.

Walday, M. and Kroglund, T. (2002) '*Europe's Biodiversity-biogeographical Regions and Seas*', *Seas Around Europe: The Baltic Sea-the Largest Brackish Sea in the World*. European Environment Agency.

Wang, L. (2018) 'Microbial control of the carbon cycle in the ocean', *National Science Review*, 5(2), pp. 287-291.

Wasmund, K., Pelikan, C., Watzka, M., Richter, A., Noel, A.C., Hubert, C.R., Rattei, T., Hofmann, T., Herbold, C.W. and Loy, A. (2019) 'DNA-foraging bacteria in the seafloor', *bioRxiv*, p. 528695.

Watanabe, M., Sasaki, K., Nakashimada, Y., Kakizono, T., Noparatnaraporn, N. and Nishio, N. (1998) 'Growth and flocculation of a marine photosynthetic bacterium Rhodovulum sp', *Applied microbiology and biotechnology*, 50(6), pp. 682-691.

Watanabe, M., Suzuki, Y., Sasaki, K., Nakashimada, Y. and Nishio, N. (1999) 'Flocculating property of extracellular polymeric substance derived from a marine photosynthetic bacterium, Rhodovulum sp', *Journal of Bioscience and Bioengineering*, 87(5), pp. 625-629.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and Mattick, J.S. (2002) 'Extracellular DNA required for bacterial biofilm formation', *Science*, 295(5559), pp. 1487-1487.

Whittaker, R.H. (1977) 'Evolution of species diversity in land communities', *Evol. Biol.*, 10, pp. 1-67.

Wilson, T.W., Ladino, L.A., Alpert, P.A., Breckels, M.N., Brooks, I.M., Browse, J., Burrows, S.M., Carslaw, K.S., Huffman, J.A. and Judd, C. (2015) 'A marine biogenic source of atmospheric ice-nucleating particles', *Nature*, 525(7568), p. 234.

Worden, A.Z., Follows, M.J., Giovannoni, S.J., Wilken, S., Zimmerman, A.E. and Keeling, P.J. (2015) 'Rethinking the marine carbon cycle: factoring in the multifarious lifestyles of microbes', *Science*, 347(6223), p. 1257594.

Wurl, O., Miller, L. and Vagle, S. (2011) 'Production and fate of transparent exopolymer particles in the ocean', *Journal of Geophysical Research: Oceans*, 116(C7).

Wurl, O., Stolle, C., Van Thuoc, C., Thu, P.T. and Mari, X. (2016) 'Biofilm-like properties of the sea surface and predicted effects on air–sea CO 2 exchange', *Progress in Oceanography*, 144, pp. 15-24.

Xavier, M., Passow, U., Migon, C., Burd, A.B. and Legendre, L. (2016) 'Transparent Exopolymer Particles: Effects on carbon cycling in the ocean', *Progress in Oceanography*.

Xu, C., Chin, W.-C., Lin, P., Chen, H., Chiu, M.-H., Waggoner, D.C., Xing, W., Sun, L., Schwehr, K.A. and Hatcher, P.G. (2019) 'Comparison of microgels, extracellular polymeric substances (EPS) and transparent exopolymeric particles (TEP) determined in seawater with and without oil', *Marine Chemistry*, p. 103667.

Yamada, Y., Fukuda, H., Inoue, K., Kogure, K. and Nagata, T. (2013) 'Effects of attached bacteria on organic aggregate settling velocity in seawater', *Aquatic Microbial Ecology*, 70(3), pp. 261-272.

Yamada, Y., Fukuda, H., Tada, Y., Kogure, K. and Nagata, T. (2016) 'Bacterial enhancement of gel particle coagulation in seawater', *Aquatic Microbial Ecology*, 77(1), pp. 11-22.

Yawata, Y., Cordero, O.X., Menolascina, F., Hehemann, J.-H., Polz, M.F. and Stocker, R. (2014) 'Competition–dispersal tradeoff ecologically differentiates recently speciated marine bacterioplankton populations', *Proceedings of the National Academy of Sciences*, p. 201318943.

Yildiz, F.H. and Visick, K.L. (2009) 'Vibrio biofilms: so much the same yet so different', *Trends in microbiology*, 17(3), pp. 109-118.

Yuan, B., Wang, X., Tang, C., Li, X. and Yu, G. (2015) 'In situ observation of the growth of biofouling layer in osmotic membrane bioreactors by multiple fluorescence labeling and confocal laser scanning microscopy', *Water research*, 75, pp. 188-200.

Yung, C.-M., Ward, C.S., Davis, K.M., Johnson, Z.I. and Hunt, D.E. (2016) 'Insensitivity of diverse and temporally variable particle-associated microbial communities to bulk seawater environmental parameters', *Appl. Environ. Microbiol.*, 82(11), pp. 3431-3437.

Zeng, R.Y., Xiong, P.J. and Wen, J.J. (2006) 'Characterization and gene cloning of a cold-active cellulase from a deep-sea psychrotrophic bacterium Pseudoalteromonas sp DY3', *Extremophiles*, 10(1), pp. 79-82.

Zhang, R., Neu, T.R., Zhang, Y., Bellenberg, S., Kuhlicke, U., Li, Q., Sand, W. and Vera, M. (2015) 'Visualization and analysis of EPS glycoconjugates of the thermoacidophilic archaeon Sulfolobus metallicus', *Applied microbiology and biotechnology*, 99(17), pp. 7343-7356.

Zhang, Y., Xiao, W. and Jiao, N. (2016) 'Linking biochemical properties of particles to particle-attached and free-living bacterial community structure along the particle density gradient from freshwater to open ocean', *Journal of Geophysical Research: Biogeosciences*, 121(8), pp. 2261-2274.

Ziervogel, K., Steen, A. and Arnosti, C. (2010) 'Changes in the spectrum and rates of extracellular enzyme activities in seawater following aggregate formation', *Biogeosciences*, 7(3), pp. 1007-1015.

Appendices

Appendix A

I. The effect of nucleases on the disintegration of natural MGPs

DNase I against Natural MGPs

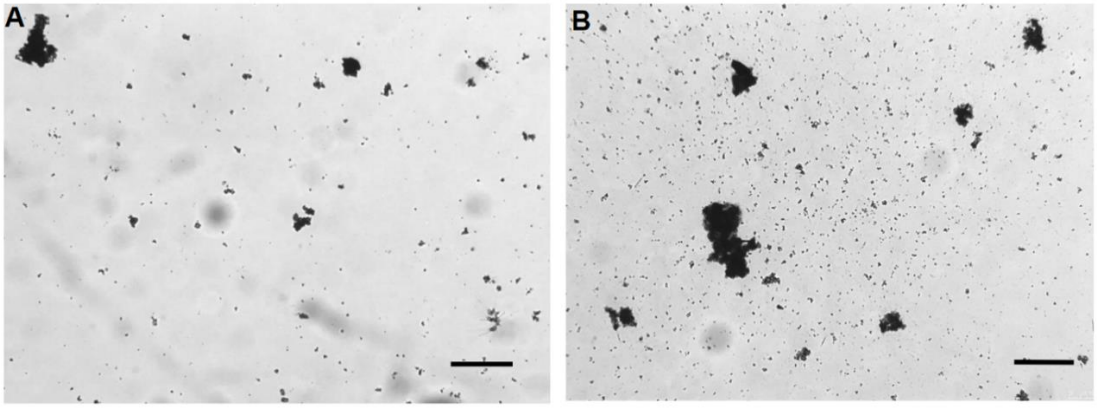

Figure 1 The first observations of the effect of DNase I on natural MGP 0-24 hours (A) and after 120 hours (B). Scale bar represents 50 µm.

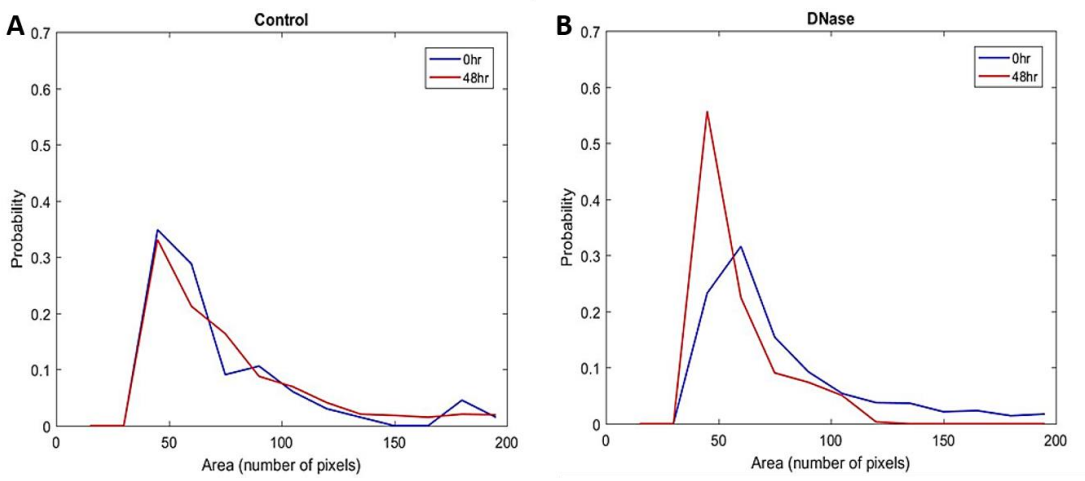

Figure 2 MATLAB analysis of marine aggregates incubated in sterile MilliQ water as a control (A) and in DNase I (B).

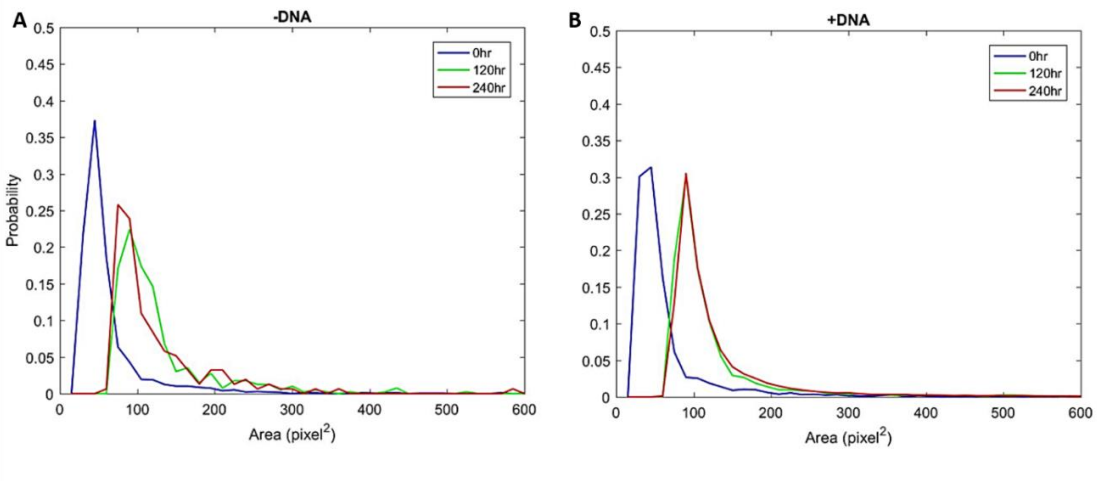

Figure 3 Filtered seawater at 0.4 µm incubated with bacterial DNA 1 mg/ ml.

1. MNase against natural MGPs

A time-lapse image using CLSM (Leica SP8) was acquired to investigate the dispersal of MGP by the addition of MNase (2x 10^6 gel U) at 37ºC. This resulted in no change in the particles over the 18-hour experiment. This would be due to deactivation of the enzyme by seawater salts.

2. MNase against *Pseudoalteromonas atlantica* EPS

Preliminary results of the effect of MNase on the dispersal of *P. atlantica* EPS aggregates.

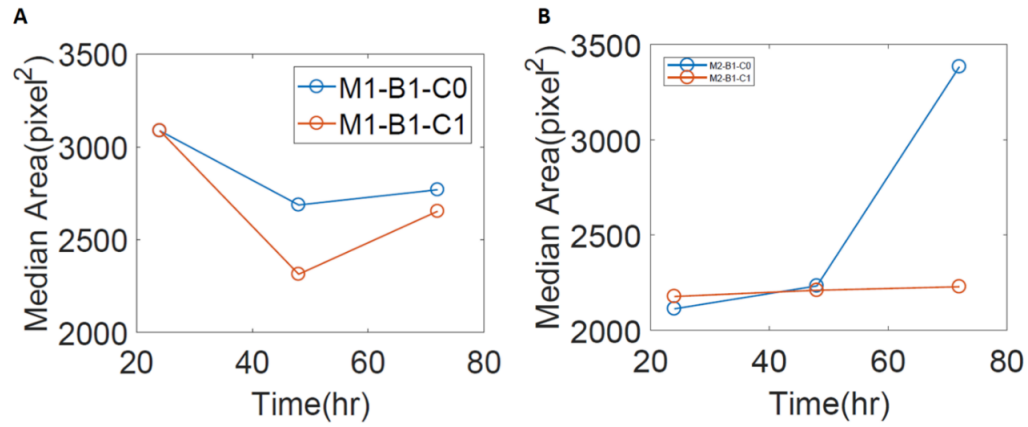

Figure 4 *P. atlantica* EPS (MGP model) in artificial seawater incubated with C0= no enzyme and C1= 200 gel U/ ml (A) and *P. atlantica* EPS (MGP model) incubated in 0.2 µm filter sterile MilliQ water with C0= no enzyme and C1= 200 gel U/ ml.

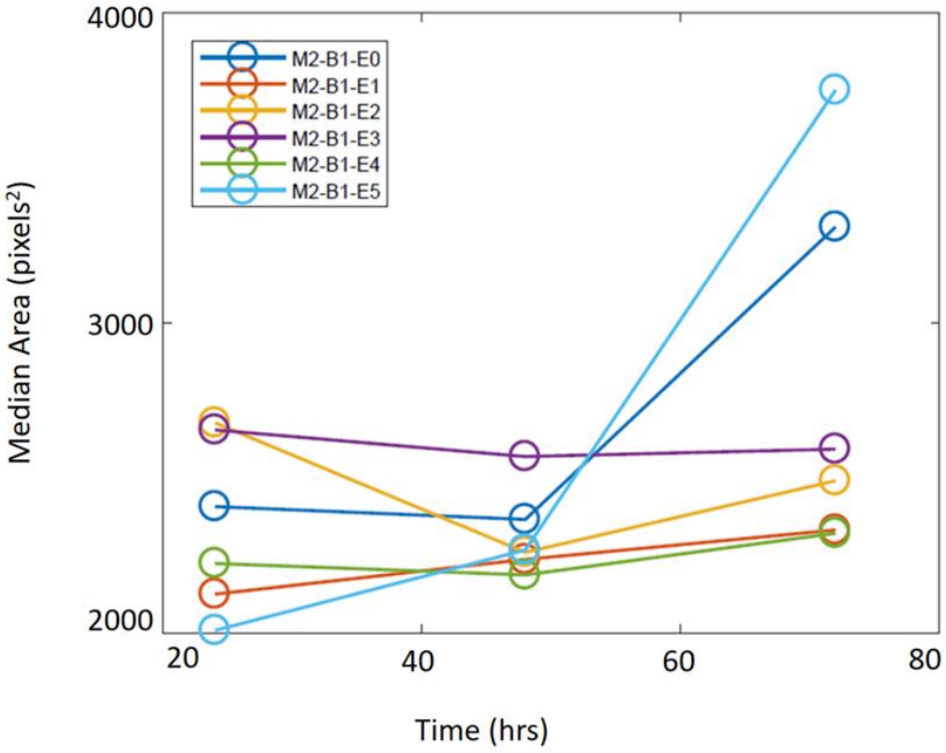

Figure 5 *P. atlantica* EPS (MGP model) incubated with: E0= MilliQ water, E1= MNase 2x106 gel U/ ml, E2= G22 (supernatant of DNase active bacteria isolated from MGPs), E3= X2 (supernatant of DNase active bacteria isolated from MGPs), E4= AW2 supernatant of DNase active bacteria isolated from marine sediments, E5= *P. atlantica* in 0.2 µm filter sterile MilliQ water.