Microbiology of Salt Marshes in the UK: Insights into Life in Gradient-Rich Environments

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This thesis is submitted to Edge Hill University in partial fulfilment for the degree of Doctor of Philosophy

January 2021

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Presented for the degree of Doctor of Philosophy

Microbiology of Salt Marshes in the UK: Insights into Life in Gradient-Rich Environments

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Abstract

Gradual changes in physico-chemical factors occurring along spatial and temporal scales in different ecosystems are described as environmental gradients. Salt marshes, an example of such gradient-rich environments, are intertidal marshy zones characterised by continuous alternating tidal cycles. Such dynamic environments are suitable hotspots for studying microbial biodiversity. The microbes of these environments could be fruitful ecological or biotechnological resources of bioactive agents that remain functional even in extreme conditions, although there are only a limited number of cultured microbes described from salt marsh environments including those in the UK. Thus, the scientific and economic value of most of these marsh sites has largely remained unexplored.

This study aimed to explore culturable diversity, and novel and potentially beneficial microbes within salt marshes, alongside the development of a promising and rapid technique for the cultivation of microbes from gradient-rich environments. Samples were obtained from salt marshes at RSPB Marshside and Fingringhoe Wick Nature Reserve, situated at the geographically and climatically distinct North-West and South-East regions of England. Pure microbial isolates obtained via cultivation-based analysis were cryopreserved, phylogenetically identified using their 16S rRNA genes, and screened for novel species as well as antimicrobial production. A full characterisation of two of the novel isolates was performed through polyphasic tests and draft genome analysis. Additionally, based on the diffusion concept, 'gel-stabilised gradient plates' were developed to mimic the physico-chemical components of gradient-rich ecosystems.

This combined approach of cultivation- and molecular-based studies with biotechnological screening identified the cultivable microbial diversity and made the initial steps to describe this diversity in the salt marshes of the UK. The present study additionally suggested the antimicrobial production by the isolates and discovered the novel microbes *Halomonas aestuarensis* and *Pseudoalteromonas belisamaea* isolated from study marsh sites. The gel-stabilised gradient plate also confirmed its significance in direct and wider applications in microbial culturing.

Keywords: Environmental gradients, Salt marshes, Microbial cultivation, Microbial isolation, Gradient-plate diffusion

Chapter 1

Introduction

1.1 ENVIRONMENTAL GRADIENTS

Environmental gradients are described as gradual changes in physico-chemical factors (e.g. pH, salinity, temperature, humidity, oxygen saturation) which occur along spatial and temporal scales in different ecosystems. Typical examples of gradient-rich environments include coniferous forests and tropical forests (Baldeck *et al.* 2016), grasslands (Tardella *et al.* 2016), fire-dependent ecosystems (Kirkman *et al.* 2001), fresh water and coastal habitats (e.g. rivers, ponds, lagoons, estuaries, salt marshes, Bernhard *et al.* 2010; Cao *et al.* 2008), as well as extreme environments like geothermal lakes (Weltzer *et al.* 2013) and deep-sea brines (Antunes *et al.* 2011; Antunes *et al.* 2019).

Environmental gradients generated at these ecosystems through both biotic and abiotic interactions supply multiple substrates and favourable conditions for the subsistence and physiological functions of diverse communities and thus lead to their species' distribution, abundance and diversity (Smith *et al.* 2002; Marton *et al.* 2015; Doebeli and Dieckmann 2003; Namba *et al.* 2020). Such habitats also control global warming and environmental pollution (Lasco 1997; Nielsen *et al.* 2016; Lima *et al.* 2019) and maintain the recreational and aesthetic value of these ecosystems (Friess *et al.* 2020; Morgan *et al.* 2009; Millennium Ecosystem Assessment 2005). Gradient-rich environments are also hotspots of microbial diversity (Signori *et al.* 2014; Chen *et al.* 2017). The co-existence of diverse microorganisms within these environments supports elemental recycling (Geyer *et al.* 2017; Bernhard *et al.* 2010), bioremediation (Bender *et al.* 1995; Basu *et al.* 2017; Lima *et al.* 2019) and morphodynamics preservation (Stal *et al.* 1994; Van Gemerden 1993).

1.2 SALT MARSHES

Environmental gradient habitats across coastal areas such as salt marshes are known to have a significant influence on the marine ecosystem (Bolhuis *et al.* 2013; Gray 1992). Salt marshes occur worldwide, particularly in the middle to high latitudes, and are common habitats of estuaries. These are the coastal wetlands; upper coastal intertidal marshy zones which exist between open sea and land. Its components are tidal flats (mudflats), tidal creeks (channels), low marsh and high marsh areas (**Fig 1.1**), which are formed according to differences in elevation, varying salinity, and patterns of halophyte vegetation zones (Kim *et al.* 2010a; Brewer *et al.* 1998; Li and Pennings 2016).

Salt marshes are characterised by continuous alternating tidal cycles of flooding and drainage of sea water (Hemminga *et al.* 1993). With their alternating wet and desiccated phases, salt marshes encounter frequent environmental changes which create unique horizontal and vertical physico-chemical gradients across these habitats e.g., of salinity (Kim *et al.* 2012), pH (Nelson *et al.* 2009; Rey 1992), redox potential (Rand 2000) and moisture content (Leeuw *et al.* 1990). These sites are also described as a pronounced sink and a source of organic and inorganic components (Cook *et al.* 2004; Gallagher *et al.* 1974). Biochemical components deposited in salt marsh via native salt tolerant macrophytes (halophytes), algae, fungi and benthic animals, and by the ocean surge and upland water bodies are decomposed by microbial communities into inorganic, mineral forms, which are then released to coastal waters (Rocha 1998; Cook *et al.* 2004).

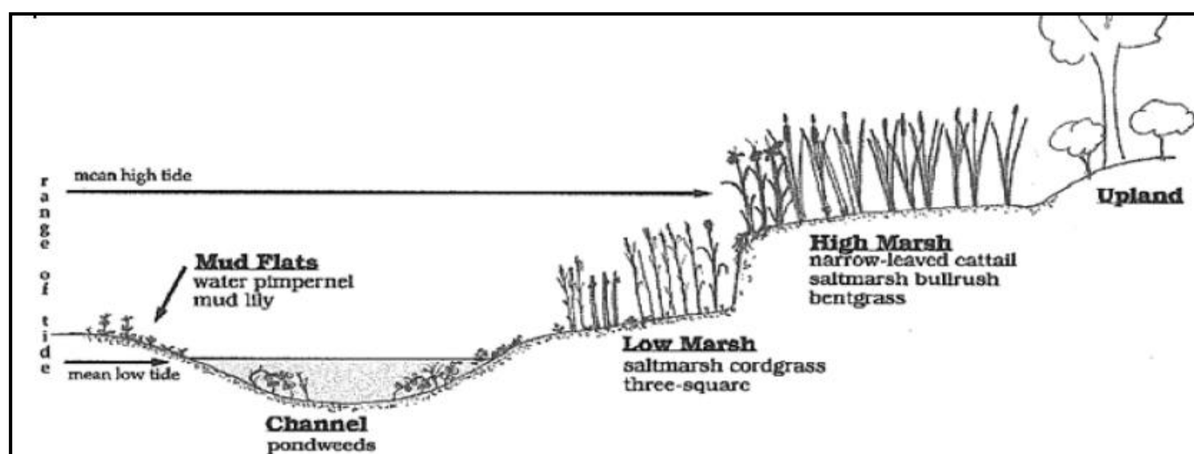

Figure 1.1 Salt marsh components - tidal flats (mudflats), tidal creeks (channels), low marsh and high marsh areas (Natural Heritage and Endangered Species programs, 2016, viewed December 2020)

1.3 SALT MARSH FUNCTIONS

The ecosystem service benefits of these intertidal habitats are numerous. As water flows though the salt marsh plants, it guards shorelines from erosion by buffering wave action and trapping sediments. Salt marshes protect the upper land from over-flooding by slowing and absorbing floodwater, maintain marine water quality by absorbing and filtering hazardous run-offs from upper land, reduce global warming, and have recreational value as well (Shepard *et al.* 2011; Van 1985). The various

gradients provide distinct niches which house different plants, animals and microbes (Wilson and Whittaker 1995; Gray 1992; Dini-Andreote *et al.* 2014).

The gradients generated at salt marshes shape the microbial diversity and abundance (Bolhuis *et al.* 2013; Webster *et al.* 2015; Nelson *et al.* 2009). Beneficial roles played by these microbes in salt marsh environments include plant growth promotion and protection against phytopathogenic bacteria (Rocha *et al.* 2016); bioremediation of contaminated coastal sites (Calvo *et al.* 2002; Beazley *et al.* 2012; McKew *et al.* 2007; Coulon *et al.* 2007); enriching marsh sediments with organic and inorganic nutrients which help to protect salt marsh morphodynamics and functionality; stabilisation of soil sediments (via e.g. extracellular substances secreted by microbes, Grant and Gust 1987); restricting soil erosion (Stal *et al.* 2010; Van Gemerden 1993); and promoting biogeochemical cycling that forms the basis of food chains existing among these areas (Chmura *et al.* 2003; Yakimov *et al.* 2007). Despite these benefits, salt marshes are facing a wide range of challenges worldwide (Oaten *et al* 2018; Burden *et al.* 2020; Phelan *et al.* 2011) and restoration and artificial re-creation programs are being conducted globally (Mossman *et al.* 2012).

1.4 SALT MARSH LITERATURE STUDIES

1.4.1 Literature Studies on Salt Marsh Microbial Diversity

Literatures studies reported in salt marshes, estuaries and other coastal environments have predominantly showed high occurrence of Proteobacteria (60–70%) and the remaining bacterial population of Firmicutes, Actinobacteria and Bacteroidetes based on both cultivation-dependant and molecular-based studies (Köpke *et al.* 2005; Fidalgo *et al.* 2016; Beazley *et al.* 2012; Johnston *et al.* 2017; Lydell *et al.* 2004). Firmicutes that exist in both the endospore and vegetative cell forms *in situ* (Berrada *et al.* 2012; Yilmaz *et al.* 2016; Wu *et al.* 2010) were reported to occur in varied proportion of <1% to >5% respectively (Filippidou *et al. 2015;* Cupit *et al. 2019;* Fidalgo *et al.* 2016; Berrada *et al.* 2012; Köpke *et al.* 2005). Actinobacteria and Bacteroidetes were also detected in lower proportions in the salt marsh microbial community (Fidalgo *et al.* 2016; Nimnoi and Pongsilp 2020; Suh *et al.* 2015; Zinger *et al.* 2011; Lydell *et al.* 2004).

Amongst the major group of Proteobacteria found in salt marshes and other coastal environments, large abundance of Alphaproteobacteria and Gammaproteobacteria

was observed in previous studies under diverse environmental conditions (Fidalgo *et al.* 2016; Webster *et al.* 2010; Beazley *et al.* 2012; Ansede *et al.* 2001; Cho and Giovannoni 2004; Zinger *et al.* 2011). Anoxic ecosystems (Zinger *et al.* 2011; Madrid *et al.* 2001), seagrass *Zostera noltii* colonised sediments (Cifuentes *et al.* 2000), and microcosm experiments reviewing anoxic microbial degradation of microphytobenthic biofilm-derived organic matter in intertidal sediments (McKew *et al.* 2013) revealed increased relative proportions of Deltaproteobacteria. Minor occurrence of Betaproteobacteria was also detected in coastal and estuarine waters (Zinger *et al.* 2011; Kerkhof *et al.* 1999; Suzuki *et al.* 1997).

The transient variations to these commonly found microbial diversity and assembly were observed with respect to different inputs to the sediments of salt marshes and associated environments. Dominance of Actinobacteria and increase in relative abundance of Firmicutes compared to Proteobacteria was observed in salt marsh microcosm experiments where microbial degradation of isoprene (Johnston *et al.* 2017) and microphytobenthic extracellular polymeric substances (in anoxic conditions, McKew *et al.* 2013) were tested individually as carbon and energy sources respectively. Abundant Firmicutes were noted amongst biomineralizing marine sediment microbes (Wei *et al.* 2015) and thermophilic bacterial population (Aanniz *et al.* 2015).

Previous salt marsh literature has also shown the interactions between microbes and various salt marsh components that contribute to the maintenance of these ecosystems. The beneficial interactions between salt marsh microbes with halophytic plants has been described (Martins 2011; Gayathri *et al.* 2010; Buchan *et al.* 2003) as has that between the microbes and benthic communities (McKew *et al.* 2013). Ecologically and biotechnologically significant microbial responses to anthropogenic activities like the external input of metals (Navarro-Torre *et al.* 2016; Rocha *et al.* 2016), polycyclic aromatic hydrocarbons (Daane *et al.* 2001; Beazley *et al.* 2012; Oliveira *et al.* 2014; McGenity 2014) and fertilisers (Peng *et al.* 2013; Bowen *et al.* 2009), as well as to that of biomineralisation (Wei *et al.* 2015) and hydrolytic enzyme production (Wei *et al.* 2018), have been noted. Salt marsh studies have also described the effects of salt marsh morphodynamics (Santos *et al.* 2007; Almeida *et al.* 2001), realigned management (Berrada *et al.* 2012), and physico-chemical conditions (Zahran *et al.* 1995; Aanniz *et al.* 2015) on microbial diversity and abundance.

The microbial consortium involved in biogeochemical cycles of salt marshes and other coastal wetlands has also been studied in the literature. The biogeochemical re-cycling processes driven by microbial communities in these environments is shown in **Fig. 1.2** (Sims *et al.* 2013; Oremland and Taylor 1978; Joye and Hollibaugh 1995) and introduced in the following paragraphs.

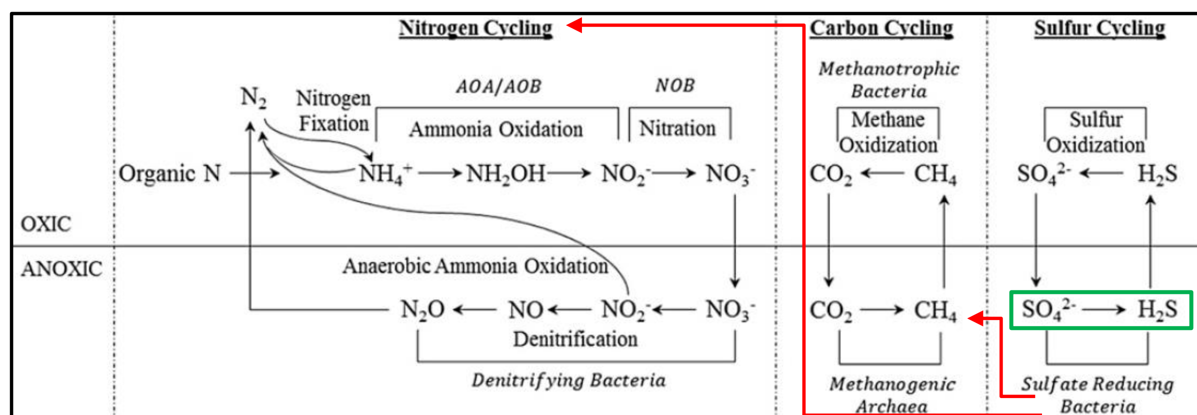

Figure 1.2 Part of microbe mediated biogeochemical cycles in hydric soils of salt marsh wetlands. Red arrows indicating inhibition of the carbon cycle (methanogenesis) and nitrogen cycle rates by sulphides (Sims *et al.* 2013; Oremland and Taylor 1978; Joye and Hollibaugh 1995)

Abbreviations: AOA = Ammonia oxidising archaea; AOB = Ammonia oxidising bacteria; NOB = Nitrite oxidising bacteria

1.4.1.1 Carbon cycle

Carbon released from decomposed organic waste is converted into methane (CH_4) by anaerobic respiring methanogenic archaea (e.g. *Methanococcus*, *Methanosarcina*, *Methanococcoides*, Purdy *et al.* 2002b) which is then used as an energy source by methanotrophs (e.g. *Methylosinus*). The comparison of methanogenesis in salt marshes with other ecosystems such as peatlands shows higher carbon accumulation (Chmura *et al.* 2003; Choi and Wang 2004) and negligible methane emission (Adams *et al.* 2012; Livesley *et al.* 2012). This is due to the presence of active sulphate reduction in coastal and salt marsh sediments, which suppresses methanogenesis and most members of the methanogenic microbial community (Senior *et al.* 1982). When methanogenic and sulphate reducing bacteria are growing on common substrates like acetate, hydrogen and CO_2, sulphate reducers outcompete methanogens. This occurs as sulphate

reducing bacteria grown thermodynamically and kinetically more favourably than the methanogens (Capone and Kiene 1988; Koizumi *et al.* 2003). In sulphate-rich tidal sediments of salt marsh environments, methanogens mainly rely on non-competitive substrates such as methanol, and methylated compounds (e.g. methylamines, methanethiol and dimethyl sulphide), so as not to be out-competed but continue their activity at low rates (Senior *et al.* 1982; Kiene 1988; Parkes *et al.* 2012). Previous studies showed that the methanogenic archaea community composition varies with environmental conditions as well as with soil organic carbon, sulphate and trimethylamine concentrations (Oliveira *et al.* 2014; Daane *et al.* 2001; Joye and Hollibaugh 1995; Capone and Kiene 1988).

1.4.1.2 Nitrogen cycle

The sequential oxidation of ammonia to nitrite and then to nitrate (nitrification) is the crucial step in the nitrogen cycle, which simultaneously channels some of the energy produced to fix CO_2 (Valiela and Teal 1979; Konneke *et al.* 2005). This process is carried out by ammonia oxidising Betaproteobacteria (e.g. *Nitrosomonas,* Peng *et al.* 2013) and Gammaproteobacteria (e.g. *Azotobacter*, Angermeyer 2016), as well as archaea (e.g. *Nitrososphaera* and *Nitrosopumilus*, Peng *et al.* 2013; Santoro *et al.* 2008). In coastal wetlands and other ecosystems, nitrification is followed by denitrification, resulting in the eventual return of nitrogen to the environment. Denitrification in salt marshes is mainly carried out by Gammaproteobacteria (e.g. *Pseudomonas denitrificans*) and *Bacillus thermodenitrificans* (Jenkins and Kemp 1984; Sebilo *et al.* 2006; Bagwell *et al.* 1998). In tidal wetland areas the fate of ammonia plays a vital role in nitrogen cycling, as the denitrification rates are greater, and the nitrogen is often the limiting element (Valiela and Teal 1979; Seitzinger *et al.* 1988; Howarth 1988). In estuarine environments, higher salinity and sulphate reduction also found to negatively affect the nitrogen dynamics (Joye and Hollibaugh 1995; Rysgaard *et al.* 1999; Giblin *et al.* 2010). Other factors of salt marsh environments such as tidal cycles, physico-chemical gradients, vegetation structure, and its spatial arrangement, are also known to have a significant impact on abundance and community composition of nitrifying microbes involved in salt marsh nitrification (Bernhard *et al.* 2010; Bernhard *et al.* 2007).

1.4.1.3 Sulphur cycle

Sulphur is also an important element for growth, but high sulphide concentrations in coastal environments have encouraged the research on these ecosystems to be conducted towards toxicity of sulphides to the marine plants and animals (Raven and Scrimgeour 1997; King *et al.* 1982). Halophytes in general are found to be resistant to sulphide-rich salt marshes (Seliskar *et al.* 2004). Sulphate reduction to sulphide is a microbial process of particular importance in the anaerobic environments created within salt marsh depths and is mainly carried out by Deltaproteobacteria (e.g. *Desulfovibrio*, *Desulfobacter*, *Desulfococcus*, *Desulfonema,* Ansede *et al.* 2001; Rooney-Varga *et al.* 1998; Purdy *et al.* 2002a). Along with methanogenesis, sulphate reduction is a major terminal oxidation step in the flow of carbon and electrons. However, sulphide generated through the sulphur cycle is found to limit the anaerobic phase of the carbon cycle (methanogenesis) and nitrogen cycle rates (Capone and Kiene 1988; Koizumi *et al.* 2003; Joye and Hollibaugh 1995; Rysgaard *et al.* 1999; Giblin *et al.* 2010).

1.4.2 Methodologies Employed in Salt Marsh Microbiology Literature Studies

Microbial consortia at different salt marshes has been analysed in the literature studies by sampling and analysing salt marsh sediments (Berrada *et al.* 2012; Aanniz *et al.* 2015), rhizosphere cores (Köpke *et al.* 2005) and halophytic tissues (Fidalgo *et al.* 2016; Bagwell *et al.* 1998) from various tidal or inter-tidal zones (McKew *et al.* 2013; Peng *et al.* 2013).

The former salt marsh studies used molecular and/or culture-dependent techniques, primarily using the former. Molecular studies were commonly based on the extraction of community DNA followed by the construction, sequencing and analysis of clone libraries (Webster *et al.* 2010; Munson *et al.* 1997; Hou *et al.* 2013). These techniques also included identifying microbial community abundance and distribution analysis by quantitative or real time PCR (Moin *et al.* 2009; Peng *et al.* 2013), fluorescent *in-situ* hybridisation analysis (FISH) (Llobet-Brossa *et al.* 1998; Oliveira *et al.* 2012), terminal restriction fragment length polymorphism (TRFLP) (Peng *et al.* 2013; Córdova-Kreylos *et al.* 2006), phospholipid fatty acid (PLFA) analysis (Córdova-Kreylos *et al.* 2006), microarray method (Beazley *et al.* 2012), denaturing gradient gel electrophoresis (DGGE) (Webster *et al.* 2010; Bowen *et al.* 2009), and isotope analysis of microbial biomarkers (Boschker *et al.*

1999). Most of these molecular-based microbial studies have used 16S rRNA gene-based primers (Purdy *et al.* 2002b; Bowen *et al.* 2009; Webster *et al.* 2010; Hou *et al.* 2013). Additional primers encoding microbes' functional genes have also been reported, e.g. nifH (Rocha *et al.* 2016) and ammonia monooxygenase subunit A (amoA) (Santoro *et al.* 2008; Peng *et al.* 2013), involved in nitrogen fixation; Hg reductase, merA gene responsible for mercury resistance (Rocha *et al.* 2016).

Cultivation-based microbial studies of salt marsh environments in literature were mostly performed by inoculating salt marsh samples into a suitable liquid or solid growth medium. Some cultivation procedures were preceded by the aim-specific or microbe-specific sample treatments e.g. surface sterilisation and sample crushing in the case of plant tissue studies (Gayathri *et al.* 2010), pasteurisation (for isolation of Gram-positive, spore-forming bacteria, Daane *et al.* 2001), and enrichments in media supplemented with specific substrates and microcosm experiments (McKew *et al.* 2011; Bohórquez *et al.* 2017). Microbes promoting plant growth were explored using growth media supplemented with chemicals such as tryptophan (to test indole acetic production, Navarro-Torre *et al.* 2016; Gayathri *et al.* 2010) and phosphate (to determine microbial phosphate solubilization, Navarro-Torre *et al.* 2016). Certain functional groups of microbes were studied by making media deprived of substrates such as a nitrogen-free medium for diazotroph (Bagwell *et al.* 1998; Bagwell *et al.* 2001) and a reduced medium for sulphate-reducers and methanogens (Rooney-Varga *et al.* 1998; McKew *et al.* 2013). Thus, in these experiments sample pre-treatment and growth media selection played an important role in the cultivation and isolation of representative isolates as well as microbes of specific physiological interests from the sampled environment.

Post-cultivation identification and characterisation of isolated microbes was mostly performed by phylogenetic identification (Cho *and Giovannoni* 2004), TRFLP (Rooney-Varga *et al.* 1998), PCR-based fingerprinting (Berrada *et al.* 2012; Fidalgo *et al.* 2016), DGGE (Johnston *et al.* 2017; McKew *et al.* 2007), and PLFA (Daane *et al.* 2001; Taylor *et al.* 2013). Additionally, physiological profiles of microbial communities were traced by either using pure isolates or sediments directly sampled from salt marshes. It was performed by inoculating the samples into biochemical miniaturized test kits such as API®, BIOLOG™ (Gilewicz *et al.* 1997; Bagwell *et al.* 1998; Bagwell *et al.* 2001) and MPN test tubes (Köpke *et al.* 2005; Lydell *et al.* 2004), and then interpreting the growth results post-incubation.

Such an array of sample analysis techniques employed in salt marsh studies have also showed some result variations due to the method selections. e.g. underestimation of *Firmicutes* in molecular-based methods (<1%) (Filippidou *et al. 2015;* Cupit *et al. 2019)* as compared to their increasing proportions (5-10%) found in culture-dependant (Fidalgo *et al.* 2016; Berrada *et al.* 2012; Köpke *et al.* 2005), microcosm (McKew *et al.* 2013) *and* dipicolinate assay methods (Fichtel *et al.* 2008; Langerhuus *et al.* 2012; Lomstein *et al.* 2012), can be related to the inability of conventional DNA extraction reagents to extract DNA from the rigid structures of Firmicutes in endospore form (Filippidou *et al.* 2015; Cupit *et al.* 2019) and estimation of their vegetative forms only. Furthermore, selective media components and growth conditions can restrict certain microbial population. e.g. an aerobic incubation of samples would limit the growth of some Bacteroidetes, sulphate reducers and methanogens as their multiplication is restricted in the presence of oxygen (Bacic and Smith 2008; Meehan *et al.* 2012; Thomas *et al.* 2011; Rooney-Varga *et al.* 1998; McKew *et al.* 2013).

1.4.3 Previous Salt Marsh Studies In the UK

According to a recent survey, the total extent of salt marshes in the UK was found to be approximately 45,500 ha (England 32,500 ha, Scotland 6,747 ha, Wales 6,089 ha, and Northern Ireland 215 ha) (JNCC 2019, viewed November 2020). These marshes mainly exist in estuaries of low-lying land in the East and North-West of England, and in Wales. A large number of very small salt marsh sites are situated at the heads of sea lochs, embayments and beaches in North-West Scotland. Smaller areas of salt marshes are also observed in the estuaries of Southern England and the firths of East and South-West Scotland (JNCC 2019, viewed November 2020).

The available literature of UK salt marshes for microbial studies evaluates their faecal pollution, microbe responses to the dynamic salt marsh environments, nutrient availability, hydrocarbons, isoprene exposures etc. as follows.

Using inoculation and membrane filtration techniques, sewage inputs were identified as critical sources of faecal population in the estuarine waters of Ribble estuary, North-West England, UK (Kay *et al.* 2005; Stapleton *et al.* 2008). Faecal indicator concentration, and its relation to the land usage data and environmental parameters, was also predicted at the Ribble estuary using a stepwise regression

model that combined water quality analyses with land cover data, predicted through digital maps. Water analysis involved screening sediment samples for total coliforms, faecal coliforms and intestinal enterococci, performed with membrane filtration and growth on suitable media, followed by the counting of colony forming units (Kay *et al.* 2005; Stapleton *et al.* 2008; Crowther *et al.* 2001). This model-based approach, which was applied to investigate the quality of coastal bathing waters within the Ribble estuary, showed the significance of combined routine monitoring of microbial and environmental data (Crowther *et al.* 2001), as well as the substantial role of physico-chemical processes (Boye *et al.* 2015), tidal activities, inputs of upstream boundaries (Gao *et al.* 2015), and sediment transport processes (Huang *et al.* 2017a) in the distribution of faecal bacteria at the estuary sites.

Furthermore, faecal pollution in the estuarian and catchment sediments of Ribble estuary and Conwy estuary, North Wales, UK, were studied through the cultivation, isolation and enumeration of coliforms (Hassard *et al.* 2017), which showed an abundance of *Escherichia coli* and *Enterococcus* spp. Physico-chemical factors such as the grain size, porosity, clay content and concentrations of Zn, K, and Al in salt marsh sediments, as well as seasonal variations, were suggested to further contribute to this observed abundance of faecal microbes (Hassard *et al.* 2017).

Microcosm enrichments reported in UK salt marsh studies to identify the responses of microbes to the fluctuating conditions of estuarine environments (McKew *et al.* 2011), microphytobenthic exopolysaccharides (Bohórquez *et al.* 2017; Taylor *et al.* 2013) and isoprene production (Exton *et al.* 2012; Johnston *et al.* 2017), and bioremediation (Coulon *et al.* 2007; McKew *et al.* 2007), are elaborated in upcoming sections. These experiments were followed by molecular-based microbe analysis such as microbial community-based DNA extraction, DGGE, RT-PCR, 16S rRNA gene cloning, sequencing etc.

McKew *et al.* (2011) studied the effects of alternative wetting and desiccating phases created by tidal cycles on benthic microbial communities at the Colne Point salt marsh (Colne estuary, UK). This experiment showed significant restructuring, resistance, and resilience of the microbial community composition as the salt marsh salinities were varied across these phases. It also detected dominating sequences of Proteobacteria, mainly Alphaproteobacteria and Gammaproteobacteria, in both desiccated and wet salt marsh sediments.

A couple of studies also highlighted the significance of different types of extracellular polymeric substances (EPS) present in the intertidal sediments of UK-based estuaries (Taylor *et al*. 2013; Bohórquez *et al*. 2017). EPS produced by diatoms and bacteria present in the microphytobenthic biofilms of intertidal sediments of Pye-fleet mudflat and Alresford Creek in the Colne Estuary were studied by Taylor *et al.* (2013) based on slurry experiments conducted in the dark. These carbohydrate rich compounds, though estimated to be only 5% of the sediments' dissolved organic carbon (DOC) pool, were recycled rapidly before other sediment-carbohydrate and DOC fractions, and thus shown to be one of the most efficient fuel sources in estuarine environments. Among the observed diverse range of bacterial 16S rRNA gene sequence clones, Alphaproteobacteria (*Azospirillum and Skermanella*) and Gammaproteobacteria (*Pseudomonas*, *Pantoea*, and some *Acinetobacter*) were found to be the dominant EPS degrading clones in this study (Taylor *et al*. 2013).

Furthermore, two different types of EPS components, colloidal EPS and the more complex hot-bicarbonate-extracted EPS, recovered from the diatom-dominated biofilms of Alresford creek mudflat of Colne Estuary, were evaluated to test their degradation rates and effects on heterotrophic bacterial communities (Bohórquez *et al.* 2017). An aerobic mudflat-sediment slurry experiment was performed in the dark with these two EPS extracted components and their degradation rate was determined, followed by pyrosequencing of bacterial 16S rRNA genes. This study showed higher degradation rate constants of colloidal EPS, whereas hot-bicarbonate-extracted EPS was an important source of regenerated inorganic nutrients. Both types of EPS components greatly altered the bacterial community composition and showed a significantly increased relative abundance of Bacteroidetes (especially *Tenacibaculum*) and *Verrucomicrobia* (Bohórquez *et al*. 2017). These studies thus improved our understanding on carbon cycling and showed the importance of diverse microbiota participating in these vital processes of estuarine sediments (Taylor *et al*. 2013; Bohórquez *et al.* 2017).

One of the most abundant hydrocarbons released into the atmosphere is a volatile, organic compound called isoprene (Sharkey *et al.* 2008). This compound is known to play important roles in providing carbon and energy benefits to marine heterotrophic bacteria (Alvarez *et al.* 2009), protection from oxidative stress (Vickers *et al.* 2009), weather patterns (Ziemann 2009; Meskhidze and Nenes

2006) etc. To identify the main biogenic sources of isoprene production, and to evaluate spatial and temporal factors affecting the availability of this compound in estuarine environments, Exton *et al.* (2012) analysed the samples from Hythe, Wivenhoe, Alresford, and Brightlingsea sites of Colne Estuary. This study showed the potential significant role of estuaries as isoprene sources, and variations in the isoprene production by their changing tidal cycles, seasons, and light availability. Intertidal microphytobenthic algal communities were found to be the main isoprene producers in this temperate estuary. Microbial degradation of isoprene in estuarine environments was further illustrated by Johnston *et al.* (2017) by sampling four sites in the UK - Wivenhoe and Hythe sites of Colne estuary, Western Channel Observatory (L4 sampling station) of Plymouth, and Penarth, South Wales. This study included sample enrichment and isolation, DNA-stable isotope probing, physiology and molecular characterization of isolated microbes, through which microbial communities, mainly Actinobacteria including genera *Mycobacterium*, *Rhodococcus*, *Microbacterium* and *Gordonia*, were observed to be the dominant isoprene degraders.

Bioremediation activities at Stanford le Hope salt marsh of Thames estuary, UK, were assessed with microcosms established using salt marsh seawater and supplemented with different petroleum hydrocarbons by McKew *et al*. (2007). When examined for the microbial communities, this experiment showed the microbial degradation of these hydrocarbons to be partitioned between different bacterial taxa. Degradation of n-decane, polycyclic aromatic hydrocarbons, pristane and n-alkane (C12–C32) containing microcosms showed dominant 16S rRNA gene sequence clones of *Thalassolituus*, *Cycloclasticus*, *Alcanivorax* and *Thalassolituus oleivorans* respectively (McKew *et al*. 2007). Another study conducted at Stanford le Hope marsh (Coulon *et al*. 2007) performed a microbial analysis of its water samples for further understanding of microbial activities and regulating parameters responsible for petroleum hydrocarbon-degradation that exist in such temperate marine environments. Microcosm models set up using these estuarine waters and crude oil exhibited pre-adapted oil-degrading microbial communities. These microbes were found to be more affected by variation in temperature than the addition of nutrients to the marsh sediments. Major clones related to *Oleispira* were found as an alkane degrader at 4 °C conditions, and *Thalassolituus*, *Cycloclasticus*, and *Roseobacter* were detected at both 4 °C and 20 °C. This indicated the thermo-versatility of these hydrocarbonoclastic organisms (Coulon *et al*. 2007). These

studies conducted on seawater samples collected from the Stanford le Hope salt marsh, which neighbours the Shell-Haven and Coryton BP oil refineries, showed the intrinsic potential of microbes in remediating petroleum hydrocarbon-impacted estuarine environments, and *Thalassolituus* as the dominant crude oil degrader (McKew *et al.* 2007).

Studies on sulphate reducing bacteria (Banat *et al.* 1981; Balba and Nedwell 1982), and molecular-based evaluation of archaeal and methanogenic microbial diversity (Munson *et al.* 1997; Purdy *et al.* 2002b) for UK-based salt marshes has been attempted before at Colne estuary. Analysis of intertidal sediments collected from the Ray Creek, Colne Point salt marsh of Colne estuary identified the coexistence of two distinct, acetate utilising and hydrogen oxidizing groups of sulphate-reducing bacteria in salt marsh sediments, which displayed differential response to sulphate reduction inhibitors (Banat *et al.* 1981). This analysis also showed no methane production as acetate, a substrate required for methanogenesis was competitively utilised by the sulphate reducers instead (Banat *et al.* 1981).

Volatile fatty acids such as acetate, propionate and butyrate that have active role in methanogenesis and sulphate reduction were analysed for their anaerobic metabolism at different depths of Colne Point salt marsh sediments via slurry experiments and gas chromatography (Balba and Nedwell 1982). In this experiment, the rate of acetate turnover was found to be far greater than that through either propionate or butyrate, and it gradually ceased with increased sediment depth. Oxidation of acetate was furthermore linked to the activity of sulphate-reducing bacteria, which are the major acetate-utilisers in salt marshes (Balba and Nedwell 1982).

The tidal creek and adjacent area of vegetative marshland of Colne Point salt marsh that showed active methanogenesis were further analysed for its 16S rRNA gene sequence-based archaeal phylogenetic diversity by Munson *et al.* (1997). This study revealed wide range of sequences related to *Euryarchaeota* and marine group II archaea, and sequences related to those of methanogens such as hydrogen utilising *Methanoculleus* and *Methanogenium*, and non-hydrogen utilising *Methanococcoides* and *Methanolobus*. However, most of the clones were indistinctly related to the reference sequences of the cultured taxa (Munson *et al.* 1997).

Unique distribution of methanogenic microbial communities and their adaptabilities to the different conditions of brackish and marine ends of Colne estuary (East Hill Bridge on the river Colne and Colne Point salt marsh respectively) was explained by Purdy *et al.* (2002b). The most common sequenced clones observed at the brackish end of Colne estuary were related to *Methanosarcina*, *Methanocorpusculum*, and obligate acetate utilising *Methanosaeta concilii*. Whereas at marine end, abundant gene libraries of marine benthic group D and halophilic archaeal genotypes, as well as clones closely related to *Methanoculleus* and *Methanococcoides* were detected.

1.5 PRESENT STUDY SITES AND INVESTIGATIONS

Salt marsh environments in England are mostly confined to the coastline and are usually developed on intertidal land within the range of the normal spring tides (Burd 1989). After the Anglian region (15,255 ha), the North-West region of England has the second largest area of salt marsh (12,018 ha) (Phelan *et al.* 2011). These regions include the study sites Fingringhoe Wick Nature Reserve and RSPB Marshside Nature Reserve respectively. These geographically distinct marshes also have clear climatic differences (Met Office, n.d., viewed December 2020).

RSPB Marshside and Fingringhoe Wick marshes are located at the Ribble and Colne estuaries respectively. Both these estuaries comprise the largest areas of salt marshes of England and are areas of Special Scientific Interest as well (Nature England, n.d.(a), viewed June 2020). As described in section 1.4.3 of this chapter, estuarian and catchment sediments of Ribble estuary were described for the microbiology studies to detect faecal pollution (Kay *et al.* 2005; Stapleton *et al.* 2008; Crowther *et al.* 2001; Hassard *et al.* 2017; Boye *et al.* 2015; Gao *et al.* 2015; Huang *et al.* 2017a). Colne Point salt marsh, Alresford Creek, Pye-fleet mudflat, as well as the Hythe, Wivenhoe, and Brightlingsea sites of Colney estuary were tested for diverse microbial evaluations (McKew *et al.* 2011; Taylor *et al.* 2013; Bohórquez et al. 2017; Exton *et al.* 2012; Johnston *et al.* 2017; Munson *et al.* 1997; Purdy *et al.* 2002b; Banat *et al.* 1981; Balba and Nedwell 1982; Purdy *et al.* 2002b). However, these literature studies clearly show the scarce documentation on cultivation and isolation-based approaches, and the absence of any study of culturable microbial diversity in UK-based salt marshes. The reasons clearly seem to be affiliated with the methodological shift towards molecular-based technologies.

As described in previous sections, the gradient-rich, extreme and dynamic environments of salt marshes embrace a plethora of phylogenetically and functionally diverse microorganisms. Features such as distinct geographical locations and climate variations, which exist at the study sites, would further contribute to the composition and richness of microbial diversity of salt marshes (Córdova-Kreylos *et al.* 2006; Chaudhary *et al.* 2018; Bardgett *et al.* 2008; Webster *et al.* 2015). Moreover, the exceptional resilience and survival success of microorganisms in these fluctuating environments (McKew *et al.* 2011; Angermeyer *et al.* 2018; Bowen *et al.* 2009) make salt marshes suitable hotspots for exploring microbial diversity (Navarro-Torre *et al.* 2016; Gayathri *et al.* 2010). Also, studies of microbes adapted to thrive in such extreme environments are beneficial as these microbes can serve as effective environmental or biotechnological resources even in extreme conditions (Sánchez-Porro *et al.* 2003; Rao *et al.* 2010; Gangola *et al.* 2018). However, the recent methodological divergence towards molecular-based studies observed in salt marsh research areas has limited the exploration of cultivable microbial diversity from these sites, and there is still a large pool of uncultured, novel microbes in these environments (Salman *et al.* 2015; Mußmann *et al.* 2005). Biochemical and physiological features of many of these microbes have also remained unknown. The hidden potential of diverse microbial populations and the scarce documentation of cultivation-based microbial findings at UK salt marshes such as the study sites have therefore motivated the present study to explore the cultivable microbial diversity of these habitats.

This knowledge gap is partially addressed in the present study by conducting microbial cultivation, isolation and phylogenetic identification experiments, aimed to isolate and analyse microbes from the two marsh sites, RSPB Marshside and Fingringhoe Wick Nature Reserve, UK. The experiments were conducted to isolate and identify the diverse microbes including any potential and/or novel species (chapter 3).

Follow up studies for the potential novel bacteria identified in the present study (chapter 4) involved full characterisation of two of the potential novel isolates (MARW_01-07-02^T and MARW_01-02-27^T) via polyphasic analysis, which was performed in accordance with the standards of prokaryotic novel species description (Arahal *et al.* 2007; Stackebrandt and Ebers 2006; Richter and Rosselló-Móra 2009; Chun *et al.* 2018).

For the novel species description the current microbial taxonomy demands the addition of good quality genomes of the novel species and their genome description (Klimke *et al.* 2011). This was accomplished in chapter 5 of the present study by sequencing and analysing the draft genomes of the two novel isolates (MARW_01-07-02^T and MARW_01-02-27^T). It was also considered beneficial to explore and highlight the virulence, adaptation, and ecological and biotechnological potential of these novel species through genome annotation.

The natural habitats of most of the microbes are dynamic and gradient-rich (Emerson *et al.* 1994; Brune *et al.* 2000). Cultivating microbes from such gradient-rich environments demands the replication of environmental gradients in the lab, for which the plate diffusion method could be an effective solution. A gel-stabilised gradient plate diffusion method was developed to mimic the environmental gradients and to extend its application as a new technique for the cultivation and isolation of microbes from gradient-rich environments, as described in chapter 6 of the present study.

Thus, the overall aims of this study can be summarized as

1) To explore the microbial diversity of salt marshes in the UK through cultivation, isolation and phylogenetic identification
2) Full characterisation of potential novel microbial isolates through polyphasic analysis
3) Draft genome sequencing of the two novel bacterial isolates and their genome description
4) Development of a promising cultivation tool that mimics the gradient-rich environments and has application in microbial cultivation and isolation

Chapter 2

General Methodology

2.1 SAMPLE COLLECTION AND PHYSICO-CHEMICAL ANALYSIS

Salt marsh water samples were collected from the marsh creek and/or small ponds, in sterile 500 ml wide mouth polypropylene bottles. Bottles were open when submerged under water, overflowed with sample to ensure air removal from the bottles and closed immediately. At Fingringhoe Wick Nature Reserve, additional 4–5 cm deep soil sediment core samples were taken from the immediate area surrounding the marsh creek or small ponds using sterile 50 ml polypropylene tubes and were stored in the same tubes. Wherever possible, these samples were taken at different salt marsh locations- lower, and mid marsh; at approximately 50 m apart from each other, to capture the ecological variation. Sample bottles and tubes were immediately covered with aluminium foil and packed individually in poly bags to prevent cross-contamination during sampling. All samples were then immediately transported to Edge Hill university, Biology lab and refrigerated in lab at 4 °C in dark until its physico-chemical and microbial analysis was performed. Majority of samples were analysed within two to three days of sample collection.

Sample coding system was used to identify each microbial isolate retrieved from these samples e.g., in case of isolate, MARW_1-7-2; MARW indicates water sample collected at RSPB Marshside, at first sample collection visit, isolated from a seventh sample, and it was a second isolate retrieved from this sample.

In-situ analysis of temperature and salinity, as well as in-house measurement of pH, salinity and other physico-chemical parameters for the collected water and soil (10 gm% (w/v) dilution in sterile distilled water) samples was performed as shown in **Table 2.1**. Phosphate, iron, ammonium, and nitrite measurements were conducted as per manufacturer’s instructions by the sequential addition of test reagents to the samples followed by respective incubation times.

Table 2.1 Physico-chemical parameter analysis

Parameters	Instruments
Temperature and pH	Portable meter (Hanna, HI-9811-5)
Salinity	Hand refractometer (Atago, S-28E, Japan)
Phosphate, Iron, Ammonium, Nitrite	Sea water test kits (Merck, 1.14661.0001, 1.14660.0001, 1.14657.0001, 1.14658.0001 respectively)

2.2 MICROBIAL CULTIVATION, ISOLATION AND CULTURE MAINTENANCE

For inoculation, 100 µl of water samples (dilution 10^{0}–10^{-4} in 0.85% sterile saline) and 10% (w/v) soil sediment samples (dilution 10^{-1}–10^{-3} in 0.85% sterile saline) were taken. Samples' inoculation and subsequent microbial isolation was performed using sterile growth media; R2A agar (R2A, Oxoid™, CM0906), nutrient agar (NA, Oxoid™, CM0003) and marine agar (MA, Difco™, 2216). Compositions of all these media are mentioned in **Tables 2.2, 2.3 and 2.4** respectively. These media were prepared in two different sets to cover the pH and salinity range observed at study salt marsh sites. One media set was prepared with 4% (w/v) sodium chloride (NaCl) and pH 7.5 (as per MA, Difco™, 2216 instructions), and second set at NaCl concentration 2.5% (w/v) and pH 8. MA provided most of the minerals and nutrients required for the growth of heterotrophic marine bacteria. The nutrient limited R2A agar stimulated the growth of slow growing, stressed microbes from salt marsh ecosystem and NA supported growth of a wide range of non-fastidious organisms (Culotti and Packman 2014; Reasoner and Geldreich 1985).

Additionally, for the isolation of oligotrophic microbes, water samples collected from the RSPB Marshside in June 2017 were inoculated with different concentrations of peptone (Oxoid, LP0037) and yeast extract (BD, 288620) in two sets (**Table 2.5**) and incubated at 35 °C in shaker incubator for 24 h, at 250 rpm. Post incubation these samples were streaked on R2A, NA and MA plates and incubated at 35 °C for 2-3 days. While streaking, 50µl of serially diluted (10^{-1}–10^{-4}) enriched sample was spread inoculated in one quadrant of the plate, allowed to settle aseptically for 10–15 min. From this first quadrant, sample was then streaked into second, third and fourth quadrant while sterilizing the wire loop in-between. This modified streaking method was employed to get the maximum and diverse isolated colonies from the incubated samples.

After performing the initial inoculation experiments for the first batch of samples collected from the RSPB Marshside, MA medium was exclusively chosen in further cultivation experiments for its ability to produce greater number of diverse and well isolated colonies. Other media were not absorbing enough (somewhat of hydrophobic nature) to uniformly spread inoculate the marsh samples and insufficient to give isolated, diverse colonies.

Post incubation, microbial isolates with diverse colony characteristics (e.g., colour, shape, size, opacity, consistence, texture, margin, elevation) were selected from each sample inoculated media plates. Individual isolates were then passed through a series of sub-culturing on their respective grown medium until it imparts the pure culture for three consecutive plating. 20% glycerol (Benchmark Supplies, GL2878) stocks of these pure isolates were prepared in their respective liquid growth medium with required NaCl concentration and stored at Tech hub biotechnology lab, Edge Hill University in -80 °C deep freezers. Their post-cryopreservation viability and purity was confirmed by re-growing them on suitable agar medium.

Table 2.2 Composition of R2A agar (Oxoid™, CM0906), components g/L

Yeast extract	0.5
Proteose peptone	0.5
Casein hydrolysate	0.5
Glucose	0.5
Starch	0.5
Di-potassium phosphate	0.3
Magnesium sulphate	0.024
Sodium pyruvate	0.3
Agar	15.0

Table 2.3 Composition of nutrient agar (Oxoid™, CM0003), components g/L

Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0 / as per requirement
Agar	15.0

Table 2.4 Composition of marine agar (Difco™, 2216), components g/L

Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45 / as per requirement
Magnesium chloride	5.9
Magnesium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15 .0

Table 2.5 Peptone and yeast extract concentrations adjusted for enrichment of samples collected from RSPB Marshside in June 2017, components g/L of sea water

	Set 1	**Set 2**
Peptone	0.05	0.005
Yeast extract	0.02	0.002

2.3 CYTOLOGICAL AND PHYSIOLOGY OBSERVATIONS

All cytology and physiology tests were performed on MA medium unless specifically mentioned. For quality control, *Escherichia coli* (DSM 30083^T) and *Micrococcus luteus* (NCTC 2665^T) were processed as per the procedures mentioned for each test.

2.3.1 Gram Staining

Gram character of the isolated microbes was confirmed following Hucker's modified Gram staining method 'A' (Hucker and Conn 1923). Crystal violet (0.5% alcoholic solution), Grams Iodine, 95% ethyl alcohol, Safranin were used as a primary stain, mordent, decolorizing reagent and counterstain respectively. A single young colony emulsified in sterile saline was smeared on a clean, grease free slide, air-dried and passed over the Bunsen burner flame two to three times with the smear-side up. Overheating was avoided at this stage as it can cause distortion of cellular morphology. Smear was then flooded with crystal violet and gently rinsed with distilled water after 1 min. Later smear was covered with Gram's iodine for 45 s, gently rinsed with distilled water and decolorised using 95% ethyl alcohol. During decolourisation, slide was slightly tilted and applied with the alcohol drop by drop for 5 to 10 s until the alcohol runs almost cleared. Decolorized smear immediately rinsed with water and flooded with safranin for 1 min. Smear again rinsed with distilled water, allowed to air dry and observed under a light-microscope using oil-immersion 100x objective lens.

2.3.2 Motility test

Hanging drop method was performed to observe the motility of the isolates (Gerhardt *et al.* 1981). Vaseline cream was placed at the four corners of square glass coverslip. A small drop of freshly prepared saline suspension of single young colony was placed on the middle portion of it. A cavity glass-slide was then placed on this coverslip, lifted and inverted gently so that a microbial culture formed a hanging drop in the cavity of the slide. The outer margin of this drop was located and observed for motility under a light-microscope using 10x and 40x objective lenses.

2.3.3 Catalase test

Isolates were tested for the presence of an enzyme catalase by aseptically transferring a small amount of freshly grown biomass onto a glass slide already containing 2–3 drops of freshly prepared 3% hydrogen peroxide (H_2O_2, 6%w/v L.R., purity 5.75%, Timstar Lab Supplies LTD., 681234). Care was taken to transfer the culture using a disposable plastic loop. The observed rapid evolution of oxygen (within 5–10 s) as evidenced by bubbling was considered a positive test for catalase. Negative reaction (absence of catalase enzyme to hydrolyse the H_2O_2)

was reported for no bubbles. A weak positive test was allocated for weak effervescence appeared after 5–10 s (Reiner 2010).

2.3.4 Oxidase test

Determination of bacterial cytochrome oxidase was performed using TestOxidase™ (Pro-Lab diagnostics, PL 390) reagent. 1–2 drops of this reagent were added to a clean cotton bud stick. Using a disposable plastic inoculating loop, a medium size colony was removed from the surface of an agar medium and rubbed onto the reagent-saturated area of the cotton bud. The production of a distinct blue or purple colour developed on the cotton bud within 30 s was considered as positive oxidase test. Negative reaction was noted for the absence of a distinct blue or purple colour.

While performing oxidase test precautionary measures such as use of fresh bacterial cultures; not older than 48 h, results interpretation within 30 s and use of plastic disposable loops were followed. Inoculation loops of nickel-base alloy wires containing chromium and iron (nichrome) were not used as this can give false positive results. Simultaneously, the oxidase test was performed from the medium containing no fermentable sugar, in this study for e.g., from MA. Fermentation of a carbohydrate results in acidification of the medium (e.g., lactose in MacConkey agar or sucrose in Thiosulphate-citrate-bile salts-sucrose agar), and a false negative oxidase test may result if the surrounding pH is below 5.1.

2.4 OPTIMUM GROWTH REQUIREMENT

Optimum growth conditions of potential novel isolates were tested in this study at different temperatures, NaCl concentrations, and pH as shown in **Table 2.6**.

These test conditions were set for the individual isolates with reference to the species description literature of their closest phylogenetic relatives (Lee *et al.* 2005; Arahal *et al.* 2002a; Diéguez *et al.* 2020; Gauthier *et al.* 1995b; Ivanova *et al.* 2002c).

For MARW_1-7-2^T, growth condition studies were conducted in liquid growth medium A containing (w/v) 0.5% yeast extract, 0.3% sodium citrate, 2% MgSO4.7H2O, 0.2% KCl (Poli *et al.* 2013). pH and NaCl concentrations were adjusted as per requirement. Medium A was used in this experiment because it is described in species description of *Halomonas smyrnensis* (Poli *et al.* 2013); which

is the closest phylogenetic relative of MARW_1-7-2^T on NCBI's blastn tool and its transparency allowed accurate spectrophotometer readings of the bacterial growth. For MARW_1-7-2^T, to further evaluate its dependence on NaCl and other marine salts, an additional experiment was conducted by observing its growth in a nutrient broth (NB) medium of pH 6.5–7; supplemented with 0% and 3% (w/v) NaCl.

MARW_1-2-27^T growth studies were conducted in a liquid medium containing synthetic sea water supplemented with 0.3% (w/v) peptone and 0.1% (w/v) yeast extract. Artificial sea water was prepared in lab and contained (w/v) 0.5% $MgCl_2$, 0.4% Na_2SO_4, 0.1% $CaCl_2$, 0.07% KCl, 0.02% $NaHCO_3$, 0.01% KBr, 0.003% H_3BO_3 (Lyman and Fleming 1940; prepared without 0.024% $SrCl_2$). NaCl concentration and pH of growth medium was adjusted as per requirement. This medium was selected for MARW_1-2-27^T as it is described for one of its closest phylogenetic relative *Pseudoalteromonas piscicida* (Bein 1954) and its transparency was suitable for spectrophotometric growth studies.

NaCl and pH adjustments were performed using a hand refractometer (Atago, S-28E, Japan) and pH electrode meter (Mettler Toledo) respectively. Temperature of the shaker incubator was adjusted to the required value by a thermometer. The growth studies at different pH values were conducted by supplementing their growth media with 1M hydrochloric acid (HCl) or 1M sodium hydroxide (NaOH) and buffering agents: MES (Sigma M1317, useful for pH range 4-6), HEPES (Sigma 83264, for pH 6–8), CAPSO (Sigma C2278, for pH 9–10) and CAPS (Sigma C2632, pH 9.5–11) at final concentration of 30 mM. Media ingredients were dissolved in distilled water in double strength, adjusted to the required pH value and autoclaved. 60 mM of required buffer was prepared separately in sterile distilled water. Autoclaved medium and its respective buffer were mixed in equal volumes and filter sterilised in aseptic conditions. For every batch of pH experiment, buffered media were freshly prepared and used for the growth studies as soon as they were filter sterilised.

For every batch of growth study experiment, a fresh stock of microbial inoculum of OD_{600nm} 0.08–0.1 was prepared in its respective medium. 4–5 ml of this inoculum was distributed in multiple glass tubes (tube volume approx. 50 ml) and immediately allowed to incubate on a shaker incubator at the required growth temperatures. Use of these multiple glass tubes maintained the required growth conditions throughout

the experiment even while withdrawing the samples from the incubator for turbidity measurement at different time intervals.

At different time intervals of incubation, three sample tubes were withdrawn and measured for their bacterial growth in terms of turbidity using spectrophotometer (VWR, UV-1600PC) at 600nm wavelength. During these measurements, respective sterile liquid growth medium was used for zeroing of the spectrophotometer and for the dilution of incubated cultures if required. An average of these three absorbance values was taken for the final growth analysis. This observed optical density was then plotted against the total incubation time and the resultant curves were observed at semi-log scale for optimum growth at all test parameters.

Table 2.6 Optimum growth parameters tested for the potential novel isolates MARW_1-7-2^{T} and MARW_1-2-27^{T}

Isolates	Growth parameters	Tested parameter values	Other growth conditions
MARW_1-7-2^{T}	Temp. (°C)	5-10-15-20-25-30-35-37-40-45-50-55	Growth medium NaCl 10% (w/v) and pH 7
	NaCl % (w/v)	0-1-3-5-10-12-15-20-25-30	Temp. 35 °C and growth medium pH 7
	pH	4.2-5-6-6.3-6.6-7-7.4-8.3-8.7-9-10	Temp. 35 °C and growth medium NaCl 3% (w/v)
MARW_1-2-27^{T}	Temp. (°C)	10-15-20-25-30-35-37-40-45-50	Growth medium NaCl 4% (w/v) and pH 7.5
	NaCl % (w/v)	0-0.5-1-2-3-5-6-7-8-9-10-12-13-14-15	Temp. 35 °C and growth medium pH 7.5
	pH	5-5.5-6-6.3-7-7.5-8-8.5-9-9.6	Temp. 35 °C and growth medium NaCl 1% (w/v)

2.5. BIOCHEMICAL TESTS

To confirm the novel status of MARW_1-7-2^{T} and MARW_1-2-27^{T}, additional physio-chemical tests were performed in triplicate by incubating the isolates in commercially available miniaturized biochemical test strips. Acid production from various carbohydrates was analysed using an API® 50 CH bacterial identification kit (bioMérieux SA, 50 300). The substrate oxidation patterns and chemical sensitivity were examined using BIOLOG GEN III MicroPlates™ (Biolog Inc., 1030). Other physiological properties and enzyme activities were assayed by API® 20 E (bioMérieux SA, 20 100) and API® ZYM kits (bioMérieux SA, 25 200).

Some preliminary experiments were performed to decide the culture density and incubation time required by each isolate for these test kits. The manufacturer's standard test protocol was followed with some minor modifications to make it suitable for the testing of study cultures. These modifications were implemented after its discussion with the test strip manufacturer's research advisors. Individual tests for each isolate were carried out aseptically using freshly grown pure culture (on MA) at similar test conditions of density of inoculum, incubation time and temperature. Before conducting these tests, the test kits, and their inoculation media were brought to the room temperature. The respective sterile inoculation media were supplemented with autoclave sterilized NaCl powder to get the final salinity of 3% (w/v) and 1% (w/v), which were the optimum salt requirement of MARW_1-7-2^{T} and MARW_1-2-27^{T} respectively. Once inoculated with the isolate, all test plates were incubated at 35 °C.

During test procedures care was taken to make the inoculum free of clumps, not to carry over the growth medium (MA) into inoculation fluid, avoid air bubble formation while dispensing the specimen into wells and avoid cross-contamination of substrates in between the test-wells by inoculating the culture using separate micropipette tip for each well.

2.5.1 API® 50 CH Test

API® 50 CH test strip (bioMérieux SA, 50 300) comprising 49 biochemical tests is designed to study the carbohydrate (and its derivatives) metabolism of microorganisms (**Table 2.7**). It requires API® 50 CHB/E medium (bioMérieux SA, 50430, designed for Gram-negative rods and Bacillus sp.) for inoculum preparation. Once inoculated and incubated with the specimen; positive test result is revealed through the colour change of the substrates in the microtubes of the test strips. This colour is developed though the detection of acid (product of the microbial carbohydrate fermentation) by the pH indicator phenol red present in the inoculation medium.

API® 50 CH test procedures were performed in a sterile environment, following the steps mentioned ahead. Test strip was placed in an incubation box made up of tray and lid (provided by the manufacturer). About 10 ml of sterile distilled water was distributed into the honey-combed wells of the tray to create a humid atmosphere during incubation.

For the specimen preparation and inoculation of API® 50 CH strips, several identical microbial colonies of the isolate were suspended in an API® 50 CHB/E medium, homogenized carefully by pipetting to obtain a final turbidity equivalent to 0.8–1 at 600nm. Using a sterile micropipette tips 150 µL of freshly prepared specimen was dispensed in each tube of the test strip. Post inoculation tray was covered with the lid and incubated for 6–7 days.

Results of the inoculates test strips were recorded every after 10–12 h of total incubation. A positive test was confirmed by the colour developed due to acidification and was revealed by the colour change of the phenol red indicator contained in an API® 50 CHB/E medium from red to yellow. For the esculin test, a positive result was confirmed by a change in media colour from red to black.

Table 2.7 The composition of the API® 50 CH Test strips (bioMérieux SA, 50 300)

Strip 0-9			Strip 10-19			Strip 20-29		
Tube no.	Test	Active ingredients	Tube no.	Test	Active ingredients	Tube no.	Test	Active ingredients
0		control	10	GAL	D-galactose	20	MDM	methyl-αD-mannopyranoside
1	GLY	glycerol	11	GLU	D-glucose	21	MDG	methyl-αD-glucopyranoside
2	ERY	erythritol	12	FRU	D-fructose	22	NAG	N-acetylglucosamine
3	DARA	D-arabinose	13	MNE	D-mannose	23	AMY	amygdalin
4	LARA	L-arabinose	14	SBE	L-sorbose	24	ARB	arbutin
5	RIB	D-ribose	15	RHA	L-rhamnose	25	ESC	esculin ferric citrate
6	DXYL	D-xylose	16	DUL	dulcitol	26	SAL	salicin
7	LXYL	L-xylose	17	INO	inositol	27	CEL	D-cellobiose
8	ADO	D-adonitol	18	MAN	D-mannitol	28	MAL	D-maltose
9	MDX	methyl-ßD-xylopyranoside	19	SOR	D-sorbitol	29	LAC	D-lactose (bovine origin)

Strip 30-39			Strip 40-49		
Tube no.	Test	Active ingredients	Tube no.	Test	Active ingredients
30	MEL	D-melibiose	40	TUR	D-turanose
31	SAC	D-saccharose (sucrose)	41	LYX	D-lyxose
32	TRE	D-trehalose	42	TAG	D-tagatose
33	INU	inulin	43	DFUC	D-fucose
34	MLZ	D-melezitose	44	LFUC	L-fucose
35	RAF	D-raffinose	45	DARL	D-arabitoL
36	AMD	amidon (starch)	46	LARL	L-arabitoL
37	GLYG	glycogen	47	GNT	potassium gluconate
38	XLT	xylitol	48	2KG	potassium 2-ketogluconate
39	GEN	gentiobiose	49	5KG	potassium 5-ketogluconate

2.5.2 API® 20 E Test

API® 20 E test strip (bioMérieux SA, 20 100) comprising of 20 microtubes of dehydrated substrates was used to predict the physico-chemical profile of potential novel isolates. Microbial break down of these substrates produced colour changes that are either spontaneous or in some microtubes revealed after the addition of an extra reagents. These extra reagents used were 10 gm% (w/v) ferric chloride ($FeCl_3$ prepared in lab, Timstar, IR3240), Kovac's reagent (Sigma Aldrich, 67309), VP 1 + VP 2 reagents (bioMérieux, 70422), NIT 1 + NIT 2 reagents (bioMérieux, 70442), Zn powder (SciChem, Z1015), and mineral oil (bioMérieux, 70100) (**Table 2.8**). Long with the novel isolates, *Bacillus thuringiensis* (DSM2046^T) was tested as a QC strain because its API® 20 E test results are well documented in the literature and were used for quality check in this study (Logan and Berkeley 1984).

To start with the test, an incubation box consisting of tray and lid was prepared to accommodate test strip. About 5 ml of sterile distilled water was distributed into the honey-combed wells of the tray to create humid atmosphere during incubation.

To prepare a bacterial suspension well isolated, pure colonies from 18-24 old culture plate were carefully emulsified in a sterile saline solution. Homogenous bacterial suspension of OD_{600nm} 0.8–1 was prepared and used immediately for the inoculation of the test strips. In the test strips, CIT, VP and GEL labelled wells were filled in both tube and cupule with the bacterial suspension, whereas for other tests only tube (and not the cupule) was filled. Mineral oil was overlaid in the test wells labelled with ADH, LDC, ODC, H_2S and URE to create anaerobic condition during incubation. The incubation box was then closed and incubated for 2–3 days or until at least 3 positive results (before adding the extra reagents) are obtained.

According to the test procedure, once the incubation was over, a range of extra reagents were added to specific wells which required them for result development (**Table 2.8)**. The indole production test and nitrate reduction tests were performed at the last since these reactions release gaseous products which interfere with the interpretation of other tests of the strip. Finally, the test results were interpreted by referring the developed colours of the test wells to the reading **Table 2.9**.

Table 2.8 Reagents required for API® 20 E test strips (bioMérieux SA, 20 100)

Test wells	Extra reagent added	Result development
TDA (Tryptophan Deaminase)	1 drop of 10% $FeCl_3$	Immediate
VP (Voges Proskauer)	1 drop each of VP 1 and VP 2 reagents	At least 10 min
IND (Indole)	1 drop of Kovac's reagent	Immediate
GLU (Glucose; to check reduction of nitrates to nitrites and N_2 gas)	1 drop each of NIT 1 and NIT 2 reagents for nitrites detection	2 to 5 min
	Further after 5 min, 2 to 3 mg of Zn reagent added for N_2 test	5 min

Table 2.9 Reading table for API® 20 E test strips (bioMérieux SA, 20 100)

Result interpretation: (1) A very pale yellow was considered positive; **(2)** An orange colour after 36-48 h incubation considered negative; **(3)** Reading made in the cupule (aerobic); **(4)** Fermentation begins in the lower portion of the tubes and oxidation begins in the cupule; (**5)** A slightly pink colour after 10 min was considered negative

Tube no.	Tests	Active ingredients	Reactions/Enzymes	Results	
				Negative	**Positive**
1	ONPG	2-nitrophenyl-ßDgalactopyranoside	ß-galactosidase (Ortho nitrophenyl-ßDgalactopyranosidase)	colourless	yellow (1)
2	ADH	L-arginine	arginine dihydrolase	yellow	red/orange (2)
3	LDC	L-lysine	lysine decarboxylase	yellow	red/orange (2)
4	ODC	L-ornithine	ornithine decarboxylase	yellow	red/orange (2)
5	CIT	trisodium citrate	citrate utilization	pale green/yellow	blue green/blue (3)
6	H_2S	sodium thiosulphate	H_2S production	colourless / greyish	black deposit/thin line
7	URE	urea	urease	yellow	red/orange (2)
8	TDA	L-tryptophane	tryptophane deaminase	yellow	reddish brown
9	IND	L-tryptophane	indole production	colourless pale green / yellow	pink
10	VP	sodium pyruvate	acetoin production (Voges Proskauer)	colourless	pink / red (5)

Table 2.9 Continued…

Tube no.	Tests	Active ingredients	Reactions/Enzymes	Results	
				Negative	Positive
11	GEL	gelatin (bovine origin)	gelatinase	no diffusion	diffusion of black pigments
12	GLU	D-glucose	fermentation/oxidation (glucose) (4)	blue / blue green	yellow / greyish yellow
13	MAN	D-mannitol	fermentation/oxidation (mannitol) (4)	blue / blue green	yellow
14	INO	inositol	fermentation/oxidation (inositol) (4)	blue / blue green	yellow
15	SOR	D-sorbitol	fermentation/oxidation (sorbitol) (4)	blue / blue green	yellow
16	RHA	L-rhamnose	fermentation/oxidation (rhamnose) (4)	blue / blue green	yellow
17	SAC	D-sucrose	fermentation/oxidation (saccharose) (4)	blue / blue green	yellow
18	MEL	D-melibiose	fermentation/oxidation (melibiose) (4)	blue / blue green	yellow
19	AMY	amygdalin	fermentation/oxidation (amygdalin) (4)	blue / blue green	yellow
20	ARA	L-arabinose	fermentation/oxidation (arabinose) (4)	blue / blue green	yellow
Extra	nitrate reduction: GLU tube- reduction of nitrates to nitrites (NO_2) and N_2 gas	potassium nitrates	NO_2 production	yellow	red

2.5.3 API® ZYM Test

API® ZYM (bioMérieux SA, 25 200); a semi-quantitative method is designed for rapid study of 19 constitutive enzymatic reactions (as mentioned in **Table 2.10**). This system consists of a strip with 20 microwells coated at the base with enzymatic substrates and its buffer. Inoculation of dense microbial suspension into these microwells allows reaction between the microbial enzyme and well substrate. Metabolic end products generated during incubation period are then detected through the coloured reactions revealed after addition of ZYM A and ZYM B reagents. In the present study, API® ZYM enzymatic assay was performed as mentioned ahead.

About 5 ml of sterile distilled water was distributed into the honey-combed wells of the tray to create a humid atmosphere before placing the test strip. Test strips were then inoculated with the microbial suspension, which was prepared in sterile saline with final optical density of 0.8–1 (measured at 600 nm wavelength). Using a sterile pipette, 110 µL of freshly prepared specimen were dispensed into each cupule. After inoculation, the tray covered with the lid was incubated for 2–3 days away from light.

To read the results post-incubation, 1 drop of ZYM A and ZYM B reagent was added to each cupule and allowed to stand for 5–10 min for colour development. At this stage, the strip was placed under a direct light source as per manufacturer's instructions to get confirmed and comparable results. The reactions were then interpreted according to the colour development chart mentioned in **Table 2.10**.

Table 2.10 Reading table for API® ZYM test strips (bioMérieux SA, 25 200)

Abbreviations: * = Colourless or colour of the control if the strip was not exposed to an intense light source after addition of the reagents; if the strip was exposed to intense light, a very pale-yellow colour was obtained

No.	Enzyme assayed for	Substrate	Result	
			Positive	Negative
1	control		colourless or colour of the sample if it has an intense coloration	
2	alkaline phosphatase	2-naphthyl phosphate	violet	colourless or very pale yellow *
3	esterase (C4)	2-naphthyl butyrate	violet	
4	esterase lipase (C8)	2-naphthyl caprylate	violet	
5	lipase (C14)	2-naphthyl myristate	violet	
6	leucine arylamidase	L-leucyl-2-naphthylamide	orange	
7	valine arylamidase	L-valyl-2-naphthylamide	orange	
8	cystine arylamidase	L-cystyl-2-naphthylamide	orange	
9	trypsin	N-benzoyl-DL-arginine-2-naphthylamide	orange	
10	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	orange	

Table 2.10 Continued…

No.	Enzyme assayed for	Substrate	Result	
			Positive	Negative
11	acid phosphatase	2-naphthyl phosphate	violet	colourless or very pale yellow *
12	naphthol-AS-BI-phosphohydrolase	naphthol-AS-BI-phosphate	blue	
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	violet	
14	ß-galactosidase	2-naphthyl-ßD-galactopyranoside	violet	
15	ß-glucuronidase	naphthol-AS-BI-ßD-glucuronide	blue	
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	violet	
17	ß-glucosidase	6-Br-2-naphthyl-ßD-glucopyranoside	violet	
18	N-acetyl-ß-glucosaminidase	1-naphthyl-N-acetyl-ßD-glucosaminide	brown	
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	violet	
20	α-fucosidase	2-naphthyl-αL-fucopyranoside	violet	

2.5.4 BIOLOG GEN III MicroPlate™

The BIOLOG GEN III MicroPlate™ (Biolog Inc.,1030) used in this study assayed microbial growth against 71 carbon sources and sensitivity against 23 chemicals (**Fig. 2.1)**. Tetrazolium redox dye pre-coated into these plates calorimetrically indicated the microbial growth in response to utilization of the carbon sources or resistance to inhibitory chemicals. Upon inoculation and incubation of these plates with the bacterial isolates, microbial growth or increased microbial respiration caused reduction of this indicator dye, forming a purple colour which indicated a positive result. The layout of this Biolog plate is as shown in **Fig. 2.1** and all test procedures are mentioned ahead.

For inoculum preparation, 'Protocol A' of Biolog testing was used which is applicable to most bacterial species. This protocol required Inoculation Fluid A (IF-A, Biolog Inc., 72401) as an inoculation medium. Bacterial suspension of cell density 85–95 % transmittance (T) was prepared from freshly grown cultures of the novel isolates. Pure culture colonies were picked using a sterile cotton tipped inoculator swab (Inoculatorz™, 3321) and dispended into IF-A by rubbing the swab against the bottom of tube. Required turbidity was adjusted with turbidimeter (Biolog Inc., 3587) which was pre-calibrated with an appropriate turbidity standard. 100 µl of this uniform bacterial suspension was added into each well. Inoculated plates were then incubated for 2–3 days. During incubation, humid conditions were maintained by placing the inoculated plate assembly in a plastic tray containing moistened tissue paper. The strips were observed for colour change every after 12–24 h for up-to 2–3 days. Negative control well A-1 and positive control well A-10 of the incubated test strip were used as a reference to interpret the results.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butryric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Activate W

Figure 2.1 Chemical layout of BIOLOG GEN III MicroPlate^{TM} (Biolog Inc., 1030). Cells highlighted with grey colour indicates tests for biochemical sensitivity

2.6 CHEMOTAXONOMIC CHARACTERISATION

2.6.1 Respiratory Quinones and Polar Lipids Analysis

Respiratory quinones and polar lipids analysis of novel isolates were performed at Leibniz Institute - German collection of microorganisms and Cell Cultures (DSMZ; attended via the Transnational Access program of European Marine Biological Research Infrastructure Cluster (EMBRIC).

A two-stage method was used to extract the respiratory quinones followed by the separation of polar lipids. 150 mg freeze dried biomass of each novel isolate was allowed to react sequentially with hexane-methanol (1:2), 0.3% (w/v) NaCl solution and later with cold n-hexane. After magnetic stirring and centrifuging this mixture, upper clear phase and bottom precipitate were separated, and kept in dark for respiratory quinones and polar lipids analysis respectively.

The upper, clear phase obtained in the previous steps, was analysed for respiratory quinones as described by Franzmann and Tindall (1990). The supernatant was evaporated under a nitrogen gas stream (1.2 bar). The resultant dried component was re-suspended in 200 µl tertiary butyl methyl ether. Respiratory quinones were then separated from this suspension using one-dimensional thin-layer chromatography (TLC) on silica gel, where sample loaded silica plates were developed in TLC chambers containing hexane and tertiary butyl methyl ether mixture (9:1) in dark. Post development, silica plates were dried, observed under ultraviolet (UV) light to spot the ubiquinones and were recovered by scratching the powdered material on the marked spot. This material was then taken into a glass filter tube, through which methanol-hexane mixture (2:1) was passed. This filtered reagent was collected in a separate tube, mixed well with 200 µl ice cold n-hexane and 4 –5 drops of ice cold 0.3% (w/v) NaCl, and then kept on ice for 4–5 min. The upper supernatant of this mixture was transferred to a separate fresh tube and evaporated under a nitrogen gas stream (1.2 bar). Further, the dried component in this tube was re-suspended in 300 µl of HPLC (high performance liquid chromatography) grade methanol and mixed gently. 5 µl of this quinone test mixture was then taken in a HPLC tube, re-diluted with 25 µl of HPLC grade methanol and analysed for quinones on HPLC machine (Agilent Technologies, 1260 Infinity II System) using a reverse phase column.

Next, extraction and recovery of polar lipids was conducted as described ahead (Bligh and Dyer 1959; Tindall 1990a; Tindall 1990b). To the precipitate obtained in the previous steps, 2–5 ml chloroform and 3 ml methanol were added, stirred overnight in brown bottle and centrifuged. If required, extra methanol was added to this mixture and re-centrifuged to get clear separation of upper and bottom phase. The clear supernatant was transferred to a separate tube, mixed with 1.25 ml chloroform and 1.25 ml of 0.3% (w/v) NaCl, and re-centrifuged. Post centrifugation, tube was observed for clear layer at the bottom. If required, extra 1 ml of chloroform was added to this tube and re-centrifuged. Clear bottom layer was then transferred to a fresh tube and completely evaporated under the nitrogen gas stream (1 bar). The dried component in this tube was re-suspended in a chloroform-methanol mixture (2:1). This extract of polar lipids was inoculated on four silica gel plates and analysed by two-dimensional TLC to separate polar lipids from the extracted mixture. The first TLC development in freshly prepared chloroform-methanol-water mixture was followed by the second development in chloroform-methanol-acetic acid-water mixture. Total lipids were then detected on one of the TLC plates by spraying 5% molybdophosphoric acid prepared in ethanol. Additionally, on the remaining three TLC plates ninhydrin reagent, phosphate reagent, and naphthol-sulphuric acid (Molisch's) reagents were sprayed individually for the detection of lipids containing free amino groups, phosphates, and sugars respectively (Tindall *et al.* 2007). All these four TLC plates with the developed chromatogram were interpreted for polar lipid contents at DSMZ. Total lipids were detected as blueish-black stained spots on yellowish-green background of developed TLC plate. Lipids with free amino groups were detected as red-purple or yellow to orange spots. Phosphate-containing lipids were spotted within 15 min as blue spots while sugar containing lipids were detected as a red-purple spots.

2.6.2 Fatty Acid Profile

As one of the recommended tools for biochemical characterisation (Arahal *et al.* 2007), cellular fatty acids of the potential novel isolates and their closest phylogenetic relatives were analysed by gas chromatography (GC). The isolates were processed for extraction of fatty acid methyl esters (FAMEs, MIDI protocol, Sherlock, Microbial Identification System), followed by its detection on GC machine (Agilent GC, 6890).

Both MARW_1-7-2^T and MARW_1-2-27^T were inoculated on MA growth medium and incubated for 24 h at 28 and 37 °C, respectively. Initially, for the preparation of GC-ready extracts these organisms went through a five-step process (**Fig. 2.2**), harvesting, saponification, methylation, extraction, and base wash using five different reagents, prepared as explained in **Table 2.11** (Sherlock, Microbial identification system, version 6.3, operating manual September 2015). Microbial colonies were harvested from the 3rd quadrant of the freshly grown culture plates. Growth was gently scraped using a 4 mm sterile inoculating loop and coated onto the lower inner surface of the glass culture tubes. 1.0±0.1 ml of Reagent 1, the methanolic base added to the culture tubes, vortexed for 5–10 s and heated in a water bath (VWR, VWB 12) at 95 –100 °C for 30 min. After these 30 min of saponification, tubes were removed and kept in a pan of cold tap water to cool. Then 2.0±0.1ml of reagent 2, the methylation reagent was added to each tube. After vortexing, tubes were transferred to 80±1 °C water bath, removed after 10±1 min and quickly cooled by placing tubes in a tray of cold tap water. After addition of 1.25±0.1 ml reagent 3; the extraction solvent, tubes were tightly capped, placed in a laboratory rotator and gently mixed end-over end for 10 min. After mixing, upper phase was transferred to a fresh tube, 3.0±0.1 ml of reagent 4; the base wash was added and tightly capped tubes were then gently rotated end-over-end for 5 min. 2/3rd of the top phase was removed and transferred to the GC vial. In case, if the top phase is turbid, few drops of saturated NaCl solution was added and rotated end-over-end.

Once this final extract was injected into the GC machine, FAMEs present in the sample mixture passed through the column to the detector. The flame ionization detectors burned the FAMEs, creating a proportional signal that was later stored in ChemStation. During this step, the program in GC ramped the temperature from 170 to 270 °C at 5 °C per min. At the completion of analysis, a ballistic increase to 300 °C allowed cleaning of the GC column.

Upon completion of the run, the retention time, response, and the area/height ration of each fatty acid peak were calculated by the Agilent ChemStation. This data is then transferred to the Sherlock Library (TSBA6) for peak naming and further processing. The resultant fatty acid peaks of MARW_1-7-2^T and MARW_1-2-27^T isolates in their respective chromatograms were identified by matching its

composition with the fatty acid profiles of the known microorganisms stored in the standard Sherlock library (TSBA6).

As per manufacturer's instructions, *Bacillus subtilis* 6633^T was used for QC validation, since it is considered to be an extremely sensitive microbes towards variations in FAME analysis and often would be the first indicator of issues with sample preparation and instrument functioning.

Table 2.11 Reagents required for the preparation of GC-ready cell extract in fatty acid analysis

Reagents	Purpose	Chemicals	Weight / Volume to process 100 samples	Preparation details
Reagent 1	Saponification- Lysis of the cells to liberate fatty acids from the cellular lipids	methanol (HPLC grade)	50 ml	Methanol and deionized water in given quantities were combined. NaOH pellets were then added to this solution and stirred until completely dissolved.
		deionized water	50 ml	
		NaOH (Certified ACS)	15 g	
Reagent 2	Methylation - Formation of fatty acid methyl esters (FAMEs)	6M HCl	108.33 ml	HCl added to the methanol while stirring.
		methanol (HPLC grade)	91.66 ml	
Reagent 3	Extraction - Transfer of the FAMEs from the aqueous phase to the organic phase	methyl tert-butyl ether (HPLC grade)	66.66 ml	Methyl tert-butyl ether added to the hexane and stirred well.
		hexane (HPLC grade)	66.66 ml	
Reagent 4	Base Wash - Aqueous wash of the organic extract prior to the chromatographic analysis	NaOH (Certified ACS)	3.6 g	NaOH pellets were added to the deionized water and stirred until dissolved completely.
		deionized water	300 ml	
Additional Reagent	Saturated NaCl: Dissolve 40g ACS NaCl in 100 ml distilled H_2O			

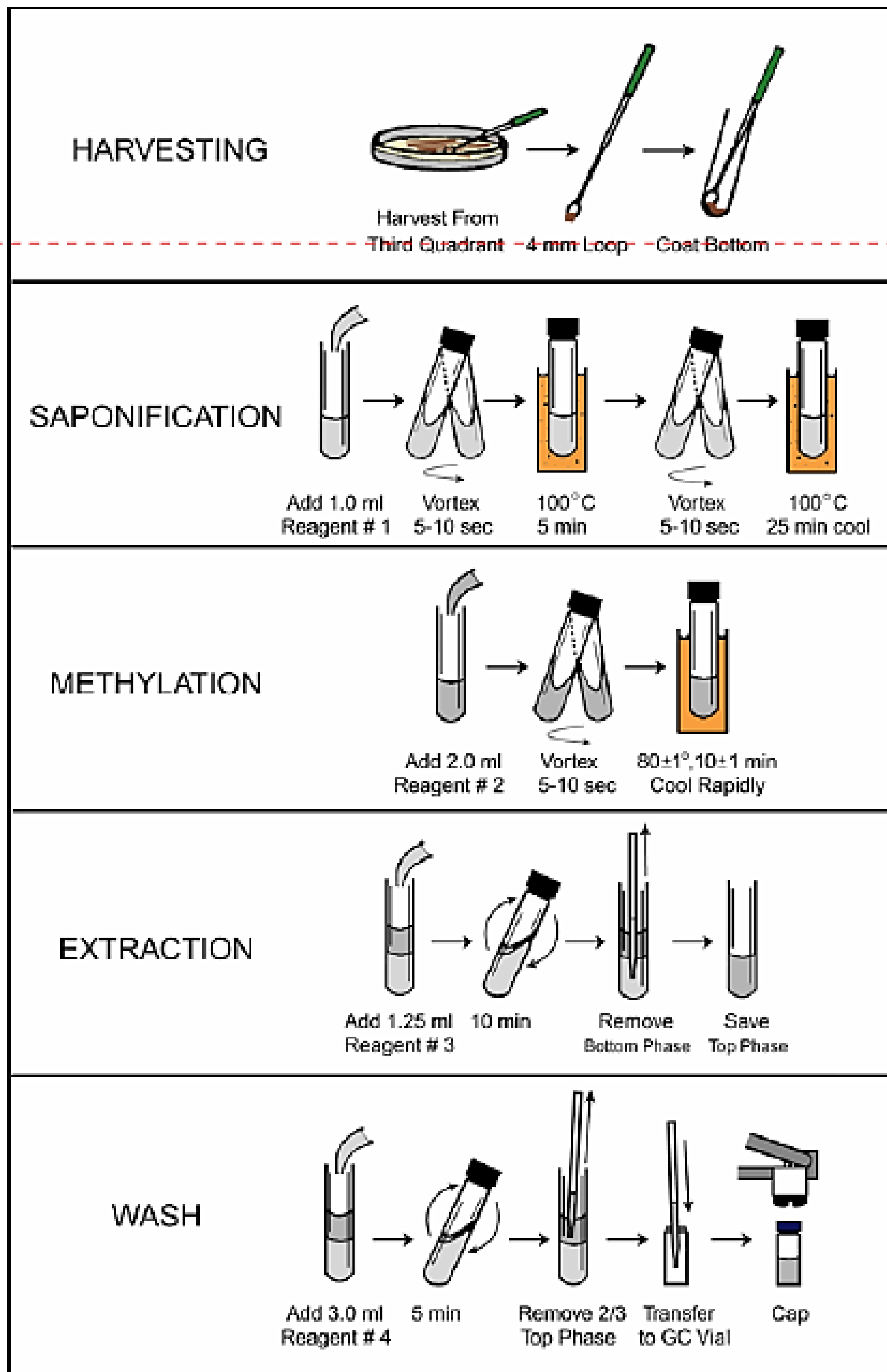

Figure 2.2 GC extract preparation steps for fatty acid characterisation (Source-Figure 2-4, Sherlock, Microbial identification system, version 6.3, operating manual, September 2015)

2.7 PHYLOGENETIC IDENTIFICATION

The phylogenetic identification of isolated bacterial cultures was performed by following steps of DNA extraction, amplification and sequencing of 16S rRNA gene and 16S rRNA gene sequence data analysis.

2.7.1 DNA Extraction

DNA extraction method mentioned by Griffiths *et al.* (2000) was followed in the present study. This procedure mainly consists of three steps; reagent preparation, Zirconia beads washing followed by actual DNA extraction procedure.

2.7.1.1 Reagent preparation

Before proceeding to actual test procedures, all required materials such as Eppendorf tubes, micropipette tips, bead beating sample tubes, and deionized water were autoclaved.

Required reagents; 5% CTAB/phosphate buffer, 30% polyethylene glycol 6000 (PEG 6000, Fisher, p/3677/08) in 1.6M NaCl solution and 70% v/v ethanol were prepared as mentioned in **Table 2.12**. MilliQ ultrapure deionized water prepared in the lab was used throughout for the reagent preparation. Phenol:Chloroform:Isoamyl alcohol (25:24:1, P:C:I, Acros Organics, ACRO327111000) was procured from the vendor.

Table 2.12 Reagents prepared for DNA extraction

Reagent	Chemicals	Preparation
10% CTAB in 0.7 M NaCl (Reagent A)	NaCl cetyl trimethylammonium bromide (CTAB, Fisher Scientific, 10203330) deionized water	0.7M NaCl was prepared by diluting 4.09 g NaCl to final volume of 100 ml with deionized water. 10 g CTAB taken in a 100 ml volumetric flask mixed with 0.7M NaCl up-to volume mark
240mM potassium phosphate buffer (pH 8) (Reagent B)	K_2HPO_4 dibasic (Fisher Scientific, EC 231-834-5) KH_2PO_4 (Fisher Scientific, P/4800/53) deionized water	8.71 g K_2HPO_4 was diluted to 50 ml final volume with deionized water to make 1M K_2HPO_4. 1.36 g of KH_2PO_4 was diluted to 10 ml final volume with deionized water to make 1M KH_2PO_4. 22.56 ml of 1 M K_2HPO_4 solution then mixed with 1.44 ml of 1M KH_2PO_4 solution in another bottle and final volume was made up to 100 ml with deionized water. pH was checked and adjusted if required.
5% CTAB/phosphate buffer (120 mM pH 8)	10% CTAB in 0.7 M NaCl 240 mM potassium phosphate buffer	Solutions prepared in previous steps; 100 ml each of reagent A and reagent B were mixed and autoclaved.
30% PEG 6000 in 1.6 M NaCl solution	NaCl polyethylene glycol (PEG) 6000 deionized water	4.67 g NaCl was diluted to 50 ml final volume with deionized water to make 1.6 M NaCl solution. 15 g of PEG 6000 then mixed with 50 ml 1.6M NaCl, and autoclaved.
70% (v/v) ethanol	absolute ethanol (Fisher bioreagents, BP2818-500) deionized water	70 ml of absolute ethanol was mixed with 30 ml of autoclaved deionized water in a sterile bottle.

2.7.1.2 Zirconia beads washing

Zirconia beads (Stratech, 11079101Z-BSP, 0.1 mm diameter) used for cell disruption during DNA extraction were washed and de-contaminated. Required amount of Zirconia beads taken in a glass tray were soaked in 0.5% (v/v) liquid bleach solution. After 1 h of soaking, bleach was removed, and beads were washed 4-5 times with plain distilled water or until discarded waste showed pH of plain distilled water. Washed beads were dried overnight at 60 °C and the bead crust formed was broken. These beads were then baked at 150–200 °C for 4 h. These beads were then stored aseptically, and care was taken not to touch these beads with bare hands.

2.7.1.3 DNA extraction steps

In bio-safety cabinet rice grain sized freshly grown, pure bacterial culture was suspended in 0.5 ml CTAB buffer, mixed well with pipetting, and transferred to the bead beating tube (tube- Fisher Scientific, 11681350, with caps of O-ring Fisher scientic 11621270) containing approx. 0.5 gm (1/4th of the tube) pre-washed zirconia beads.

Further extraction steps were carried out in a fume hood. 0.5 ml P:C:I (bottom layer) was added to the bacterial suspension tubes prepared in previous steps. During DNA extraction procedure, care was taken while adding P:C:I as phenol drips quickly. After every usage, the P:C:I bottle lid was tightly closed, and all P:C:I contaminated waste was disposed of in a segregated phenol waste container.

These P:C:I added microbial tubes were then allowed to bead-beat in a lab homogenizer (MP Biomedicals) for two runs of 20 s at 4 m/s (with a pause of 30 s in between two runs), immediately cooled on ice for 4–5 min and centrifuged at 13000 rpm for 5 min. Up to 450 ul of the upper aqueous layer was transferred to a fresh, sterile Eppendorf tubes, mixed with 1 ml of 30% PEG 6000 prepared in 1.6 M NaCl and inverted 3–4 times. To precipitate the DNA, these tubes were left on bench undisturbed (minimum 2 h but best if kept overnight).

Next day, these tubes were centrifuged (13000 rpm, for 5 min) to get the DNA pellet. During centrifugation, the hinge of the tubes was always faced outward, to know where the pellet will be. Post centrifugation, tubes were removed carefully, and the supernatant was discarded directly without using the micropipette as some supernatant remained behind in the tube has no effect on DNA recovery. Further,

after addition of 1 ml of 70% v/v ice cold ethanol these tubes were inverted 5–6 times, and re-centrifuged at 13000 rpm for 2 min. Ethanol was removed from these tubes as much as possible with a 1000 µl pipette without touching the walls or bottom of the tube. After giving a short spin to the empty tubes (13000 rpm for 1 min), extra ethanol is removed very carefully with a 100 µl pipette. Tubes were then air dried in the bio-safety cabinet for 10–15 min. Over drying of the DNA pellet was avoided at this step. To re-suspend this dried DNA, 50 µl of sterile deionized water was added.

Before proceeding into next steps of molecular studies, all extracted DNA and PCR amplicon products (5 µl) were analysed by gel electrophoresis on 1% agarose gel (Electran DNA grade, BDH Prolabo, 438792U) prepared in 1X Tris Borate EDTA (TBE, Sigma-Aldrich, T4415) buffer and 2 µl of ethidium bromide (Sigma-Aldrich, E1510). Sample loaded gel was run at 96 volts for 25 min. DNA Ladder mix (GeneRuler DNA Ladder mix, Thermo Scientific, SM0333) was also run as a reference. Post run, the DNA bands developed on agarose gel were observed under ultraviolet light. The required appropriate dilution of the extracted DNA to be taken for PCR cycle and the quality of PCR amplicons were decided from their band intensity and quantity observed in their gel electrophoresis. Pure DW was also tested as a negative control and QC validation of DNA extraction and PCR reagents.

2.7.2. Amplification and Sequencing of the 16S rRNA Gene

DNA extraction was followed by polymerase chain reaction (PCR) and sequencing of the bacterial 16S rRNA gene from an extracted DNA using two universal bacterial primers (Sigma), forward primer 27f (5' AGAGTTTGATCCTGGCTCAG) and reverse primer 1492r (5' TACGGYTACCTTGTTACGACGAC). These primers are known to amplify approximately 1400-1550 bp length of the bacterial 16S rRNA gene (Frank *et al.* 2008; Wilson *et al.* 1990). The PCR reagent mixture used in the PCR reaction per sample tube contained 0.1 µl of each forward and reverse primer (of stock concentration 100µM), 12.5 µl of 2x PCRBIO Taq Mix red (PCRBiosystems, PB10.13-02), 11.8 µl of PCR grade water and 0.5 µl of extracted DNA to make final total volume of 25 µl. For PCR reaction, the appropriate dilution of an extracted DNA was mixed with reagents and was subjected to PCR cycle in a PCR minicycler. The PCR parameters of minicycler were set with initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for

1 min and 72 °C for 4 min, with a final extension at 72 °C for 8 min. The PCR machine lid was maintained at 105 °C.

Further, PCR amplified products were purified using GenElute^{TM} PCR clean-up kit (Sigma-Aldrich, NA1020) according to the manufacturer's instructions and the purified 16S rRNA PCR amplicons were then taken for Sanger sequencing. For majority of the samples, sequencing service was outsourced to Eurofins Genomics (GATC Biotech), Germany using their 96 well plate and 50 tube analysis service. GATC service for 96 well plate offered Sanger sequencing up to 1100 nucleotides in Phred20 quality. Some of the samples were also sequenced from Core Genetics Service at University of Sheffield, and School of Life Sciences, University of Nottingham, UK.

2.7.3 Analysis of the 16S rRNA Gene Sequence Data

The 16S rRNA gene sequence chromatograms obtained through Sanger sequencing were manually checked at Edge Hill University for weak/small or mis-placed nucleotide peaks and 'N' calls (uncalled bases), followed by trimming of low-quality bases present at both the ends of the sequence. A dataset was created of these quality-checked 16S rRNA gene sequences. Putative phylogenetic identification of bacterial isolates was performed by comparing this dataset against 16S rRNA gene sequences of the type strains available on NCBI's blastn (National Center for Biotechnology Information, n.d., viewed June 2020) and EzBioCloud BLAST (EzBioCloud, ChunLab, May 2020, viewed June 2020) web portals.

Microbial isolates were processed for these steps of DNA extraction, sequencing and phylogenetic identification in batches during the period 2017–2019, additionally their BLAST results were reconfirmed altogether in June 2020 before putting into this report.

The obtained BLAST results were also analysed for novel species identification. In novel species characterisation, the BLAST search performed for the potential novel isolates MARW_1-7-2^{T} and MARW_1-2-27^{T} showed their phylogenetic similarity to the genus *Halomonas* and *Pseudoalteromonas* respectively. For MARW_1-7-2^{T} and MARW_1-2-27^{T}, further confirmation of these BLAST results was carried out by constructing an independent phylogenetic tree using 16S rRNA gene sequence retrieved from their draft genome data (**Chapter 4**).

Chapter 3

Cultivation, Isolation and Identification of Microbes from Salt Marsh Environments in the UK

3.1 INTRODUCTION

Salt marshes are upper coastal intertidal marshy zones which exist between open sea and land and are characterised by continuous alternating tidal cycles of inundation and drainage with seawater (Hemminga *et al.* 1993) and development of a salinity gradient across these areas (Joye *et al.* 1995; Kim *et al.* 2010a; Sauret *et al.* 2016). The pronounced sink of organic and inorganic components further contributes to the generation of chemical gradients across these ecosystems (Cook *et al.* 2004; Gallagher *et al.* 1974). Varying concentrations of these components in different seasons (Bolhuis *et al.* 2011), elevations (Smith *et al.* 2002; Marton *et al.* 2015) etc. are known to shape microbial diversity and abundance. Diverse group of microbes residing in salt marshes have active roles in protecting salt marsh morphodynamics and functionality (Grant *et al.* 1987; Stal et al. 1994; Van gemerden 1993; Stal et al. 2010; Van Gemerden 1993), promoting various geochemical cycles (Parkes *et al.* 2012; Bernhard *et al.* 2010; Senior *et al.* 1982; Banat *et al.* 1981), plant growth (Rocha *et al.* 2016), bioremediation (Oliveira *et al.* 2014) etc.

3.1.1 Study Sites

Two UK-based marsh sites- RSPB Marshside and Fingringhoe Wick Nature Reserve, situated at North-West and Anglian region of England respectively (**Fig. 3.1**) were evaluated for microbial analysis in the present study. These geographically separated salt marshes have a distinct climate throughout the year (**Fig. 3.2**). The RSPB Marshside Nature Reserve area is generally cooler and wetter. It has a cool summer and mild climate in winter. Whereas Fingringhoe Wick Nature Reserve has comparatively warm and dry summer and a mild winter (Met Office, n.d., viewed December 2020).

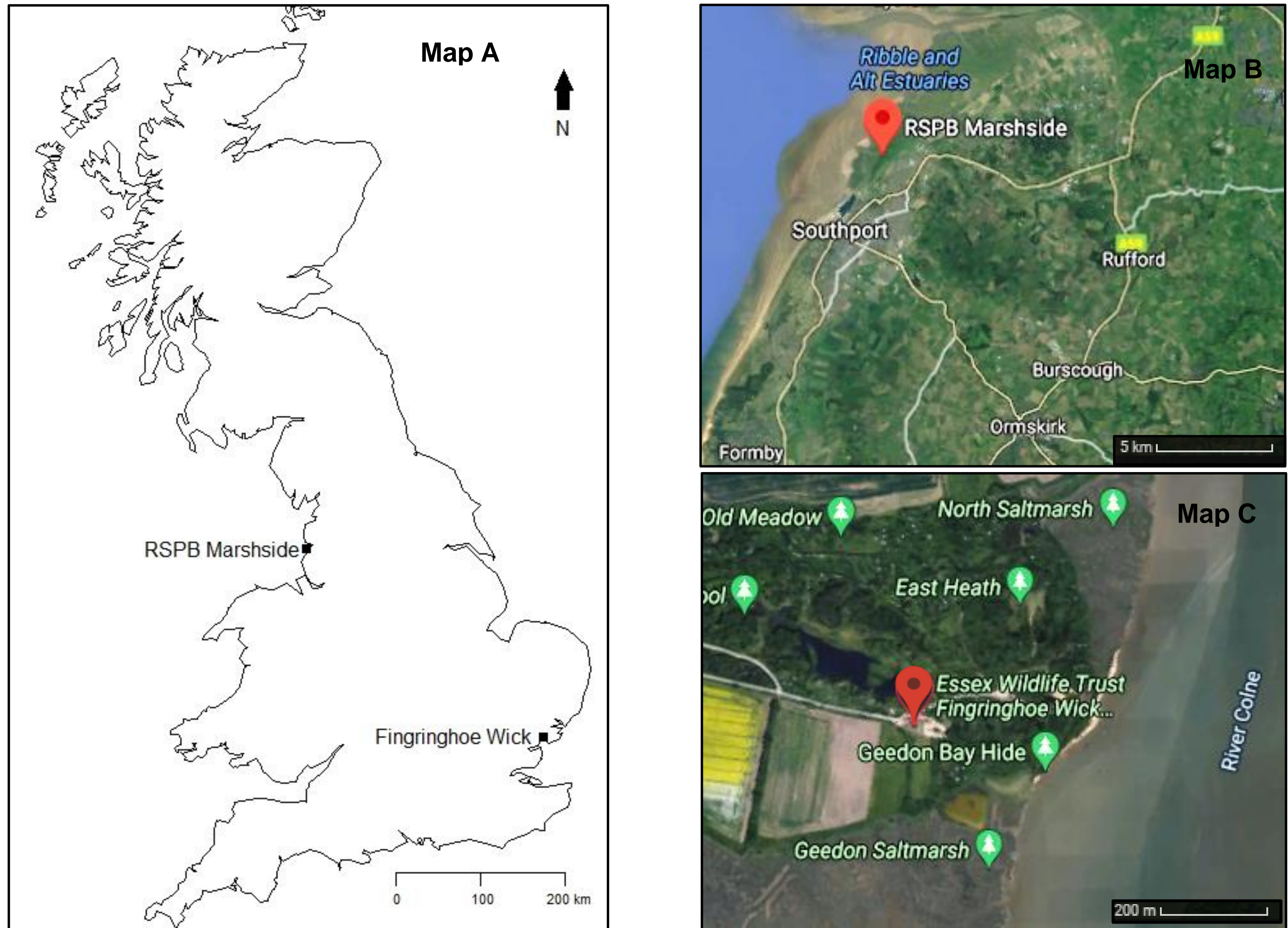

Figure 3.1 Map A, Salt marsh sample collection sites in England, UK. Map B, RSPB Marshside Nature Reserve. Map C, Fingringhoe Wick Nature Reserve (North salt marsh and Geedon salt marsh)

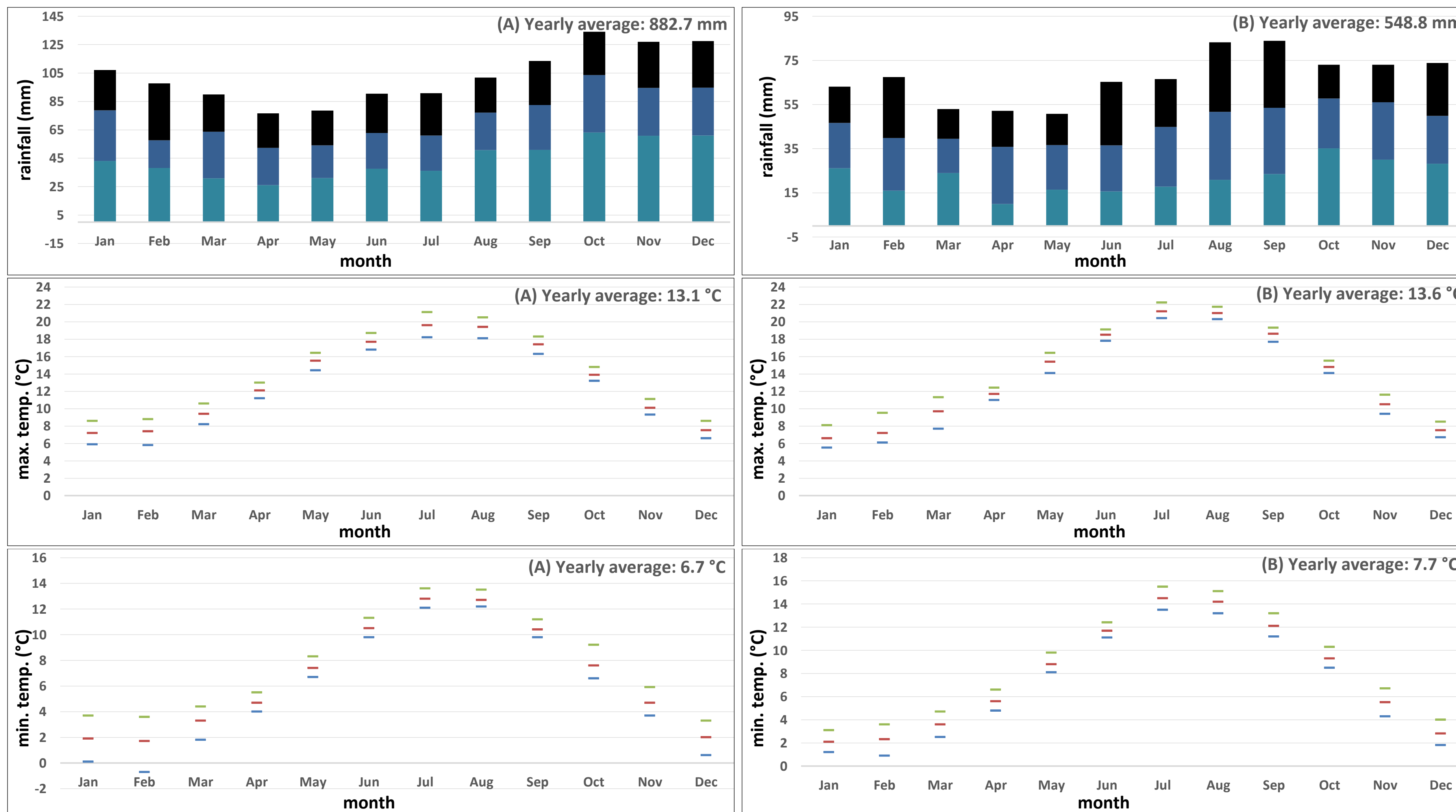

Figure 3.2 Climate comparison between study site locations, RSPB Marshside ('A' graphs) and Fingringhoe Wick Nature Reserve ('B' graphs) for the period 1981-2010. Each data point represents 20th and 80th percentiles, and the average of monthly observations (Met Office, n.d., viewed December 2020).

RSPB Marshside and Fingringhoe Wick Nature Reserve are located at the coastal wetlands of Ribble and Colne estuary respectively, have Site of Special Scientific Interest (SSSI) designation (Nature England, n.d.(a), viewed June 2020; Nature England, n.d.(b), viewed June 2020), considerable marsh areas and are known for their restoration success.

3.1.1.1 The RSPB Marshside Nature Reserve

The RSPB Marshside Nature Reserve was established on the Ribble estuary, North-West of England in 1994. The Ribble estuary (total area 9348.70 ha) is located on the Lancashire coast, West of Preston between Southport and Lytham St. Annes and extends inland to Longton (Nature England, n.d.(a), viewed June 2020; Ribble estuary, 1984). This estuary has extensive intertidal sand silt flats and has one of the largest green marshes in Great Britain. This estuary constitutes an actively developing salt marsh of 'Littoral sediment' and 'Neutral Grassland – Lowland' habitat types and covers 2000 ha area in total (Nature England, n.d.(a), viewed June 2020; Ford *et al.* 2012).

Since 1994, salt marsh of RSPB Marshside has undergone significant restoration as a result of RSPB management activities such as improving the extent and quality of salt marsh grassland biodiversity by re-planting halophytic plants, infrequent grazing and topping, as well as regular monitoring for sward height, cattle numbers, and water quality and levels. To increase the extent of open water, new ditches and small pools are being created followed by their rotational cleaning and re-profiling. Meadows were restored with an open structure. As a result, it now protects 110 ha of coastal grassland and pools, and 230 ha of salt marsh on the shores of the Ribble estuary. RSPB Marshside is also providing a nesting place and habitat for birds which are declining elsewhere (such as lapwings and redshanks) and a diverse range of halophytic plants (Baker 2009; Nature England, n.d.(a), viewed June 2020; The Royal Society for the Protection of Birds, n.d.).

A number of studies were previously published describing various aspects of coastal areas near the Ribble estuary. They include benefits of coastal managed realignment such as flood risk protection observed at Hesketh Out Marsh West (MacDonald *et al.* 2020); recommendations for water management practices (Watson and Howe 2006); descriptions of faunal growth and development (Thompson *et al.* 1990); the spread and movements of fauna in the estuary being

affected by the sediment salinity, mud (Popham 1966) and dissolved oxygen (Priede *et al.* 1988); seasonal variations as the key drivers of greenhouse gas emissions in the salt marshes rather than the grazing patterns (Ford *et al.* 2012); the assessment of radioactivity concentrations received at Ribble estuary from Sellafield and Springfield discharges (Mamas *et al.* 1995; Brown 1997; Brown *et al.* 1999; Sneddon 2017). The few microbial studies conducted at the Ribble estuary have been restricted to the detection of bacterial contamination in the surface water in terms of occurrences of faecal indicators (Kay *et al.* 2005; Wither *et al.* 2005; Gao *et al.* 2015; Hassard *et al.* 2017). However, no study has ever assessed phylogenetic and physiological microbial diversity at RSPB Marshside or any other Ribble estuary sites.

3.1.1.2 Fingringhoe Wick Nature Reserve

The Essex coastline in the South-East of England has approximately 5,000 ha of salt marshes (Harmsworth and Long 1986). It includes an internationally important intertidal habitat called Fingringhoe Wick Nature Reserve situated on the Colne estuary, South of Colchester (Fautley and Garon 2004). The Colne estuary is a typical narrow funnel-shaped estuary, approximately 12 km long, and has branching with five tidal arms that flow into the main channel of the River Colne with a total area of 2,335 ha. Its intertidal area covers 2,002 ha comprising 700 ha of mudflats and 300 ha of salt marsh. Currently, the largest extent of salt marsh in the Colne estuary lies within the Fingringhoe as well as Brightlingsea and Mersey triangle (Thomson *et al.* 2011). Fingringhoe Wick Nature Reserve land was previously used for gravel extraction and farming which was later purchased and developed into a Nature Reserve by Essex Wildlife Trust in 1975, creating a new habitat for rare and endangered species and protecting the local wildlife. Its tidal channels and marshland stretch into an enormous expanse of salt marshes, which is comprised of Geedon salt marsh and North salt marsh. Both are a littoral sediment type of habitat (Fautley and Garon 2004; Nature England, n.d.(b), viewed June 2020). Its semi-improved grassland is divided by the straight drainage ditches, water filled fleets and creeks (Colchester Borough Council 2020, viewed June 2020). Fingringhoe Wick Nature Reserve now protects approximately 273.9 ha of salt marsh area.

‘Coastal squeeze’ that is erosion along the edges of the main channels and creeks were reported at salt marshes in Essex. Between 1988 and 1998 these marshes

were also reported to have lost an area of over 300 ha, including a loss of 49.5 ha in the Colne estuary (Cooper *et al.* 2001; Leeds 2016). Due to the existing vulnerability to flooding and poor agricultural value, the need for managed realignment was identified at Fingringhoe Wick marsh site. As a result, an additional 22 ha of new wetland was created in 2015 by partially breaching its enclosed sea wall to cause flooding and development of a new area of salt marshes, mudflats and saline lagoons (Leeds 2016).

Microbial studies at Colne Point salt marsh, Alresford Creek, Ray Creek, Pye-fleet mudflat, and Hythe, Wivenhoe, and Brightlingsea sites of Colne Estuary (except Fingringhoe Wick Nature Reserve) are reported earlier for their archaeal studies (Munson *et al.* 1997) and active roles of microbial populations in carbon and sulphur turnover (Purdy *et al.* 2002a; Purdy *et al.* 2002b), microbial responses to anthropogenic activities (Coulon *et al*. 2007; Purdy *et al.* 2002; McKew *et al.* 2007; Balba and Nedwell 1982; Exton *et al*. 2012; Johnston *et al.* 2017) etc.

However, all previous documented studies involving the Fingringhoe Wick Nature Reserve have been non-microbiology studies, such as describing the importance of canopy complexity as a habitat for spiders and beetles (Ford *et al.* 2017) and the dynamics of soil erosion in relation to plant diversity (Ford *et al.* 2016). At the time of writing, no studies describing the salt marsh microbial diversity of Fingringhoe Wick have been reported.

Thus, from this description of the Ribble and Colne estuaries it is clear that more microbiology literature is available on the Colne estuary compared to the Ribble estuary at the time of writing this chapter. Additionally, cultivation-based studies of RSPB Marshside and other Ribble estuary environments which could explore the innate microbial or physiological diversity of these sites have not been previously reported.

3.1.2 The Present Study

As salt marshes support enriched microbial diversity (Navarro-Torre *et al.* 2016; Gayathri *et al*. 2010), various natural and anthropogenic factors further shape both composition and physiological processes of these ecosystems and the diversity that it accommodates (Córdova-Kreylos *et al*. 2006; Chaudhary *et al*. 2018; Bardgett *et al.* 2008; Webster *et al*. 2015). Despite their potential roles in the maintenance of ecosystems and biotechnological applications (Sánchez-Porro *et al.* 2003; Rao *et*

al. 2010; Gangola *et al*. 2018), many salt marshes in the UK remain untouched by microbial evaluations. Available UK literature studies are focused on a few specific marsh sites and are limited to molecular-based approaches, due to which there is still a large pool of uncultured microbes in these environments which remain unexplored (Salman *et al.* 2015; Mußmann *et al.* 2005).

Considering the existence of diverse microbial populations and the deficit of cultivation-based microbial findings in the former salt marsh literature at study sites, the present study is aimed at addressing this knowledge gap. This was achieved by exploring its bacterial niches, with an emphasis on a cultivation and isolation technique that also included molecular-based 16S rRNA gene phylogenetic identification of isolated microbes. With this approach it became possible to describe the isolated microbes up to genus level and discover novel microbes that may be of great scientific interest. An additional work was also performed in the present study to screen the salt marsh microbes of potential biotechnological benefits. Isolates were tested for antimicrobial production against both Gram-positive and Gram-negative bacteria by the agar diffusion method. These study findings are expected to further extend our knowledge of these gradient-rich saline environments.

Thus, the aims of this study can be summarised as

1) To explore culturable microbial diversity of study sites via microbes' 16S rRNA gene-based phylogeny, cytology and physiology examinations
2) Discover potential novel microbes based on phylogenetic identification
3) Analyse salt marsh isolates for salt tolerance and antimicrobial production

3.2 METHODS

3.2.1 Sample Sites

Two salt marsh sites were sampled in this study, RSPB Marshside, Southport, and Fingringhoe Wick Nature Reserve, Essex (**Fig. 3.1)**. Permission to access the former site was granted by the site warden of the Ribble Reserves, whereas Fingringhoe Wick Nature Reserve was accessed through the induction conducted at School of Life Sciences, Essex University.

3.2.2 Sample Collection and Physico-chemical Analysis

Three sampling visits were conducted at RSPB Marshside on 8th June 2017, 25th October 2017 and 18th July 2018. Water samples collected in 2017 were processed for microbial cultivation and physico-chemical parameters, while the samples collected in 2018 were included for additional measurement of physico-chemical parameters. At Fingringhoe Wick, an almost equal number of samples were collected from each of North salt marsh and Geedon salt marsh on 3rd November 2017. Water and soil samples from these sites were also processed for physico-chemical and microbial analysis.

The number of samples collected at these sites and their GPS co-ordinates are described in **Table 3.1**. GPS co-ordinates were not taken at Fingringhoe Wick salt marsh, but water samples 1–2, and soil samples 1–3 were collected from North salt marsh. Water sample 3–4 and soil samples 4–5 were collected from Geedon salt marsh (**Fig. 3.1, Map C**). The sampling spots within the RSPB Marshside are shown in **Fig. 3.3**.

Further procedural details on sample collection and physico-chemical analysis are described in **chapter 2, section 2.1**.

Table 3.1 Sampling details

Abbreviations: MARW_1, MARW_2, MARW_3 = Water samples collected from RSPB Marshside on 8th June 2017, 25th October 2017 and 18th July 2018 respectively; FINW_1, FINS_1 = Water and soil samples respectively collected from Fingringhoe Wick on 3rd November 2017; * = Sample collected from low marsh area; N/A = Not available

Salt marsh site	Sample collection date	Sample size		Sample no.	Actual sample ID	GPS coordinates
		Water	Soil			
RSPB Marshside, Southport	8th June 2017	10	-	1	MARW_1-1*	53°40'44.0"N 2°59'59.0"W
				2	MARW_1-2*	53°40'46.0"N 2°59'56.0"W
				3	MARW_1-3*	53°40'46.0"N 2°59'57.0"W
				4	MARW_1-4	53°40'47.0"N 2°59'49.0"W
				5	MARW_1-5	53°40'46.0"N 2°59'45.0"W
				6	MARW_1-6	53°40'45.0"N 2°59'44.0"W
				7	MARW_1-7	53°40'46.0"N 2°59'45.0"W
				8	MARW_1-8	53°40'45.0"N 2°59'39.0"W
				9, 10	MARW_1-9, MARW_1-10	53°40'47.0"N 2°59'48.0"W
	25th October 2017	2	-	-	MARW_2-1*	53°40'45.0"N 2°59'59.0"W
					MARW_2-2*	53°40'46.0"N 2°59'56.0"W
	18th July 2018	3	-	-	MARW_3-1*	53°40'42.2"N 2°59'54.4"W
					MARW_3-2*	53°40'45.1"N 2°59'59.7"W
					MARW_3-3*	53°40'46.1"N 2°59'58.4"W
Fingringhoe Wick, Essex	3rd November 2017	4	5	-	FINW_1-1*	N/A
					FINW_1-2*	
					FINW_1-3	
					FINW_1-4	
					FINS_1-1*	
					FINS_1-2*	
					FINS_1-3*	
					FINS_1-4	
					FINS_1-5	
Total		19	5	-	-	-

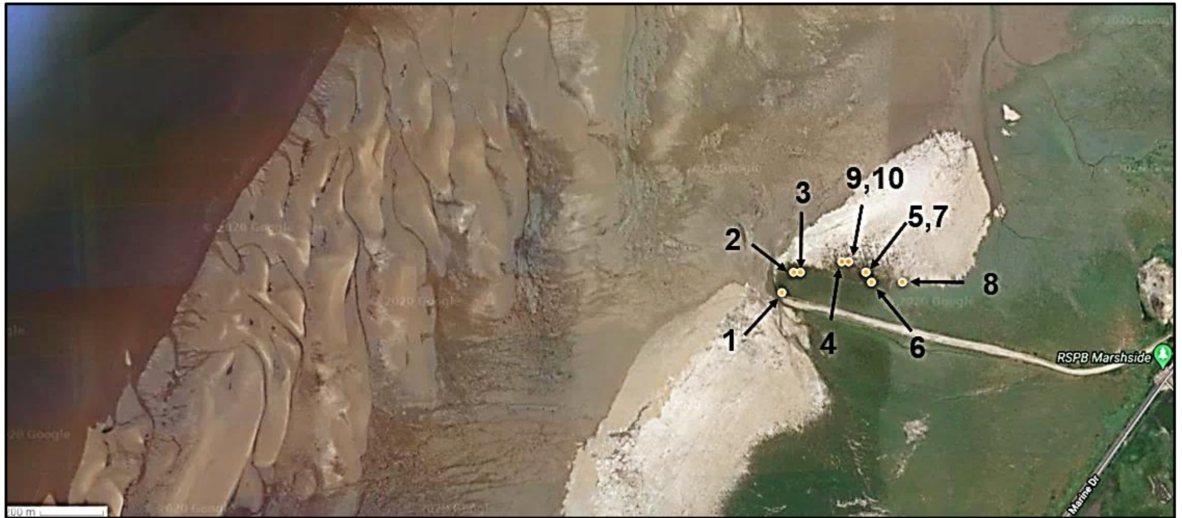

Figure 3.3. Sampling spots within RSPB Marshside Nature Reserve. Samples collected from low marsh (samples 1–3) and mid marsh (samples 4–10). Map scale, 200m

3.2.3 Microbial Cultivation, Isolation and Culture Maintenance

Collected samples were analysed for microbial cultivation, isolation and culture cryopreservation as per the procedure elaborated in **chapter 2, section 2.2**. In summary, original and diluted water and soil (10% w/v) samples were inoculated on different growth media such as marine agar (MA, Difco™, 2216), R2A agar (R2A, Oxoid™, CM0906) and nutrient agar (NA, Oxoid™, CM0003). Each media was prepared with two different sets of pH and NaCl concentrations. Additionally, for the RSPB Marshside water samples, oligotrophic growth experiments were performed by incubating samples in two different sets of lower concentrations of peptone and yeast extract followed by inoculations on solid MA, R2A and NA media plates. After primary inoculation experiments, MA medium was exclusively chosen for further sample cultivation studies.

Individual representative colonies picked from the agar plates were cryopreserved and later processed for cytology, physiology and salt tolerance studies as well as for DNA extraction and phylogenetic identification by Sanger sequencing, and antimicrobial screening.

3.2.4 Cytology and Physiology Observations

While processing the salt marsh microbial isolates for cryopreservation, a portion of the isolates that resembled diverse colony morphologies were picked for cellular and physiological characterisation. Thirty two isolates were studied for Gram-staining by Hucker's modified method 'A' (Hucker and Conn 1923), motility test by the hanging drop method (Gerhardt *et al.* 1981), catalase test using 3% H_2O_2 (Reiner 2010) and oxidase test using TestOxidase™ (Pro-Lab diagnostics, PL 390) reagent. For quality control (QC) of all these procedures, *Escherichia coli* (DSM 30083^T) and *Micrococcus luteus* (NCTC 2665^T) cultures were used. A detailed explanation of these procedures and their test result interpretations are described in **chapter 2, section 2.3**.

3.2.5 Salt Tolerance Study

A subset of the microbial isolates cultivated from RSPB Marshside were also identified for their halotolerance and were further investigated regarding their phylogenetic identification in October 2017. For this purpose, 46 isolates were chosen from the available culture pool, in the order that they were originally cryopreserved. A salt tolerance study of these isolates was performed by inoculating freshly revived pure cultures on MA growth media plates supplemented with 2, 4, 8, 12 and 16% (w/v) NaCl concentrations at pH 7.6±0.2. Inoculated cultures were allowed to grow at 30 °C and were checked for the presence or absence of microbial growth throughout the total incubation period of 13–15 days. Of these isolates screened at Edge Hill University, 15 halotolerant isolates were also taken to School of Life Sciences, Essex University for DNA extraction and phylogenetic identification as a part of my planned induction program (sequencing outsourced to the University of Nottingham).

3.2.6 Phylogenetic Identification

Study salt marsh isolates including halotolerant microbes were phylogenetically identified by performing a procedure as described in detail at **chapter 2, section 2.7**.

In summary, approximately 190 cryopreserved isolates were taken for phylogenetic identification, of which about 60% were water isolates cultured from RSPB Marshside and remaining 25% and 15% were soil and water isolates from Fingringhoe Wick respectively. These isolates from both the sites were chosen in

the order they were originally cryopreserved and also those who showed maximum halotolerance. These cryopreserved microbes were revived by passaging twice on a growth media (on which they were originally isolated) and then sequentially analysed for DNA extraction (Griffiths *et al.* 2000), PCR and purification of PCR amplicons (GenElute^{TM} PCR clean-up kit, Sigma-Aldrich, NA1020) at Edge Hill University, Biology lab. The concentration and purity of all extracted DNA and PCR amplicons was confirmed via their gel electrophoresis on 1% agarose gel in 1X TBE buffer. The sequencing of PCR purified amplicons of the isolates was outsourced to three institutes, Eurofins Genomics (former GATC Biotech), Germany, as well as to Core Genetics Service at University of Sheffield, and School of Life Sciences, University of Nottingham, UK. Two universal primers specific for the bacterial 16S rRNA gene, forward primer 27f and reverse primer 1492r were employed for PCR (Frank *et al.* 2008; Wilson *et al.* 1990). Both these primers were also used in sequencing, except at School of Life Sciences, University of Nottingham, UK where only reverse primer was used.

A putative phylogenetic identification of isolates was performed by comparing their quality checked sequences with the 16S rRNA gene database of type strains available on two webtools, NCBI blastn (National Center for Biotechnology Information, n.d., viewed June 2020) and EzBioCloud web portals (EzBioCloud, Chunlab, May 2020, viewed June 2020). Though DNA extraction and subsequent phylogenetic identification was performed in batches during the period 2017–2019, their BLAST results were reconfirmed altogether in June 2020 before putting into this report. The BLAST results were also re-analysed for novel species identification.

Phylogenetic tree analysis based on 16S rRNA gene sequence was conducted for isolates collected from both sites with MEGA7.0 (Kimura 1980; Kumar *et al.* 2016). 16S rRNA gene sequence of their closest phylogenetic relatives were also included to represent the broadest diversity of bacteria. The evolutionary history and evolutionary rate differences among sites were inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model and discrete Gamma distribution model respectively. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. In aligned

sequence, all positions containing gaps and missing data were eliminated. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

3.2.7 Screening for Antimicrobial Production

A total of 69 isolates were selected from the pool of phylogenetically identified microbes of this study. They were additionally screened to test their potential for antimicrobial production. Each isolate was assayed in triplicate using the double-layer agar diffusion method (modified method of Zhu *et al.* 2014). The antimicrobial inhibition was tested against both Gram-positive and Gram-negative indicator bacteria, *Escherichia coli* (DSM 30083^T) and *Micrococcus luteus* (NCTC 2665^T) respectively.

Freshly grown salt marsh isolate was spot inoculated at the centre of sterile media plate using a sterile wire-loop. Growth media for the isolates was prepared with 0.9 % (w/v) marine broth,1.5% (w/v) agar, 4% (w/v) NaCl and pH 7.6±0.2. Marine agar was prepared at its $1/4^{th}$ strength of what is normally suggested by the manufacturer, to create nutritionally depleting environment that was expected to initiate microbial antibiotic production (Falkinham *et al.* 2009). The inoculated plates were then incubated for 15–17 days at 30 °C. The longer incubation period favoured the nutrient depletion, creation of stressed environment and hence offered increased chances of antimicrobial production.

After 15–17 days of incubation, the indicator strains were prepared for overlay. The freshly grown broth cultures of indicator strains *E. coli* and *M. luteus* were individually mixed in a 15 ml sterile, molten nutrient agar butt (with 0.7% agar and pH 7.4±0.2) at final concentration of 0.03–0.05 OD_{650nm}. Sterile molten agar butt containing one of the indicator strains then overlaid on the isolate's incubated plates, surrounding the isolates growth area. After the media was set properly, all the plates were incubated for 24–48 h, at 37 °C and 30 °C for indicator strains *E. coli* and *M. luteus* respectively. Antibiotic activity was assessed by observing the inhibition zone of the indicator strain around a test colony. Media controls were kept to check the phage or bacterial contamination.

3.3 RESULTS

3.3.1 Sample Collection and Physico-chemical Analysis

Physico-chemical analysis results of salt marsh samples collected at RSPB Marshside and Fingringhoe Wick Nature Reserve are presented in **Table 3.2.**

Table 3.2 Physico-chemical parameters of samples collected at the salt marshes of RSPB Marshside and Fingringhoe Wick Nature Reserves. The cumulative physico-chemical results of multiple samples were reported as the observed range with its mean written in parenthesis, except for MARW_2, FINW_1 and FINS_1 (mid marsh) samples where only two samples were collected and analysed

Abbreviations: MARW_1, MARW_2, MARW_3 = Water samples collected from RSPB Marshside on 8th June 2017, 25th October 2017 and 18th July 2018 respectively; FINW_1, FINS_1 = Water and soil samples respectively collected from Fingringhoe Wick on 3rd November 2017; N/A = Not available

Parameters	**MARW_1**		**MARW_2**	**MARW_3**	**FINW_1**		**FINS_1**	
	Low marsh	**Mid marsh**	**Low marsh**	**Low marsh**	**Low marsh**	**Mid marsh**	**Low marsh**	**Mid marsh**
Salinity (g/100g)	2.4–3 (2.6)	2–2.8 (2.5)	0.8, 1.8	3.6–4 (3.73)	3.3, 3.7	3.7, 3.7	0.4, 0.4, 0.4 (0.4)	0.2, 0.4
pH	7.9–8.2 (8.1)	7.5–8.8 (8.0)	7.6, 7.6	7.5–7.6 (7.5)	7.5, 7.7	7.4, 7.7	N/A	N/A
Phosphate (PPM)	0.5–0.75 (0.67)	0.25–0.87 (0.76)	0.5, 0.5	0.75–1.5 (1)	0.5, 0.5	0.5, 0.5	0–0.25 (0.08)	0.25, 0.75
Iron (PPM)	0.05–0.3 (0.18)	0.1–0.6 (0.24)	0.6, 1.2	0.6–0.8 (0.7)	0.1, 1.2	0.2, 0.4	0–0.1 (0.05)	0, 0.4
Ammonium (PPM)	0–0.5 (0.33)	0–0.5 (0.11)	0.5, 0.5	0.5–1 (0.5)	0.5, 0.5	0.5, 0.55	0–5 (2.66)	0.5, 0.3

3.3.2 Microbial Cultivation and Isolation

After the samples were spread inoculated on the growth media, microbial growth was observed after four to five days of incubation. A few cultures exhibited pleomorphism, such as a change in the colony colour or colony consistency, when incubated or refrigerated for more than a week. Isolates cultivated from study sites were mostly retrieved from marine agar plates (226 out of 287 isolates), whereas the rest were grown on R2A and nutrient agar media.

The total number of pure isolates retrieved from each site are as mentioned in **Table 3.3.** These are now cryopreserved and kept in -80 °C deep freezer at the Biotechnology Laboratory, Technology Hub, Edge Hill University.

Table 3.3 Details of the number of samples collected and microbial isolates cultivated from these samples
Abbreviation: N/A = Not applicable

Sampled salt marsh sites	Sample collection date	Sample size		No. of pure microbial isolates obtained from samples	
		Water	Soil	Water	Soil
RSPB Marshside, Southport	Jun. 2017	10	N/A	145	N/A
	Oct. 2017	2	N/A	39	N/A
Fingringhoe Wick, Essex	Nov. 2017	4	5	46	57
Total		**16**	**5**	**230**	**57**

3.3.3 Cytology and Physiology Observations

The observations of cytology and physiology tests performed on 32 selected isolates are shown in **Table 3.4**.

Table 3.4 Cytology, motility and physiology observations of microbes from cultured salt marsh population

Abbreviations: + = Positive results; - = Negative results; W+ = Weak positive results

Isolate ID	Gram character	Motility	Oxidase	Catalase
MARW_1-1-1	Gram negative rods	-	+	-
MARW_1-1-2	Gram negative rods	+	+	-
MARW_1-1-3	Gram positive rods	+	+	W+
MARW_1-1-4	Gram positive cocci	+	-	+
MARW_1-1-5	Gram negative rods	-	-	+
MARW_1-1-6	Gram positive rods	-	+	+
MARW_1-1-7	Gram positive rods	+	-	-
MARW_1-1-8	Gram positive rods	-	+	-
MARW_1-1-18	Gram negative rods	-	+	-
MARW_1-2-1	Gram positive cocci	+	-	+
MARW_1-2-2	Gram negative rods	+	-	-
MARW_1-2-3	Gram positive rods	+	+	-
MARW_1-2-27	Gram negative straight rods	+	+	W+
MARW_1-3-1	Gram positive cocci	+	+	-
MARW_1-3-2	Gram negative rods	-	+	-
MARW_1-7-2	Gram negative straight rods	-	+	W+
MARW_1-8-1	Gram negative rods	+	+	+
MARW_1-8-2	Gram positive rods	-	+	+
MARW_1-8-3	Gram positive rods	-	+	+
MARW_1-8-4	Gram positive cocci	+	-	+
MARW_1-8-5	Gram positive rods	+	-	-
MARW_1-8-6	Gram negative rods	-	+	+
MARW_1-8-7	Gram negative rods	-	+	W+
MARW_1-8-8	Gram positive cocci	-	-	+
MARW_1-8-9	Gram positive rods	+	+	+
MARW_1-8-10	Gram negative rods	+	+	+
MARW_1-8-11	Gram negative rods	-	+	+
MARW_1-8-12	Gram positive cocci	+	-	-
MARW_1-8-22	Gram negative rods	-	+	-
MARW_1-10-5	Gram negative rods	-	+	+
MARW_1-10-7	Gram positive cocci	+	+	W+
FINS_1-4-5	Gram negative rods	-	+	W+

3.3.4 Salt Tolerance Study

The salt tolerance capacity exhibited by 46 salt marsh isolates when grown at different NaCl concentrations in MA growth media is shown in **Table 3.5**. All were able to grow up to 8% (w/v) NaCl concentration. Thirty two (70%) could tolerate 2–12% (w/v) NaCl and 18 (39%) were able to grow with NaCl concentration up to 16% (w/v). Fourteen of the isolates which sustain up to 16% (w/v) NaCl, however took one to two weeks to grow at these higher salt concentrations.

Table 3.5 Salt tolerance ability of salt marsh isolates observed on MA growth media supplemented with varying NaCl concentrations

Abbreviations: ++ = Abundant growth observed within 2–3 days of incubation; ++7d = Abundant growth observed within 7 days of incubation; ++13d = Abundant growth observed within 13 days of incubation; +13d = Visible scanty growth observed after 13 days of incubation; - = No growth

Isolate ID	NaCl concentration % (w/v)				
	2	4	8	12	16
MARW_1-1-5	++	++	++	-	-
MARW_1-1-9	++	++	++ 13d	-	-
MARW_1-1-14	++	++	++	++	-
MARW_1-2-4	++	++	++	++	+ 13d
MARW_1-2-10	++	++	++	+ 13d	+ 13d
MARW_1-2-15	++	++	++	++	++ 7d
MARW_1-2-18	++	++	++	+ 13d	+ 13d
MARW_1-2-21	++	++	++	+ 13d	+ 13d
MARW_1-2-22	++	++	++	++ 13d	-
MARW_1-2-26	++	++	++ 13d	-	-
MARW_1-3-1	++	++	++ 13d	-	-
MARW_1-3-2	++	++	++ 13d	-	-
MARW_1-3-3	++	++	++	++ 13d	-
MARW_1-4-1	++	++	++	++	++
MARW_1-4-2	++	++	++	++	-
MARW_1-4-3	++	++	++	++ 13d	-
MARW_1-4-7	++	++	++	++	++ 7d
MARW_1-4-9	++	++	++	++	++
MARW_1-4-10	++	++	++	-	-
MARW_1-4-16	++	++	++	++	++ 13d
MARW_1-4-17	++	++	++	++	++ 7d
MARW_1-5-2	++	++	++	++	-

Table 3.5 continued…

Isolate ID	NaCl concentration % (w/v)				
	2	4	8	12	16
MARW_1-5-4	++	++	++	++	-
MARW_1-5-6	+	+	+	-	-
MARW_1-6-1	++	++	++	++	++ 7d
MARW_1-6-3	++	++	++	++	++
MARW_1-6-10	++	++	++	++	++ 7d
MARW_1-6-11	++	++	++	++	
MARW_1-7-1	++	++	++	++	++ 13d
MARW_1-7-2	++	++	++	++	++
MARW_1-8-4	++	++	++	++	-
MARW_1-8-9	++	++	++	++	-
MARW_1-8-13	++	++	++	++	++ 7d
MARW_1-8-15	++	++	++	++	-
MARW_1-8-16	++	++	++	-	-
MARW_1-8-17	++	++	+ 13d	-	-
MARW_1-8-20	++	++	++	-	-
MARW_1-8-22	++	++	++	++	-
MARW_1-9-3	++	++	++	-	-
MARW_1-10-1	++	++	++	-	-
MARW_1-10-3	++	++	++	-	-
MARW_1-10-5	++	++ 7d	++ 13d	++ 13d	++ 13d
MARW_1-10-6	++	++	++	+ 13d	-
MARW_1-10-7	++	++	++	++	++ 7d
MARW_1-10-8	++	++	++	++ 7d	-
MARW_1-10-9	++	++	++	-	-

3.3.5 Phylogenetic Identification Based on 16S rRNA Gene Analysis

One hundred and thirty-eight good quality PCR purified amplicons were obtained out of 190 isolates processed for DNA extraction and PCR. When these 16S rRNA gene amplicons were analysed on gel electrophoresis along with DNA ladder, the amplified product showed a band at approximately 1500 bp (**Fig. 3.4**). Approx. 70% of these PCR purified amplicons delivered sequences which were then manually checked to extract good quality nucleotide sequences. Post quality check, nucleotide length of sequences ranged from 150 to 1210 bp with an overall average length of 600 bp (3rd column from the left of **Table 3.6)**. It was also observed that sequences obtained using Eurofins Genomics' 96 well plate method produced shorter length sequences (150–710 bp, mean 490 bp) compared to those analysed by other sequencing services (400–1210 bp, mean 920 bp). A detailed sequencing log of salt marsh isolates is shown in **Table 3.6**.

From the quality checked (QCed) sequence files, a 16S rRNA gene sequence dataset was generated that included sequences of approx. ≥ 500 bp nucleotide length. Phylogeny results of this dataset on EzBioCloud BLAST for the bacterial isolates of RSPB Marshside and Fingringhoe Wick salt marsh sites are given in **Tables 3.7 and 3.8** respectively.

The taxonomic distribution and evolutionary relationship of the isolates obtained in this study are represented in the phylogenetic trees (**Fig. 3.5 and 3.6)**. They also provide an overview of the general frequency of isolation of retrieved microbial taxa.

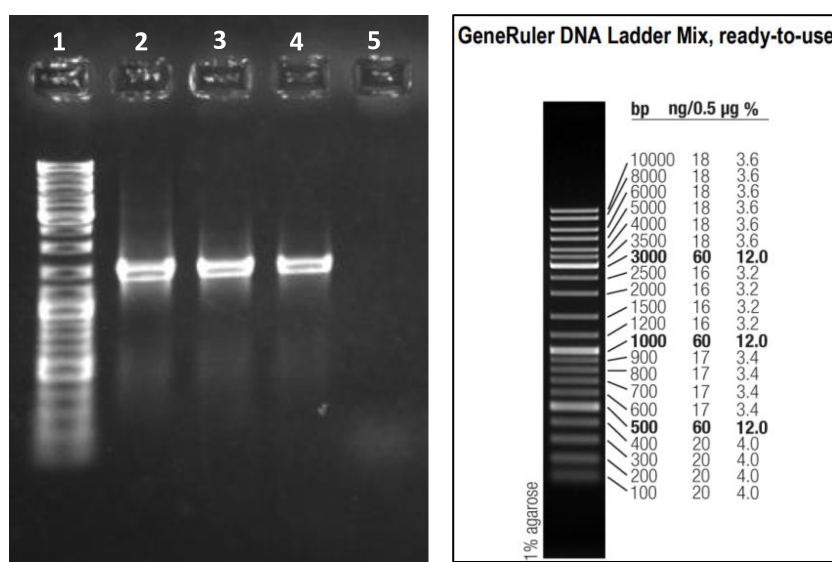

Figure 3.4 Gel electrophoresis on 1% (w/v) agarose, observed under U. V. light. Well 1, DNA ladder mix.; Well 2-4, purified PCR amplicons of isolates; Well 5, reagent control. Amplicons' band match at approx. 1500 bp lane of DNA ladder.

Table 3.6 16S rRNA gene sequencing log of salt marsh isolates. Nucleotide lengths are mentioned in brackets

Abbreviations: ¥ = Sequencing using 96 well plate service; * = Sequencing using tube method; QCed = Quality checked

Sequencing service used	No. of PCR amplicons processed for sequencing	No. of good quality sequences	No. of QCed sequences processed for BLAST and further data analysis
Eurofins Genomics¥	94	77 (150–710 bp, mean 490 bp)	43 (510–710 bp, mean 596 bp)
Eurofins Genomics*	18	6 (660–930 bp, mean 780 bp)	6 (660–930 bp, mean 780 bp)
Uni. of Sheffield*	9	7 (888–985 bp, mean 950 bp)	7 (888–985 bp, mean 950 bp)
Uni. of Nottingham*	17	9 (400–1210 bp, mean 985 bp)	8 (821–1210 bp, mean 1055 bp)
Total	**138**	**99**	**64**

Table 3.7 Phylogenetic identification of pure bacterial cultures isolated from different regions of RSPB Marshside salt marsh, based on comparative analysis of their partial 16S rRNA gene sequences on EzBioCloud BLAST tool

Putative novel species isolates are highlighted in **bold**

Abbreviations: MARW_1, MARW_2 = Water samples collected from RSPB Marshside on June 2017 and October 2017 respectively; T = Type strain species; * = showed >99% phylogenetic similarity to non-type strain of *Marinobacter* sp. BW6 on BLAST

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud BLAST	Similarity %	Variation ratio	Phylum	Class	Order	Family
Low marsh isolates								
MARW_1-1-9	985	*Alcaligenes faecalis subsp. Parafaecalis G(T)*	99.8	2/976	Proteobacteria	Betaproteobacteria	Burkholderiales	*Alcaligenaceae*
MARW_1-1-14	888	*Bacillus licheniformis ATCC 14580(T)*	99.44	5/887	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-1-16	568	*Exiguobacterium marinum DSM 16307(T)*	99.30	4/568	Firmicutes	Bacilli	Caryophanales	Caryophanales, no family
MARW_1-1-17	602	*Exiguobacterium marinum DSM 16307(T)*	100	0/602	Firmicutes	Bacilli	Caryophanales	Caryophanales, no family
MARW_1-1-18	806	*Pseudidiomarina sediminum c121(T)*	99.75	2/806	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*
MARW_1-1-22	646	*Halomonas aestuarii Hb3(T)*	100	0/646	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-1-23	550	*Alkalihalobacillus hwajinpoensis SW-72(T)*	99.45	3/550	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-2-4	829	*Planococcus maritimus DSM 17275(T)*	99.88	1/829	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
MARW_1-2-15	1102	*Marinobacter maroccanus N4(T)*	99.82*	2/1102	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-2-21	1092	*Zooshikella ganghwensis JC2044(T)*	99.54	5/1092	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Hahellaceae*
MARW_1-2-22	633	*Rhodovulum sulfidophilum DSM 1374(T)*	100	0/633	Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*
MARW_1-2-23	682	*Planomicrobium flavidum ISL-41(T)*	99.12	6/682	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
MARW_1-2-24	588	*Marinobacter goseongensis En6(T)*	99.83	1/588	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-2-25	589	*Exiguobacterium marinum DSM 16307(T)*	99.83	1/589	Firmicutes	Bacilli	Caryophanales	Caryophanales, no family

Table 3.7 continued…

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud BLAST	Similarity %	Variation ratio	Phylum	Class	Order	Family
Low marsh isolates								
MARW_1-2-26	**576**	***Erythrobacter marinus KCTC 23554(T)***	**98.44**	**9/576**	**Proteobacteria**	**Alphaproteobacteria**	**Sphingomonadales**	***Erythrobacteraceae***
MARW_1-2-27	**663**	***Pseudoalteromonas piscicida JCM 20779(T)***	**99.68**	**2/633**	**Proteobacteria**	**Gammaproteobacteria**	**Alteromonadales**	***Pseudoalteromonadaceae***
MARW_2-2-1	572	*Bacillus pumilus ATCC 7061(T)*	99.83	1/572	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_2-2-2	630	*Rheinheimera baltica DSM 14885(T)*	100	0/630	Proteobacteria	Gammaproteobacteria	Chromatiales	*Chromatiaceae*
MARW_2-2-6	678	*Erythrobacter litoralis DSM 8509(T)*	99.26	5/678	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Erythrobacteraceae*
MARW_2-2-7	642	*Thioclava dalianensis DLFJ1-1(T)*	99.69	2/641	Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*
Mid marsh isolates								
MARW_1-4-1	953	*Marinobacter flavimaris SW-145(T)*	98.16*	19/951	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-4-9	1062	*Halomonas aestuarii Hb3(T)*	99.06	10/1062	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-4-16	**964**	***Marinobacter sediminum R65(T)***	**98.23**	**17/963**	**Proteobacteria**	**Gammaproteobacteria**	**Alteromonadales**	***Alteromonadaceae***
MARW_1-4-17	941	*Marinobacter maroccanus N4(T)*	99.15	8/939	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-5-2	638	*Planococcus maritimus DSM 17275(T)*	100	0/638	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
MARW_1-5-5	590	*Henriciella litoralis DSM 22014(T)*	100	0/589	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Hyphomonadaceae*
MARW_1-6-1	821	*Alkalihalobacillus hwajinpoensis SW-72(T)*	99.51	4/821	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-6-3	615	*Halomonas denitrificans M29(T)*	99.19	5/615	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-6-4	608	*Halomonas aestuarii Hb3(T)*	99.84	1/607	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-6-6	690	*Marinobacter flavimaris SW-145(T)*	98.41*	11/690	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-6-8	591	*Planococcus maritimus DSM 17275(T)*	99.83	1/591	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*

Table 3.7 continued…

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud BLAST	Similarity %	Variation ratio	Phylum	Class	Order	Family
Mid marsh isolates								
MARW_1-6-9	697	*Halomonas aestuarii Hb3(T)*	99.86	1/697	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-6-10	974	*Marinobacter flavimaris SW-145(T)*	98.67*	13/974	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-6-12	715	*Halomonas aestuarii Hb3(T)*	99.86	1/715	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-6-14	600	*Marinobacter flavimaris SW-145(T)*	100	0/600	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-6-17	498	*Marinobacter maroccanus N4(T)*	100	0/498	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-7-1	595	*Alkalihalobacillus hwajinpoensis SW-72(T)*	99.66	2/595	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-7-2	**1200**	***Halomonas taeanensis BH539(T)***	**98.42**	**19/1200**	**Proteobacteria**	**Gammaproteobacteria**	**Oceanospirillales**	***Halomonadaceae***
MARW_1-7-3	559	*Marinobacter goseongensis En6(T)*	99.82	1/559	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-7-4	665	*Alkalihalobacillus hwajinpoensis SW-72(T)*	99.4	4/665	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-8-13	1209	*Marinobacter maroccanus N4(T)*	99.83	2/1209	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-8-19	612	*Marinobacter flavimaris SW-145(T)*	98.2*	11/612	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-8-20	661	*Alcaligenes faecalis subsp. Parafaecalis G(T)*	100	0/661	Proteobacteria	Betaproteobacteria	Burkholderiales	*Alcaligenaceae*
MARW_1-8-21	640	*Citromicrobium bathyomarinum JF-1(T)*	99.84	1/640	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*
MARW_1-8-22	584	*Pseudidiomarina sediminum c121(T)*	99.66	2/584	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*
MARW_1-8-23	497	*Exiguobacterium oxidotolerans JCM 12280(T)*	99.6	2/497	Firmicutes	Bacilli	Caryophanales	Caryophanales, no family
MARW_1-9-5	598	*Pseudidiomarina sediminum c121(T)*	99.16	5/598	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*
MARW_1-10-1	549	*Halomonas saccharevitans AJ275(T)*	99.09	5/549	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
MARW_1-10-5	547	*Alcanivorax gelatiniphagus MEBiC08158(T)*	99.63	2/546	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Alcanivoracaceae*
MARW_1-10-7	1137	*Halomonas alkaliphila 18bAG(T)*	100	0/1137	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*

Table 3.8. Phylogenetic identification of pure bacterial cultures isolated from different regions of Fingringhoe Wick salt marsh, based on comparative analysis of their partial 16S rRNA gene sequences on EzBioCloud BLAST tool

Putative novel species isolates are highlighted in **bold**
Abbreviations: FINW_1, FINS_1 = Water and soil samples respectively, collected from Fingringhoe Wick on November 2017; T = Type strain species

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud BLAST	Similarity %	Variation ratio	Phylum	Class	Order	Family
Low marsh isolates								
FINW_1-1-1	590	*Pseudoalteromonas mariniglutinosa KMM 3635(T)*	99.83	1/590	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Pseudoalteromonadaceae*
FINW_1-1-6	510	*Halomonas venusta DSM 4743(T)*	99.61	2/510	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
FINS_1-1-9	555	*Paracoccus marcusii DSM 11574(T)*	100	0/555	Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*
FINS_1-1-11	667	*Bacillus mycoides DSM 2048(T)*	100	0/667	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-2-2	**808**	***Pleionea mediterranea MOLA:115(T)***	**98.27**	**14/808**	**Proteobacteria**	**Gammaproteobacteria**	**Oceanospirillales**	***Alcanivoracaceae***
FINS_1-2-5	547	*Shewanella marisflavi SW-117(T)*	100	0/546	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Shewanellaceae*
FINS_1-3-1	559	*Alkalihalobacillus berkeleyi KMM 6244(T)*	100	0/557	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-3-2	595	*Halobacillus halophilus DSM 2266(T)*	99.5	3/595	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-3-5	934	*Alkalihalobacillus hwajinpoensis SW-72(T)*	99.79	2/934	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-3-6	544	*Halomonas nitroreducens 11S(T)*	99.82	1/544	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
Mid marsh isolates								
FINS_1-4-4	**501**	***Alkalihalobacillus hwajinpoensis SW-72(T)***	**98.8**	**6/500**	**Firmicutes**	**Bacilli**	**Caryophanales**	***Bacillaceae***
FINS_1-4-5	**681**	***Halomonas taeanensis BH539(T)***	**98.38**	**11/681**	**Proteobacteria**	**Gammaproteobacteria**	**Oceanospirillales**	***Halomonadaceae***
FINS_1-5-2	484	*Planococcus maritimus DSM 17275(T)*	100	0/484	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
FINS_1-5-4	475	*Alkalihalobacillus berkeleyi KMM 6244(T)*	100	0/472	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*

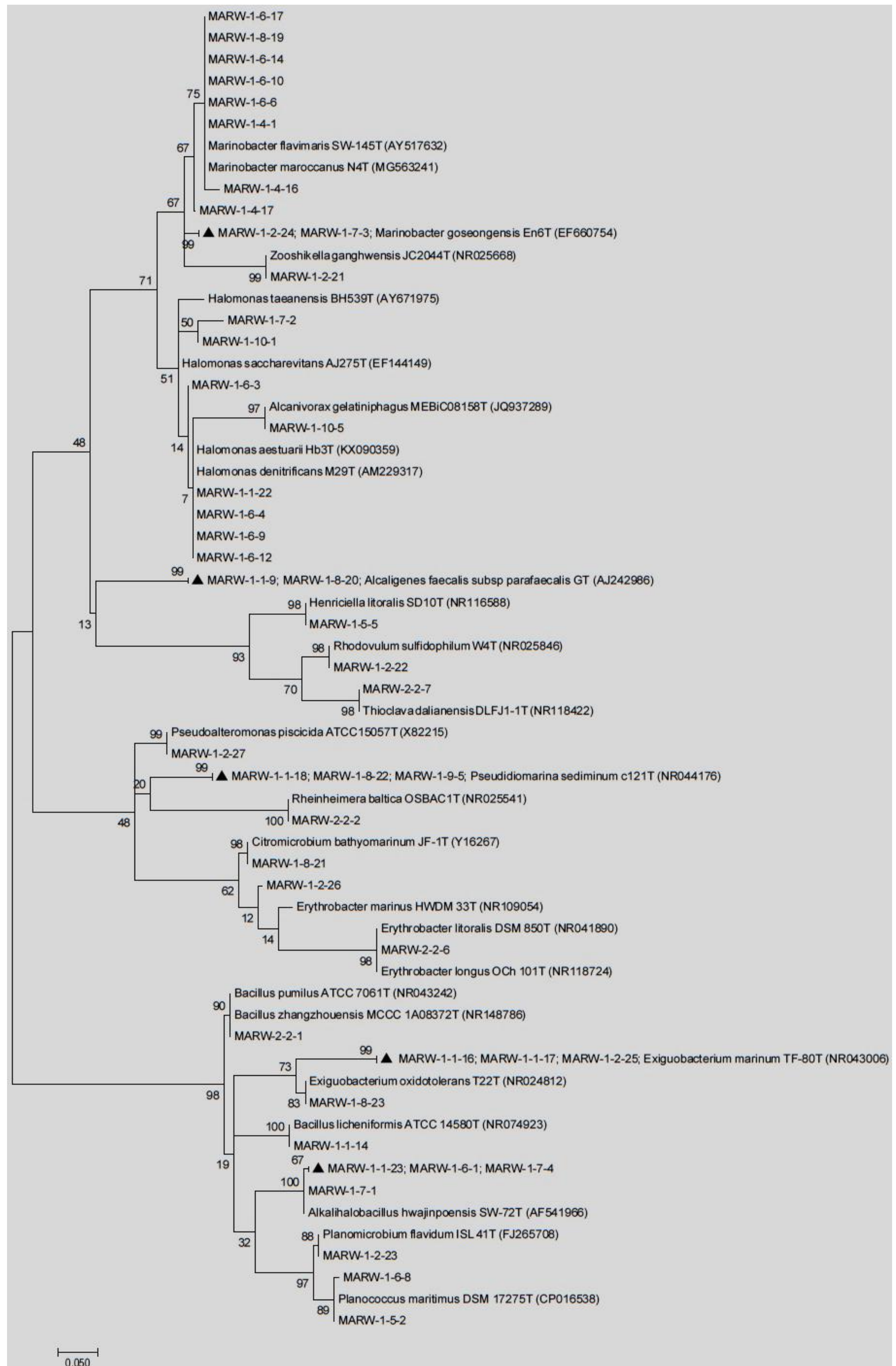

Figure 3.5 Phylogenetic tree providing an overview of bacterial isolates from RSPB Marshside Nature Reserve. The tree was constructed using the Maximum-Likelihood algorithm in MEGA7.0. It comprised of 16S rRNA sequences of isolates (500-1210 bp) and their closest phylogenetic relatives mentioned with their type strain IDs and accession numbers. Bootstrap values (n = 1000 replicates) are indicated at the nodes. Isolates' branches representing similar phylogenetic identity are collapsed to a single node and are indicated with a triangle. The scale below shows substitution per site**.**

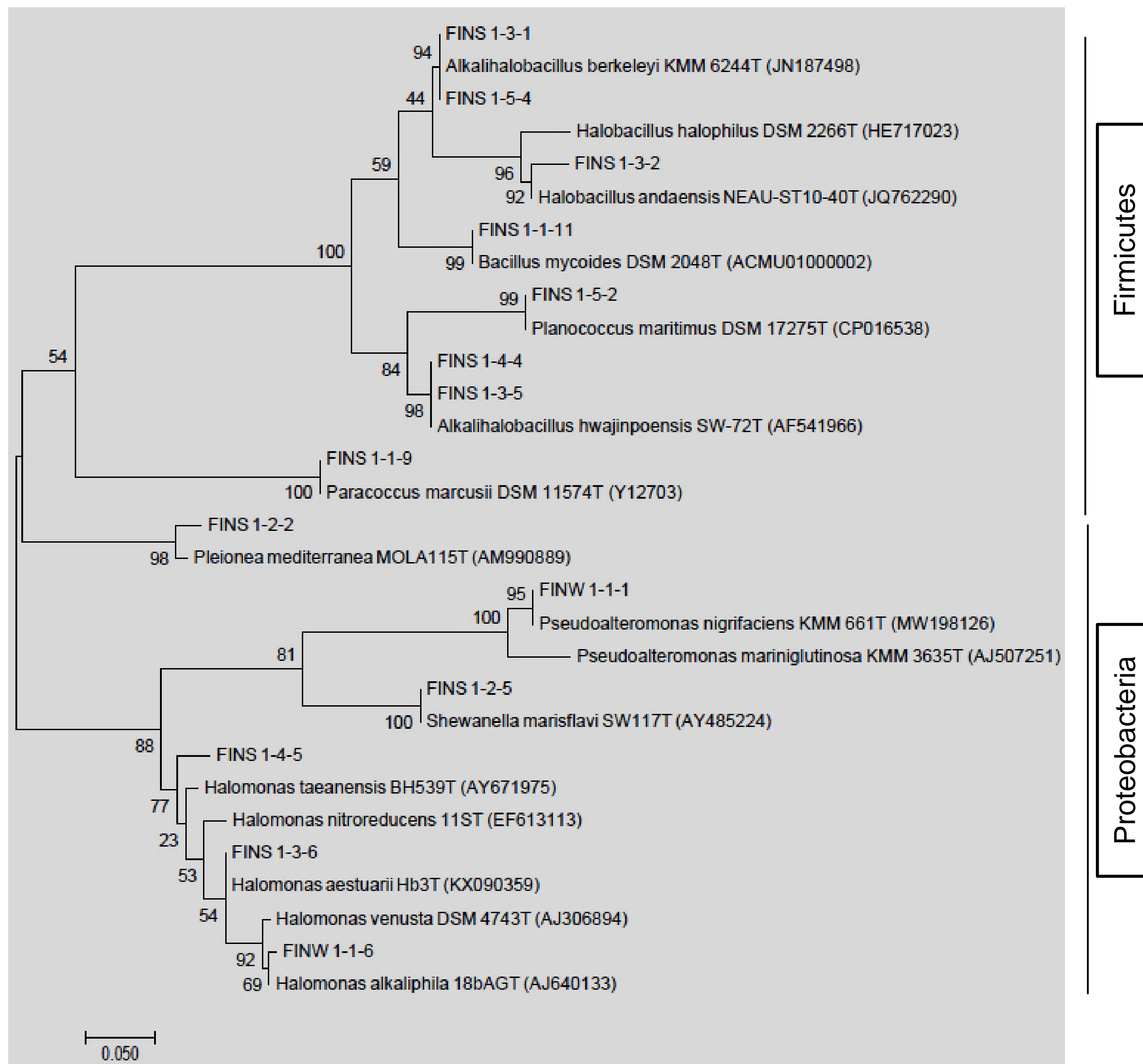

Figure 3.6 Phylogenetic tree providing an overview of bacterial isolates obtained from Fingringhoe Wick Nature Reserve. The tree was constructed using Maximum-Likelihood algorithm in MEGA7.0. It comprised of 16S rRNA sequences of isolates (500-935 bp) and their closest phylogenetic relatives mentioned with their type strain IDs and accession numbers. Bootstrap values (n = 1000 replicates) are indicated at the nodes. The scale below shows substitution per site.

3.3.6 Taxonomic Hierarchical Description of Isolates

The distribution of the phylogenetically identified isolates was categorised into different taxa and taxonomic hierarchies (**Tables 3.7 and 3.8** and **Fig. 3.5 and 3.6),** which are collectively described as follows.

Bacterial isolates identified from RSPB Marshside and Fingringhoe Wick Nature Reserve were largely classified as members of the phyla Proteobacteria (43 out of 64 isolates) and Firmicutes (21 out of 64). At class level, the greater proportion of microbial isolates were phylogenetically identified as Gammaproteobacteria (34 out of 64), Bacilli (21 out of 64), and some proportion as Alphaproteobacteria (7 out of 64). Betaproteobacteria class was assigned to only 2 isolates from the samples of RSPB Marshside. This resulted in positioning these isolates into two major families *Halomonadaceae* (12 out of 64) and *Bacillaceae* (12 out of 64). Abundant isolates belonging to the family *Alteromonadaceae* were also detected at RSPB Marshside (12 isolates). At both sites, some fraction of the identified fraction of the isolates were *Caryophanaceae* (9 out of 64), while rest of the bacterial families identified among test isolates were as mentioned in **Tables 3.7 and 3.8**.

Microbes isolated from both salt marsh study sites when phylogenetically identified on EzBioCloud BLAST tool, they were cumulatively grouped closed to 22 different genera, with overall 16S rRNA gene similarity ratio of 98–100%. A predominance of the genera *Marinobacter* and *Halomonas* was observed in the sequenced isolates from RSPB Marshside, whereas more *Alkalihalobacillus* and *Halomonas* were observed in the Fingringhoe Wick sequenced isolates (**Tables 3.7 and 3.8, Fig. 3.5 and 3.6**).

3.3.7 Novel Species Identification

Based on results obtained from EzBioCloud BLAST tool of phylogenetic identity and 16S rRNA gene similarity ratio to the closest relatives, seven isolates were identified as putative novel species in the present study. These are highlighted in bold font in **Tables 3.7 and 3.8**.

3.3.8 Screening for Antimicrobial Production

Four out of 69 salt marsh isolates screened for antimicrobial production showed positive results as shown in **Table 3.9**.

Table 3.9 Salt marsh isolates showing positive antimicrobial activity, when tested against *E. coli* (DSM 30083) and *M. luteus* (NCTC 2665) using double-layer agar diffusion method

The measured zones of inhibitions are mentioned in brackets
Abbreviations: + = Positive results; - = Negative results

Indicator strains	**Salt Marsh Isolates**			
	MARW_1-2-21	**MARW_1-2-22**	**MARW_1-8-20**	**FINS_1-1-11**
E. coli	-	+ (3 mm)	+ (3 mm)	-
M. luteus	+ (2–3 mm)	+ (2 mm)	+ (6 mm)	+ (1–2 mm)

3.4 DISCUSSION

To the best of my knowledge this is the first study that has cultivated and identified microbial diversity from the UK salt marshes, RSPB Marshside and Fingringhoe Wick Nature Reserve. A total of 287 microbial isolates were cultured from these sites, of which 64 bacterial isolates were successfully identified using Sanger sequencing method based on their 16S rRNA gene. A portion of this cultured microbial population was also tested for its phenotypic characterisation, salt tolerance and antimicrobial production assay. The availability of pure cultures and simultaneous assessment of this cultured community composition have allowed an exploration of the microbial population of these salt marsh sites.

3.4.1 Cytology, Physiology and Salt Tolerance study

A consortium of morphological and physiological differences was revealed in the studied salt marsh isolates (**Tables 3.4–3.5**). Isolates showed highly variable and broad-ranged salt tolerance and were considered as moderate halophilic because they grew best at 4% NaCl (w/v) and most of them have a maximum salt tolerance of 12–16% NaCl (w/v) (**Table 3.5**). These study findings though reiterate high salt tolerance of microbes of marine environments, it extends this knowledge to the

isolates of this study, including potential new species and shows that they are well adapted to the varying saline conditions of the salt marshes.

Gradients of salt concentrations cause transient variations in metabolic activities of halophiles (e.g. *Halomonas halophila*, *Micrococcus halodenitrificans,* and *Pseudomonas salinaria*), that also includes microbial production of a membrane bound enzyme called cytochrome oxidase which is highly active only in the presence of elevated sodium and potassium chloride salt concentrations. Cytochrome oxidase maintains the intracellular salt contents against concentration gradient and is required for growth of these halophiles (Ventosa *et al.* 1998; Baxter and Gibbons 1956). Additionally, excess of reactive oxygen species such as hydrogen peroxide in an indigenous environment creates an oxidative stress that can be lethal to microbes by destroying their proteins, cellular membranes, and nucleic acids. Some microbes however produce a scavenging enzyme called catalase that breaks down hydrogen peroxide into nontoxic molecular oxygen and water, and thus acquire protection against oxidative stress (Imlay 2013; Loew 1900).

A number of isolates tested in the present study also showed positive cytochrome oxidase and catalase production, which indicates their capabilities to withstand osmotic and oxidative stress generated at salt marsh environments.

3.4.2 Phylogenetic Identification

The 27f and 1492r primer sets used in this study for PCR and sequencing are known to amplify the full-length sequence of the 16S rRNA gene which is 1400–1550 bp long (Frank *et al.* 2008; Wilson *et al.* 1990). However, the Sanger sequencing services (cost-effective ones) used in this study were able to provide a partial gene sequence of length 1200 bp. As a result, approx. 65% of the QCed sequences obtained from the isolates of this study had a nucleotide length of 500–1200 bp (mean 700 bp) (**Tables 3.7–3.8)**. On the other hand, the remaining QCed sequences were of a shorter length (150–500 bp, mean 330 bp), most of which were obtained through a 96-well plate sequencing service at Eurofins Genomics (**Table 3.6**).

Six out of the eighteen PCR amplicons analysed using a tube sequencing service at Eurofins Genomics imparted quality sequences of length 660–930 bp (mean 780

bp). Prior to this, a 96-well plate service offered by the same sequencing company produced no/poor quality sequences from these eighteen PCR amplicons.

While performing DNA extraction and PCR reactions at Edge Hill University lab, similar reagents, conditions, and quality control standards were applied for the preparation of all these PCR amplicons. The quality and purity of all PCR purified amplicons was also confirmed as they exhibited single and prominent bands on gel electrophoresis. Hence, differences in the quality and nucleotide length of the sequences obtained using 96-well plate could be either due to contaminants incorporated while transferring PCR amplicon samples from individual tubes to the 96-well sequencing plate or improper sealing of these plates Edge Hill lab, during transportation of the sample plate to the sequencing company or well-to-well contamination while samples are being sequenced (Walker 2019).

Some of the 16S rRNA gene-PCR amplicons which produced short QCed sequences (200–900 bp) in the first sequencing attempt delivered longer sequences (600–1200 bp) at the next attempt. A comparison table of the phylogenetic identities of these amplicons is as shown in **Appendix 2**. An EzBioCloud BLAST analysis of all these sequences showed that for each individual isolate, both the shorter and longer sequence resulted in the same genus level identity, with either no change or moderate variation in the similarity ratio. However, according to the quality standard decided for this phylogenetic study, the final dataset was created using only those quality-checked sequences that had at least 500 bp of nucleotide length (**Tables 3.7–3.8)**. Those isolates whose sequences did not fulfil this criterion were excluded from the phylogenetic analysis.

When quality sequences of salt marsh isolates with at least 500 bp nucleotide length of the 16S rRNA gene were examined on the EzBioCloud server, it allowed the taxonomic identification of these isolates up to the genus level (**Tables 3.7 and 3.8**). It was also observed that different phylogeny assignment tools such as EzBioCloud and NCBI blastn had the capability to capture the taxonomic information of 16S rRNA gene sequences up to genus level, though differences were observed with species identification and sequence similarity ratios (data not shown).

3.4.3 Taxonomic Hierarchical Description of isolates

Phylogenetically identified bacterial isolates mentioned in **Tables 3.7** and **3.8,** and represented in **Fig. 3.5 and 3.6**, were discussed with respect to their distribution in phylum, class and genus taxonomic levels as follows.

3.4.3.1 Isolates' phylum and class level description

<u>Diversity</u>

The bacterial isolates identified at both salt marsh sites of RSPB Marshside and Fingringhoe Wick Nature Reserve were clustered into two phyla, Proteobacteria and Firmicutes which have been previously reported to have important roles in the sulphur cycle, ammonia oxidation and hydrocarbon-degradation in salt marsh environments (Ansede *et al.* 2001; Beazley *et al.* 2012; Santoro *et al.* 2008).

Results of the present study are in general accordance with reports from previous cultivation-based and -independent microbial studies performed on the surface and subsurface sediments of salt marshes, estuaries and other coastal environments. Such prior studies grouped microbial isolates into Proteobacteria (60–70%) (Köpke *et al.* 2005; Martins 2011; Fidalgo *et al.* 2016; Beazley *et al.* 2012; Inagaki *et al.* 2003), with the remainder consisting of Firmicutes, Bacteroidetes (Beazley *et al.* 2012; Fidalgo *et al.* 2016; Lydell *et al.* 2004; Martins 2011) and Actinobacteria (Beazley *et al.* 2012; Johnston *et al.* 2017; Fidalgo *et al.* 2016).

Microcosm experiments, where the degradation potential of coastal microbes against microphytobenthic extracellular polymeric substances (McKew *et al.* 2013) and isoprene (one third of volatile organic compounds from the terrestrial and marine biosphere; Johnston *et al.* 2017) were tested individually as sources of carbon and energy respectively, showed increased dominance of Firmicutes and Actinobacteria compared to Proteobacteria. Abundant Firmicutes were also noted amongst biomineralizing marine sediment microbes (Wei *et al.* 2015) and a thermophilic bacterial population (Aanniz *et al.* 2015). This indicates the transient variations in the microbial assembly with respect to different inputs to the salt marshes and associated environments.

In the present study, most of the isolates were obtained after cultivation on marine agar and incubation under aerobic conditions at 30 °C. This setting is routinely used in marine and salt marsh cultivation studies. Marine agar supports the growth of a

number of aerobic heterotrophic marine bacteria (Jannasch and Jones 1959; ZoBell 1941), so could be seen as a potential source of over-representation of this type of microbe and potential bias in the obtained results. However, the observed dominance of Proteobacteria isolates in the present has also been observed in previous cultivation-independent studies (Cifuentes *et al.* 2000; Beazley *et al.* 2012; Webster *et al.* 2010).

Firmicutes exist in both the endospore and vegetative cell forms *in situ* and are usually considered not to be of coastal origin (Berrada *et al.* 2012; Yilmaz *et al.* 2016; Wu *et al.* 2010). They are usually detected in very low abundance (<1%) in sequence libraries generated from marine sediments (Seo *et al.* 2017; Nimnoi and Pongsilp 2020; *Beazley et al.* 2012; Cupit *et al.* 2019; Kawai *et al.* 2015). Contrastingly, increasing numbers of Firmicutes were observed (>5%) through cultivation-dependent methods (Fidalgo *et al.* 2016; Berrada *et al.* 2012; Köpke *et al.* 2005; Volpi *et al.* 2017), microcosm experiments (McKew *et al.* 2013) and through estimation of their endospore specific compound, dipicolinic acid (Fichtel *et al.* 2008; Langerhuus *et al.* 2012; Lomstein *et al.* 2012). Simultaneously, higher proportions of Firmicutes (>10%) were also observed in the culture studies of coastal sediment layers (Köpke *et al.* 2005; Martins 2011; Fidalgo *et al.* 2016) and in thermophilic diversity experiments (Aanniz *et al.* 2015). Detection of a low percentage of Firmicutes when solely applying molecular techniques is considered to be associated with the estimation of their vegetative forms only. The inability of conventional DNA extraction reagents to extract DNA from the rigid structures of Firmicutes in endospore form could be the reason behind underestimation of these communities in molecular based methods (Filippidou *et al.* 2015; Cupit *et al.* 2019). Such Firmicute endospores, when carried from the water column, may germinate into vegetative cells and thrive upon burial in the coastal soil sediments (Cupit *et al.* 2019). This could be the reason that Firmicutes were the dominating genera from the studied soil sample isolates from Fingringhoe Wick. This could also be an indication of microbial differences among the types of the salt marsh samples collected. However, more sequencing data and further analysis would be needed to support this.

Actinobacteria and Bacteroidetes are additional relevant phyla that are also normally present in lower proportions than Proteobacteria in salt marshes and other ocean environments (Fidalgo *et al.* 2016; Nimnoi and Pongsilp 2020; Suh *et al.* 2015; Seo *et al.* 2017; Zinger *et al.* 2011). The absence of these phyla in the present

study could be explained by the fact that only a fraction of total isolates was phylogenetically identified during this study, and not every isolate was picked for the isolation which might have missed these phyla. Additionally, the use of aerobic incubation could have restricted the growth of Bacteroidetes as some of them (e.g., genus *Bacteroides*) are obligate anaerobes or aerotolerant (Meehan *et al.* 2012; Thomas *et al.* 2011; Tang *et al.* 1999) and their multiplication is restricted in the presence of oxygen (Bacic and Smith 2008). Other reasons of these minor phyla remained undetected could be the suppression of these Actinobacteria and Bacteroidetes by fast growing microbes (Bodor *et al.* 2020; Chaudhary *et al.* 2019), as well as a lack of media supplements (e.g. $CaCO_3$) known to prevent the growth of fast-growing bacteria and enhance the isolation of Actinobacteria (Fang *et al.* 2017).

Potential roles

The classes of identified study isolates, such as Betaproteobacteria, Gammaproteobacteria and Bacilli are known to have an ecological potential in coastal wetlands as they play important roles in nitrification and denitrification steps of the nitrogen cycle (Peng *et al.* 2013; Angermeyer 2016; Jenkins and Kemp 1984; Sebilo *et al.* 2006; Bagwell *et al.* 1998). Generally, heterotrophic microbes found in saline habitats such as those of salt marshes, are reported to have reduced nitrogenase biosynthesis and activity at high salt concentrations and are predicted to have poorly functional nitrogen fixation ability in saline ecosystems (Zahran *et al.* 1995; Tripathi *et al.* 2002). Nevertheless, nitrogen fixation has been reported in the saline soils of salt marshes, via the occurrences of halotolerant, heterotrophic, nitrogen fixing bacteria of class Bacilli and Gammaproteobacteria (genus e.g. *Bacillus* and *Azotobacter*, Zahran *et al.* 1995; Angermeyer 2016; Diab *et al.* 1984; Dicker *et al.* 1980; Ventosa *et al.* 1998). The persistence of these bacteria in saline environments of the salt marshes thus supports the existence of potential nitrogen turnover which maintains the fertility and productivity of these saline ecosystems.

3.4.3.2 Isolates' genus level description

Diversity

Phylogenetic and systematic studies based on 16S rRNA gene sequencing have placed a large number of microbes from the identified fraction of the isolates from both study sites within the genera *Marinobacter*, *Halomonas* and *Alkalihalobacillus*

(**Fig. 3.5 and 3.6**). As described in literature, members of these genera are halotolerant or halophilic, and ubiquitous in salt marshes and various other coastal environments (Whitman 2015; Berrada *et al.* 2012; McKew *et al.* 2011; Patel and Gupta 2020). In the recently proposed novel genus *Alkalihalobacillus* (Patel and Gupta 2020), some species are described as obligately alkaliphilic, requiring an unusually highly alkaline pH range between 6 to 11. Such properties enable microbes of these genera to grow rapidly and dominate the marine microbial community.

Potential roles

These detected genera of the present study have been highlighted in the previous literature for their ecological and biotechnological potential in saline environments. For example, denitrifying *Marinobacter* are commonly associated with oil spills and pollution in these areas (Bergey and Holt 1993). Members of this genus produce biosurfactants that are capable of degrading wide variety of aliphatic or aromatic compounds and thus have an industrial application in microbe-mediated oil recovery and bioremediation processes (Tripathi *et al.* 2019; Handley and Lloyd 2013).

Also, exopolysaccharides secreted by *Halomonas* have versatile ecological and biotechnological applications such as in plant growth promotion (Upadhyay *et al.* 2011; Bergmann *et al.* 2009; Ruiz-Lozano *et al.* 2000), biofilm formation, immunomodulation, emulsification, and bioremediation of heavy metal contaminated environments (Amjres *et al.* 2015; Dworkin 2006; Quesada *et al.* 2004). Hydrolytic enzymes produced by these microbes are also studied to have role in bioremediation, and fermentation of foods (Quesada *et al.* 2004; Hinrichsen *et al.* 1994; Yoon *et al.* 2002).

Furthermore, some of the *Alkalihalobacillus* species are diazotrophic and can fix atmospheric nitrogen to ammonia (Patel and Gupta 2020). Additionally, several of these microbes were reported to have potential applications including probiotic activity; production of enzymes like cellulases, proteases, xylanases and cyclodextrin glucanotransferase for their use in laundry detergents, pulp paper industry and for manufacturing of cyclodextrin from starch respectively (Patel and Gupta 2020).

The abundance of these halo-versatile, beneficial microbes at the salt marsh sites further provides an insight on the potential scientific and economic value of these gradient-rich environments, including the salt marsh sites of the present study.

3.4.4 Novel Species Isolates

The microbial isolates were sequenced for their 16S rRNA gene in batches at different times of this study, and their sequences were initially analysed solely based on the NBCI blastn tool. Twenty six out of 64 identified microbial isolates were found to be distantly related to the species already known as their sequence similarity ratios were less than 99% (data not shown). However, later confirmation of these phylogenetic results on EzBioCloud BLAST led this study to conclude a revised compact list of potential novel species. Despite the result variations observed in between these two phylogeny tools, EzBioCloud results were acknowledged in this study for it is more commonly referred to in recent microbial phylogeny literature and contains more curated sequences as compared to the NCBI blastn tool (Park *et al.* 2012). Total six isolates, identified in EzBioCloud tool, were putatively identified as novel species based on the recent recommendation, where 98.7–99% sequence similarity ratio was proposed as a cut-off value for the identification of a new bacterial species (Stackebrandt and Ebers 2006; Kim *et al.* 2014). However, an exception to this 99% cut-off value was the inclusion of the isolate MARW_1-2-27 as a putative novel species based on the literature reported by Venkateswaran and Dohmoto (2000) and distinct polyphasic characteristics of MARW_1-2-27.

Based on initial identified 96% phylogenetic similarity to *Pseudoalteromonas sp.* on NCBI tool (analysis during the year 2017-2018), MARW_1-2-27 was taken for novel species characterisation, physico-chemical, chemotaxonomic and genome analysis. However, later confirmation on EzBioCloud BLAST tool concluded MARW_1-2-27 to have >99% similarity to *Pseudoalteromonas* sp. Also, while constructing phylogenetic tree it was noted that the 16S rRNA gene of the closest *Pseudoalteromonas* sp. to MARW_1-2-27 lacks specificity to differentiate it from other *Pseudoalteromonas* relatives which was in accord with the observation reported by Venkateswaran and Dohmoto (2000). Hence, to overcome the limitation of having lower 16S rRNA gene differences, biochemical and chemotaxonomic analysis as well as genome-based, well accepted traits like DNA-DNA hybridization and average nucleotide identity were analysed to confirm the novelty of MARW_1-

2-27 (Richter and Rosselló-Móra 2009; Ivanova *et al.* 2002c; Huang *et al.* 2017b). MARW_1-7-2 of this putative novel list was also fully characterised to confirm its novelty. The full details of these characterisation tests and results of these isolates are detailed in **chapter 4, sections 4.2.3 and 4.3.**

Time constraints did not allow characterisation of the rest of the putative novel species isolates; however, this remaining work is planned to re-start at post-PhD thesis submission

3.4.5 Screening for Antimicrobial Production

Antimicrobial compounds produced by bacterial, fungal and archaeal populations are examples of widely employed secondary metabolites. Antimicrobial compounds play an important role in maintaining environmental microbe populations and have commercial value with wider applications (Zhu *et al.* 2013; Ying *et al.* 2020). Irrespective of continual research in the field of antimicrobials, multidrug resistance has caused further demand to discover non-toxic, cost-effective, wide-spectrum antimicrobial compounds. One of the effective ways of achieving this demand has always been isolation, cultivation and surveillance of microbes from diverse natural environments. Microbes isolated from salt marsh sediments and halophytes are also promising sources of bioactive compounds and produce biocontrol agents that could have potential applications in medicine, agriculture and biotechnology (Lee *et al.* 2011; Chen *et al.* 2019; Gayathri *et al.* 2010; Kalyanasundaram *et al.* 2015; Gashgari *et al.* 2016)

The simple, rapid and widely used agar diffusion plate method employed in the present study has also revealed the salt marsh isolates' antimicrobial activity. Four out of 69 screened study microbes produced extracellular antimicrobial compounds, active against Gram-negative and/or Gram-positive microbes. Based on 16S rRNA gene analysis, these four isolates, FINS_1-1-11, MARW_1-2-21, MARW_1-8-20, and MARW_1-2-22, showed phylogenetic similarity to *Bacillus, Zooshikella, Alcaligenes* and *Rhodovulum* respectively (similarity ratio >99%) (**Tables 3.7 and 3.8**). These closest phylogenetic relatives have been also reported in literature for antimicrobial activity (Schallmey *et al.* 2004; Sethi *et al.* 2013; Lee *et al.* 2011; Kim *et al.* 2009; Zahir *et al.* 2013), except for MARW_1-2-22, whose closest relative is known for probiotic activity (Ying *et al.* 2020).

Bacillus species produce large quantities of extracellular enzymes and conduct industrially important reactions at varied pH and temperatures. Advanced gene cloning and growth media manipulations have further enhanced their application value (Schallmey *et al.* 2004; Vitullo *et al.* 2012; Carvalho *et al.* 2010). The closest relative of FINS_1-1-11, *Bacillus mycoides*, produces antifungal compounds, phenylacetic acid and methylphenyl acetate that suppress spore germination in *Fusarium oxysporum* and protects *Fusarium*-induced wilting of tomato plants (Wu *et al.* 2020). A co-culture of *Bacillus mycoides* and marine *Streptomyces* is also known to optimise the production of algicidal tryptamine derivatives which has a promising role in managing harmful algal blooms (Yu *et al.* 2015).

A phylogenetically related bacterial strain of MARW_1-2-21, *Zooshikella* sp., produces natural compounds called prodigiosin, cycloprodigiosin and their novel analogues. These compounds are tested to have antimicrobial activity against several microbial species and anticancer activity against human melanoma cells (Yi *et al*. 2003; Lee *et al*. 2011).

Productivity in traditional aquaculture farming suffers greatly from increased chemical oxygen demand (COD), nitrate levels, antibiotic resistant pathogenic bacteria etc. (Davidson *et al.* 2014; Su *et al.* 2017). However, the incorporation of a photosynthetic purple bacterium, *Rhodovulum sulfdophilum* (closest phylogenetic strain of isolate MARW_1-2-22), in marine aquaculture has shown its mitigation power by restricting COD, nitrate levels and the occurrence of sulfonamide (antibiotic) resistant bacteria (Ying *et al*. 2020).

These literature sources of phylogenetic relatives of antimicrobial producing FINS_1-1-11, MARW_1-2-21, MARW_1-8-20, and MARW_1-2-22 provide a general idea that these isolates might also provide resources of novel biocatalyst and have biotechnological applications. In comparison to other microbes, bacteria are easy to maintain in lab conditions and easy to modify for the strain improvements (Sethi *et al.* 2013). Antimicrobial producing bacteria obtained in the present study are therefore valued and can be explored further to evaluate the novelty of these compounds.

3.4.6 Concluding Remarks

These studies were conducted on a small fraction of the microbial isolates and lacked results for water isolates of Fingringhoe Wick salt marsh; due to time constraints and later shift in focus towards novel species characterisation, genome analysis of the two novel isolates, and work on the gel-stabilised gradient plates. The number of isolates taken for various analysis also varied in this study as they were processed at different times of the study and for different purposes. Despite these limitations, the discussed study results have shown the substantial culturable microbial diversity and potential of studying the salt marsh ecosystem.

Chapter 4

Characterisation of MARW_1-7-2^T and MARW_1-2-27^T, Novel Species of Genera *Halomonas* and *Pseudoalteromonas*

4.1 INTRODUCTION

Two bacterial isolates of class Gammaproteobacteria, MARW_1-7-2^T and MARW_1-2-27^T are proposed in this chapter as novel species. Molecular-based analysis have suggested their phylogenetic placement within the genera *Halomonas* and *Pseudoalteromonas* respectively and results from a polyphasic taxonomic approach have confirmed that they represent new species. These bacterial strains were isolated from the salt marsh surface sea water sampled from Royal Society for the Protection of Birds Marshside (RSPB Marshside) Nature Reserve, established on Ribble estuary, Southport, Merseyside, in the North-West of England. Extended information on RSPB Marshside and on salt marshes in general is provided in **chapter 1**.

4.1.1 The Genus *Halomonas*

4.1.1.1 Phylogeny

Based on 16S rRNA gene phylogenetic studies of *Halomonas* and *Deleya* species Franzmann *et al.* (1988) proposed the inclusion of these genera into the new family *Halomonadaceae*. Later, with further exploration of 16S rRNA gene, and the description of new taxa, the family *Halomonadaceae* was amended by the unification of the genera *Halomonas*, *Deleya* and *Halovibrio* and the species *Paracoccus halodenitrificans* into the single genus *Halomonas* (Dobson and Franzmann 1996).

Extensive studies made in recent years in hypersaline environments, have resulted in a large number of new species of genus *Halomonas* being isolated. The phenotypic heterogeneity of species within this genus (Mata *et al.* 2002), and the adoption of molecular- and genomic-based approaches such as 16S rRNA gene phylogeny, DNA-DNA hybridization (DDH) and average nucleotide identity (ANI); have also resulted in taxonomic re-positioning (e.g., Arahal 2002a) and prompted a more detailed examination of this group of bacteria (Kämpfer *et al.* 2018; Vreeland *et al.* 1980). Taking this into consideration, minimal standards for description of new taxa for the family *Halomonadaceae* were recommended (Arahal *et al.* 2007) and were followed in the present study.

4.1.1.2 Taxonomy

The taxonomic hierarchy of this genus, from domain to family can be described as Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, *Halomonadaceae* (LPSN, n.d., viewed June 2020; Parte 2018; Diéguez *et al.* 2020; Dworkin 2006).

At the time of writing, the family *Halomonadaceae* has fourteen validly published genera; *Halomonas* (Vreeland *et al.* 1980), *Aidingimonas* (Wang *et al.* 2009), *Carnimonas* (Garriga *et al.* 1998), *Chromohalobacter* (Ventosa *et al.* 1989), *Cobetia* (Arahal *et al.* 2002a), *Halotalea* (Ntougias *et al.* 2007), *Halovibrio* (Fendrich 1988), *Kushneria* (Sánchez-Porro *et al.* 2009), *Larsenimonas* (León *et al.* 2014), *Modicisalibacter* (Gam *et al.* 2007), *Pistricoccus* (Xu *et al.* 2016), *Salinicola* (Anan'ina *et al.* 2007), *Terasakiispira* (Zepeda *et al.* 2015) and *Zymobacter* (Okamoto *et al.* 1993). *Halomonas* is the type genus of the family *Halomonadaceae* and also the largest genus with a current total of 96 validly published species. Vreeland *et al.* (1980) first established this genus, with *H. elongata* defined as the type species. Distinctive characteristics e.g., extreme salt tolerance, resistance to the vibriostatic agent (0/129), negative cytochrome oxidase test, distinctive morphology cycle, glucose fermentation, flexible motility and high G+C content (60.5±0.5 mol%) placed strain 1H9^{T} in the new genus *Halomonas*, which was previously classified in the gammaproteobacterial order Vibrionales and family II *Vibrionaceae* (Buchanan *et al.* 1974).

4.1.1.3 Physiology

Cells of species belonging to the genus *Halomonas* can be described as Gram-negative, straight or curved rods. Members of the genus *Halomonas* are described as halotolerant or euryhaline halophiles i.e., able to grow in wide spectrum of saline concentrations, ranging from 0.1–32.5% (w/v). They mainly possess respiratory type of metabolism, having oxygen as the terminal electron acceptor. From a chemotaxonomic point of view, ubiquinone 9 is the significantly found respiratory quinone and C16:1, C17:0 cyc, C16:0, C18:1, and C19:0 cyc are the major fatty acids found amongst this genus (Whitman 2015; Vreeland *et al.* 1980; Diéguez *et al.* 2020).

4.1.1.4 Ecology and habitats

Halomonas is one of the most ubiquitous genera and can be found in many saline environments. Some of these members which are alkalophilic are also found in alkaline lakes and alkaline soils (**Table 4.1**).

Table 4.1 Isolation sources of selected *Halomonas* species

Species	Isolation sources	References
H. aquamarina	Marine water (Hawaii, USA)	Akagawa *et al.* 1989; Dobson and Franzmann 1996
H. campisalis	Alkali lake sediment (Washington, USA)	Mormile *et al.* 1999
H. cupida	Marine water (Hawaii, USA)	Baumann *et al.* 1983; Dobson and Franzmann 1996
H. desiderata	Sewage treatment plant (Gottingen, Germany)	Berendes *et al.* 1996
H. elongata	Solar saltern (Bonaire, Netherlands Antilles)	Vreeland *et al.* 1980
H. eurihalina	Saline soil (Alicante, Spain)	Mellado *et al.* 1995
H. halodenitrificans	Meat-curing brines	Dobson and Franzmann 1996
H. halodurans	Great Bay estuary (New Hampshire, USA)	Hebert and Vreeland 1987
H. halophila	Saline soil (Alicante, Spain)	Quesada *et al.* 1984; Dobson and Franzmann 1996
H. pantelleriensis	Hard sand (Pantelleria, Italy)	Romano *et al.* 1996
H. salina	Saline soils (Alicante, Spain)	Valderrama *et al.* 1991; Dobson and Franzmann 1996
H. subglaciescola	Organic lake (Antarctica)	Franzmann *et al.* 1987
H. taeanensis	Solar saltern (Taean, Korea)	Lee *et al.* 2005

4.1.1.5 Biotechnological and ecological significance of *Halomonas*

Various characteristics of *Halomonas* species are suggestive of their potential relevance in ecology and biotechnology and have been reviewed in detail (e.g., Ventosa 1995; Zhao 2014). Their ability to produce exopolysaccharide (Amjres *et al.* 2011), compatible solutes and hydrolytic enzymes such as amylase, DNases, lipase, protease, pullulanase (Mellado *et al.* 2004; Sánchez-Porro *et al.* 2003), degradation of aromatic compounds (García *et al.* 2004), and their role in bioremediation, and fermentation of foods (Quesada *et al.* 2004; Hinrichsen *et al.* 1994; Yoon *et al.* 2002) has been reported previously. Because of their capability to grow in diverse growth conditions, bioactive compounds produced by these halophiles can show optimal activities even in extreme conditions. e.g., their hydrolytic activities could offer an invaluable solution to treat oilfield waste where high temperature and salinity are found (Sánchez-Porro *et al.* 2003).

Several species of *Halomonas,* e.g., *H. maura* (Bouchotroch *et al.* 2001), *H. rifensis* (Amjres *et al.* 2011), *H. ventosae* (Martínez-Cánovas *et al.* 2004) can secrete exopolysaccharides (EPS). EPS are reported to have versatile biotechnological applications such as in immunomodulation, crude oil emulsification, viscosity enhancements in food products, bioremediation of heavy metal contaminated environments, and in biofilm formation (Amjres *et al.* 2015; Dworkin 2006; Quesada *et al.* 2004). In saline environments EPS is known to promote plant growth by conferring them salt tolerance through limiting the content of sodium ions available for the plant uptake (Upadhyay *et al.* 2011). EPS also creates a microbial rhizosheath surrounding the salt marsh plant roots through attachment of sandy soil and dinitrogen fixing soil bacteria to the plant roots (Bergmann *et al.* 2009). These halotolerant, EPS producing species of *Halomonas* thus could be a valuable resource for plant production systems where soils or irrigation water contain high salt concentrations (Ruiz-Lozano *et al.* 2000).

4.1.2 The Genus *Pseudoalteromonas*

4.1.2.1 Phylogeny

Based on 16S rRNA gene analysis, Gauthier *et al.* (1995b) demonstrated heterogeneity amongst a few strains of the class Gammaproteobacteria. This phylogenetic analysis re-assigned eleven *Alteromonas* and one *Pseudomonas* species and proposed to place them in the new genus *Pseudoalteromonas*. The

genus continued to expand with further addition of novel species. Ivanova *et al.* (2004) introduced the new family *Pseudoalteromonadaceae* that embraced bacteria belonging to *Pseudoalteromonas* and the genus *Algicola*.

4.1.2.2 Taxonomy

The taxonomic hierarchy of the genus *Pseudoalteromonas* from domain to family can be described as Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, *Pseudoalteromonadaceae*. (LPSN, n.d., viewed June 2020; Parte 2018).

At the date of writing, the family *Pseudoalteromonadaceae* included four validly published genera: *Pseudoalteromonas* (Gauthier *et al.* 1995b), *Algicola* (Ivanova *et al.* 2004), *Psychrosphaera* (Park *et al.* 2011) and *Salsuginimonas* (Sheu *et al.* 2017). *Pseudoalteromonas* is the type genus of the family *Pseudoalteromonadaceae* which has a total of 47 validly published species, while *P. haloplanktis* is the type species of this genus (Gauthier *et al.* 1995b).

4.1.2.3 Physiology

Pseudoalteromonas species strains are aerobic, Gram-negative, non-spore forming, straight or slightly curved, rod shaped bacteria of size 0.2–1.5 µm x 1.8–4.0 µm. They are mostly motile with single or peritrichous, sheathed or unsheathed polar flagella. These cells do not accumulate poly-beta-hydroxybutyrate and are oxidase positive, catalase positive or weak positive. *Pseudoalteromonas* requires NaCl or sea water for growth. Species within this genus utilize a variety of organic substances as energy and carbon source. They are characterised by their hydrolytic activities as they produce lipase, gelatinase, DNAses, caseinase etc. Most *Pseudoalteromonas* species are resistant to O/129 vibriostatic agent, polymyxin, penicillin and lincomycin (Dworkin 2006).

4.1.2.4 Ecology and habitats

Bacteria included in the genus *Pseudoalteromonas* are mostly isolated from diverse marine environments and biomes (Holmström and Kjelleberg 1999). Most of these species are able to tolerate 10% NaCl (w/v), and some can withstand up-to 15% (w/v) concentration (Bowman 1998). Examples of species within this genus isolated from diverse habitats are listed in **Table 4.2**.

The genus can be divided into two clades; pigmented and nonpigmented, the former possessing a larger genome (Bowman 1998; Bozal *et al.* 1997; Xie *et al.* 2012). Pigmented *Pseudoalteromonas* species tend to produce more biologically active compounds than the nonpigmented ones, whereas wider environmental tolerance is normally exhibited by the nonpigmented strains (Egan *et al.* 2002; Holmström *et al.* 1996; Holmström *et al.* 2002). *P. tunicata* which was isolated from an adult tunicate (*Ciona intestinalis*) has been shown to have antifouling activity which is related with its yellow pigmentation (Egan *et al.* 2002). Examples of other bio-antifoulant producers are *P. ulvae* (dark-purple colonies, Egan *et al.* 2001), *P. rubra* (red colonies, Gauthier *et al.* 1976b), *P. aurantia* (orange colonies, Gauthier and Breittmayer 1979) and *P. luteoviolacea* (yellowish violet, Gauthier 1982; Gauthier *et al.* 1995b). This expressed antifouling property is beneficial to producer species as it allows them to competitively colonize new surfaces. It also acts as a defence mechanism for the neighbouring higher organisms in marine environments by inhibiting colonization and growth of fouling organisms on their surfaces (Holmström *et al.* 1996).

Table 4.2 Isolation sources of selected *Pseudoalteromonas* species

Species	Isolation sources	References
P. amylolytica	Surface seawater (Arabian sea)	Wu *et al.* 2017
P. antarctica	Mud collected in the inlet of Admiralty Bay (South Shetland Islands)	Bozal *et al.* 1997
P. byunsanensis	Tidal flat sediment (South Korea)	Park *et al.* 2005
P. flavipulchra	Surface seawater (off Nice, France)	Ivanova *et al*. 2002c
P. issachenkonii	Thallus of the brown alga; *Fucus evanescens* (Kraternaya Bight, Kurile Islands, Pacific Ocean)	Ivanova *et al.* 2002a
P. luteoviolacea	Surface seawater (Mediterranean Sea near Nice, France)	Gauthier 1982; Gauthier et al. 1995b
P. maricaloris	Sponge *Fascaplysinopsis reticulata*, collected from the Coral Sea (Australia)	Ivanova *et al.* 2002c
P. piratica	Mucus from a tissue-loss disease lesion on a *Montipora capitate* (Kane'ohe Bay, Hawaii)	Beurmann *et al.* 2017
P. piscicida	Zones of red tides and surface of wounded fishes (South-West coast of Florida)	Bein 1954
P. ruthenica	Mussel; *Crenomytilus grayanus* (Sea of Japan)	Ivanova *et al.* 2002b
P. rubra	Mediterranean Sea water (Nice, France)	Gauthier *et al.* 1976b
P. shioyasakiensi	Sediment samples (Pacific Ocean, at a water depth of 2100 m, off Shioyasaki, Japan)	Matsuyama *et al.* 2014
P. tunicata	*Ciona intestinalis*; tunicate, collected from water off the (Western coast of Sweden)	Holmström *et al.* 1998
P. ulvae	Marine alga; *Ulva lactuca* (rocky intertidal zone of Sydney, East coast of Australia)	Egan *et al.* 2001

4.1.2.5 Biotechnological and ecological significance of *Pseudoalteromonas*

The ability of *Pseudoalteromonas* to occupy a wide range of marine habitats (**Table 4.2**) indicates their adaptive and survival strategies and is suggestive of their potential applications in marine biotechnology research (Bowman 2007; Holmström and Kjelleberg, 1999). e.g., *P. tunicata* and *P. issachenkonii* having antifouling activity have been proposed as an eco-friendly alternative to toxic marine anti-foulant chemical paints (Holmström and Kjelleberg 1999; Yee *et al.* 2007; Dobretsov *et al.* 2007). The potential use of *P. sagamiensis, P. undina, P. espejiana* and other *Pseudoalteromonas* species in fish farming as a biocontrol, probiotic, nutrient enhancers and as a hatchery diet has been reported in multiple studies

(Fernandes and Kerkar 2019; Imada *et al* 1985; Kobayashi *et al.* 2003; Maeda *et al.* 1997; Wang *et al.* 2018; Gorospe *et al.* 1996; Uchida *et al.* 1997). Numerous *Pseudoalteromonas* species have also been reported to exhibit bactericidal, anti-fungal and anti-algal activities, that target a range of organisms (Holmström and Kjelleberg 1999). *P. phenolica* for example exhibits bactericidal effect against methicillin-resistant *Staphylococcus aureus* and a few *Enterococcus* species and has potential as an antibiotic application (Isnansetyo and Kamei 2003). *P. rubra* produces extracellular polyanionic antimicrobial substances which interfere with the respiration of several bacterial species (Gauthier 1976b; Gauthier 1976a; Gauthier *et al.* 1995b). In marine environments, cohabitation of *Pseudoalteromonas* species with eukaryotes is commonly found (**Table 4.2**) that also shows ecologically relevant microbe-host interactions for survival of higher organisms living in its proximity (Holmström *et al.* 2002).

4.1.3 Objectives of the Proposed Study

This study followed up on cultivation and isolation of a salt marsh inhabiting bacteria and aimed to describe two isolates, MARW_1-7-2^T and MARW_1-2-27^T potentially representing new taxa. The isolates were analysed using a polyphasic approach, in accordance with standards of new species description, which integrated phenotypic tests with genotypic methods (Ramasamy *et al.* 2014; Arahal *et al.* 2007; Chun *et al.* 2018; Stackebrandt and Ebers 2006; Richter and Rosselló-Móra 2009).

4.2 METHODS

4.2.1 Sample Collection and Physico-chemical Analysis

Salt marsh surface sea water samples were collected from RSPB Marshside, Southport, UK in June 2017 during low tide and immediately processed upon return to Edge Hill University, Biology lab. The geographical location of the sampling site and collection points within this site are shown in **chapter 3, Fig. 3.2 and 3.3**. Potential novel isolates MARW_1-7-2^T and MARW_1-2-27^T; described in this study were isolated from the water sample ID seven and two with GPS coordinates 53°40'46.0"N 2°59'45.0"W and 53°40'46.0"N 2°59'56.0"W respectively.

Further procedural details on sample collection and physico-chemical analysis are as mentioned in **chapter 2, section 2.1.**

4.2.2. Microbial Isolation, Cultivation and Culture Maintenance

Collected water samples were analysed for microbial cultivation, isolation and culture's cryopreservation as per the procedure elaborated in **chapter 2, section 2.2.**

4.2.3 Test Procedures for Novel Species Characterisation

Cryopreserved isolates MARW_1-7-2^T and MARW_1-2-27^T were revived by passaging twice on a marine agar media (pH 7.6±0.2, NaCl 4% (w/v)) and were further characterised using a comprehensive polyphasic approach. All characterisation tests were performed on freshly grown; 24–48 h old cultures as described below.

4.2.3.1 Colony morphology and physiology observations

Cellular and physiological characteristics of the isolates were tested using Hucker's modified Gram staining method 'A' (Hucker and Conn 1923), motility testing by the hanging drop method (Gerhardt *et al.* 1981), catalase testing using 3% H_2O_2 (Reiner 2010) and bacterial cytochrome oxidase testing using TestOxidase™ reagent (Pro-Lab diagnostics, PL 390). Detailed explanation of these procedures and their test result interpretations is described in **chapter 2, section 2.3**.

Production of poly-β-hydroxyalkanoate (PHA) was assessed using a modified method of Lathwal *et al.* (2015). Marine agar in required NaCl concentration was supplemented with 2% (w/v) glucose (D (+), anhydrous, Timstar, GL2858), then streaked with pure cultures of MARW_1-7-2^T and MARW_1-2-27^T and incubated at 35 °C for three days. Post incubation, 2 ml of lipophilic stain Sudan Black B; 0.3% (w/v) in 70% ethanol; was poured unto the plate and left undisturbed for 30 min. The plates were finally washed with 99% ethanol that removed excess of the stain from the colonies. The colonies that retained Sudan black were recorded positive for PHA.

To confirm the oxygen requirement, inoculated marine agar plates were incubated in an anaerobic environment created inside a GasPak glass jar using GENbox anaerobic generator sachet (bioMérieux SA, 96 124) which contains activated charcoal, sodium ascorbate, and other organic and inorganic compounds that absorbs oxygen and releases carbon dioxide without addition of water or a catalyst. Once the generator is removed from its aluminium packaging cover, it is immediately placed inside the jar containing inoculated plates. The jar lid was tightly

closed and additionally sealed with silicon grease. The reaction starts as soon as the generator come in contact of air, so care was taken to minimize the time between opening the aluminium sachet containing generator and closing the jar. Anaerobic atmosphere was monitored by placing an anaerobic indicator strip (bioMérieux SA, 96 118) inside the jar without touching its inner side and was exteriorly visible. This entire system was then placed inside the incubator at 35 °C with minimum disturbance. In parallel, inoculated plates were incubated aerobically as a control. Growth was observed after 4–5 days of incubation. Isolates were tested in triplicate.

4.2.3.2 Optimum growth requirements

The detailed procedure of these measurements is explained in **chapter 2, section 2.4**.

In summary, optimum growth conditions of potential novel isolates were tested in this study at wide ranges of temperatures, NaCl concentrations, and pH. MARW_1-7-2^T and MARW_1-2-27^T growth studies were conducted in liquid 'growth medium A' (Poli *et al.* 2013), and medium containing synthetic sea water supplemented with 0.3% (w/v) peptone and 0.1% (w/v) yeast extract (Lyman and Fleming 1940; Bein 1954) respectively.

4.2.3.3 Biochemical tests

Additional physio-chemical tests for MARW_1-7-02^T and MARW_1-2-27^T were performed in triplicate using commercially available miniaturized biochemical test strips. Acid production from carbohydrates was analysed using an API® 50 CH bacterial identification kit (bioMérieux SA, 50 300**)**. The substrate oxidation pattern of carbon sources and sensitivity against different chemicals was examined using BIOLOG GEN III MicroPlate™ (Biolog Inc., 1030). Other physiological properties and enzyme activities were assayed by API® 20 E (bioMérieux SA, 20 100**)** and API® ZYM kits (bioMérieux SA, 25 200). Detailed procedures of these test kits are given in **chapter 2, section 2.5**.

4.2.3.4 Chemotaxonomic characterisation

Respiratory quinones and polar lipids analysis

A two-stage method was performed at Leibniz Institute - German collection of microorganisms and Cell Cultures (DSMZ) to extract respiratory quinones followed

by the separation of polar lipids from the potential novel isolates. 150 mg freeze dried biomass of isolates was allowed to react in sequential steps with hexane-methanol (1:2), 0.3% (w/v) NaCl and cold n-hexane. After magnetic stirring and centrifuging this mixture, upper clear phase and bottom precipitate were separated and were analysed for respiratory quinones on HPLC; Agilent Technologies 1260 Infinity II System (Tindall 1990b; Franzmann and Tindall 1990), and for polar lipids using two-dimensional TLC (Tindall *et al.* 2007; Bligh and Dyer 1959; Tindall 1990a; Tindall 1990b). These procedures are explained in detail in **chapter 2, section 2.6.1**.

Fatty acid profile

As one of the recommended tools for biochemical characterisation of novel bacterial isolates (Arahal *et al.* 2007), gas chromatography (GC) analysis of cellular fatty acid was performed in this study. Novel isolates and their closest phylogenetic strains were analysed for fatty acid contents via fatty acid methyl esters (FAMEs, MIDI, Sherlock, Microbial Identification System, version 6.3) protocol and detected on an Agilent GC, 6890 series. *Bacillus subtilis* 6633^{T} was used for QC validation as per manufacturer's instructions. Comprehensive details of fatty acid procedure is provided in **chapter 2, section 2.6.2**.

4.2.3.5 Antibiotic susceptibility test

Microbial strains were also tested for sensitivity to thirteen different antimicrobial compounds using the diffusion agar method (Mata *et al.* 2002). The antimicrobial compound discs used in this study were either commercially available (Oxoid) or prepared in lab by impregnating sterile 5 mm paper discs with the required concentration of filter sterilised antibiotic solution. For both the isolates, each antibiotic was tested in triplicate.

First, a loopful culture of freshly grown MARW_1-7-2^{T} and MARW_1-2-27^{T} was inoculated into their respective liquid growth medium (described in section 4.2.3.2) and incubated at 35 °C for 3–5 h to get a final inoculum OD_{600nm} of 0.3–0.5. The respective autoclaved growth medium containing 0.9% (w/v) agar in 18 ml volume was inoculated with 2 ml of isolates' incubated broth. This seeded agar media was then plated aseptically in a 9 mm petri plate and allowed to solidify. Antibiotic test discs were transferred aseptically onto the solidified seeded agar surface, followed

by incubation at 35 °C. To assess the antibiotic sensitivity, zones of inhibition were observed at 12, 24 and 36 h of incubation.

4.2.3.6 Genetic studies

Genetic studies such as phylogenetic identification based on 16S rRNA gene analysis and draft genome analysis were performed for novel species characterisation of isolates; MARW_1-7-2^T and MARW_1-2-27^T.

Phylogenetic identification

The steps involved in phylogenetic identification of the isolates are briefly mentioned below and explained in detail in **chapter 2, section 2.7**.

The cryopreserved isolates from the present study were revived by passaging twice on a marine agar and then sequentially analysed by DNA extraction, PCR amplification and purification of PCR amplicons at Edge Hill University, Biology lab.

DNA extraction

The DNA extraction procedure consisted of three steps; reagent preparation, Zirconia bead washing in diluted bleach solution, followed by the actual DNA extraction procedure (Griffiths *et al.* 2000).

16S rRNA gene amplification and sequencing

Amplification and sequencing of the 16S rRNA gene from extracted DNA was performed using two universal, forward and reverse bacterial primers (Sigma) (Frank *et al.* 2008; Wilson *et al.* 1990). PCR products were purified using GenElute^{TM} PCR clean-up kit (Sigma-Aldrich, NA1020) and then taken for Sanger sequencing service outsourced to Core Genetics Service at University of Sheffield, UK, and Eurofins Genomics (GATC Biotech), Germany.

16S rRNA gene data analysis

For preliminary putative identification, isolates' quality checked 16S rRNA gene sequences were BLAST against reference sequences of type microbial strains available in NCBI's blastn (National Center for Biotechnology Information, n.d., viewed June 2020) and EzBioCloud web portal (EzBioCloud, Chunlab, May 2020, viewed June 2020). In the BLAST search, MARW_1-7-2^T and MARW_1-2-27^T showed phylogenetic similarity to the genus *Halomonas* and *Pseudoalteromonas* respectively.

Construction of phylogenetic tree

To confirm the phylogenetic position of MARW_1-7-2^T and MARW_1-2-27^T to their most closely related reference strains, a phylogenetic tree was constructed according to the maximum-likelihood method, using isolate's 16S rRNA gene sequence retrieved from its draft genome sequence data. 16S rRNA gene sequences of closest refence species of *Halomonas* and *Pseudoalteromonas* were retrieved from NCBI and EzTaxon database. Affiliation of 16S rRNA gene sequences was done using the parsimony ARB tool (Ludwig *et al.* 2004).

Genome sequencing

Pure cultures of MARW_1-7-2^T and MARW_1-2-27^T freshly grown at the Edge Hill University, Biology Lab were sent to MicrobesNG for genome sequencing in March 2019. At MicrobesNG, draft genomes were sequenced on the Illumina HiSeq 2500 platform. The sequence files of genome assembly received at Edge Hill University, Biology Lab were further assessed for quality and were annotated by the Rapid Annotations using Subsystems Technology (RAST) tool to predict gene structures and functions (Seemann *et al.* 2014; Aziz *et al.* 2008). Further details on genome analysis are described in **chapter 5, sections 5.2**.

Analysis of genome sequence for novel taxa description

Genomes of the potential novel isolates obtained in this study and genome of their closest phylogenetic relatives retrieved from NCBI and EzBioCloud GenBank database were analysed for species specific genome characteristics, such as DNA G+C content, calculation of average nucleotide identity (ANI) and DNA-DNA homology (DDH).

DNA G+C (mol%) content of MARW_1-7-2^T and MARW_1-2-27^T was retrieved from their genome sequence.

ANI is a recommended genome feature in novel species characterisation for the evaluation of similarity between two genomes (Goris *et al.* 2007; Jain *et al.* 2018). For this purpose, ANI web calculator available at ChunLab's Orthologous ANI server was used in this study (Lee *et al.* 2016), where the genome sequences of potential novel isolates and their phylogenetic relatives were uploaded. Cut off value of ANI for species boundary proposed by Richter and Rosselló-Móra (2009) was used to predict these results.

For genome comparison between isolates and their closest relatives, Genome-to-Genome Distance Calculator (GGDC 2.1, Meier-Kolthoff *et al.* 2013a) bioinformatic web tool (http://ggdc.dsmz.de/) was used. This server calculates intergenomic distances between uploaded sequences, which are then converted into similarity values analogous to the wet-lab DDH results. In the present study, these DDH values were interpreted to confirm the novel status of the isolates based on the instructions provided by Tindall *et al.* (2010) and Wayne *et al.* (1987).

4.2.3.7 Differentiation from closely related taxa

To demonstrate the novel species status of MARW_1-7-2^T and MARW_1-2-27^T their morphological, physiological, genomic, chemo-taxonomical characters obtained in the present study were compared with those of their 7–8 closest phylogenetic relatives.

4.3 RESULTS

4.3.1 Physico-chemical Analysis of Salt Marsh Samples

Isolates MARW_1-7-2^T and -1-2-27^T were cultivated from the mid marsh and low marsh sea water samples 7 and 2 respectively. The physico-chemical analysis results of these water samples, along with the cumulative data of all mid marsh and low marsh samples of this marsh visit are detailed in **Table 4.3**.

Table 4.3 Physico-chemical parameters of sea water samples 7 and 2 collected at mid marsh and low marsh region of RSPB Marshside respectively. The cumulative data reported as the observed range with its mean written in parenthesis.

Parameters	Mid marsh		Low marsh	
	Sample 7	Cumulative data	Sample 2	Cumulative data
Salinity (g/100g)	2	2–2.8 (2.5)	2.4	2.4–3 (2.6)
pH	8.8	7.5–8.8 (8.0)	8.1	7.9–8.2 (8.1)
Phosphate (PPM)	0.87	0.25–0.87 (0.76)	0.75	0.5–0.75 (0.67)
Iron (PPM)	0.15	0.1–0.6 (0.24)	0.2	0.05–0.3 (0.18)
Ammonium (PPM)	0.5	0–0.5 (0.11)	0	0–0.5 (0.33)

4.3.2 Halomonas Novel Species

4.3.2.1 Colony morphology and physiology

MARW_1-7-2^T showed abundant growth when incubated for 2–3 days on marine agar at 35 °C. Colonies appeared cream white, semi-transparent, circular, convex, 2–3 mm, with smooth consistency and entire margin. Mucoid consistency observed for culture incubated or refrigerated for more than a week. Gram-stained cells were found to be Gram-negative, 1–3 µm long, thin, single, straight rods. It did not reduce nitrates, nitrites or produce H_2S. Differential test results shown by MARW_1-7-2^T are displayed in **Table 4.4**.

4.3.2.2 Optimum growth requirements

Growth displayed by MARW_1-7-2^T at different temperature, NaCl and pH conditions in comparison to its phylogenetically related close strains is described in **Table 4.5**. This isolate had shown optimum growth at temperature of 35 °C, NaCl concentration of 3% (w/v) and pH 6.5–7. Growth of MARW_1-7-2^T ceased when medium pH was less than 5, and the operating values of temperature, NaCl and pH were risen beyond 45 °C, 20% (w/v) and 9 respectively.

Growth studies of MARW_1-7-2^T performed in NB growth medium to further evaluate its dependence on NaCl and other salts, showed no sign of growth in medium deprived of NaCl, when incubated up to 70 h. Whereas growth was observed in NB added with 3% (w/v) NaCl.

4.3.2.3 Biochemical tests

Differential biochemical test results of MARW_1-7-2^T such as acid production and hydrolysis of various chemical components using API® 50 CH and API® 20 E test strips are displayed in comparative **Table 4.6**. MARW_1-7-2^T was able to utilize variety of substrates for growth and was characterised by its hydrolytic activity for esculin.

In addition, acid production was observed from D-ribose, D-fructose, D-turanose, D-salicin, potassium 5-ketogluconate and weak positive reaction from D-trehalose, when tested using API® 50 CH test strip. Incubation with following substrates did not yield acid: D-raffinose, L-rhamnose, D-cellobiose, D-melibiose, D- and L-fucose, erythritol, D-sorbitol, D-arabitol, methyl-αD-mannopyranoside, methyl-αD-

glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, glycogen, starch, and potassium 2-ketogluconate.

Microbial breakdown of substrates observed using an API® 20 E test strip displayed oxidation of trisodium citrate, and L-tryptophan (tryptophan deaminase). Whereas, L-arginine, lysine, ornithine, D-sorbitol, D-mannitol, inositol, D-glucose, L-arabinose, D-sucrose, D-melibiose, amygdalin, 2-nitrophenyl-ßDgalactopyranoside (ONPG test), were not hydrolysed. Indole and acetoin (Voges Proskauer test) were not produced.

When tested with GEN III MicroPlate™, following substrates did not support microbial growth: D-maltose, D-mannose, sucrose, D-raffinose, D- and L- fucose, L-rhamnose, gentiobiose, D-melibiose, D-cellobiose, D-trehalose, D-turanose, glycerol, myo- inositol, D-sorbitol, D-arabitol, D-gluconic acid, L-glutamic acid, D- and L-aspartic acid, N-acetyl-D glucosamine, L-arginine, L-alanine, D- and L-serine, 1% sodium lactate, guanidine HCl, niaproof 4, tetrazolium blue, p-hydroxy phenylacetic acid, bromo-succinic acid, potassium tellurite, and sodium butyrate. Whereas, microbial growth was observed in presence of D-fructose, D-galactose, D-salicin, lithium chloride, tween 40, propionic acid, acetoacetic acid and acetic acid. Sensitive of MARW_1-7-2^T towards antimicrobials e.g., nalidixic acid, fusidic acid, troleandomycin, minocycline, rifamycin SV, aztreonam, whereas resistant against vancomycin was also noted when observed on GEN III MicroPlate™.

Positive enzymatic activity of MARW_1-7-2^T was observed for alkaline phosphatase, esterase (C4), esterase, lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase when tested using an API® ZYM test strips. Enzymatic activity of lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, α- and ß- glucosidase, N-acetyl-ß-glucosaminidase, α-mannosidase, α-fucosidase, ß-glucuronidase was not observed.

Table 4.4 Colony morphology and physiological characteristics of MARW_1-7-2^T and closely related species
All taxa are Gram-negative.

Strains (data source in brackets): 1, MARW_ 1-7-2^T (this study); **2,** *H. taeanensis* BH539^T (Lee *et al.* 2005); **3,** *H. halophila* F5-7^T (Quesada *et al.* 1984); **4,** *H. salina* F8-11^T (Valderrama *et al.* 1991); **5,** *H. smyrnensis* AAD6^T (Poli *et al.* 2013); **6,** *H. elongata* 1H9^T (Vreeland *et al.* 1980); **7,** *Cobetia marina* ATCC 25374^T (Arahal *et al.* 2002a).
Abbreviations: T = Type strain; + = Positive; - = Negative; W+ = Weak positive; NG = Not given in novel species literature

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell morphology	Straight rod	Short rod	Rod	Rod	Rod	Curved and straight rod	Straight rod
Pigmentation	Cream white	Cream	Cream	Slightly yellow or cream	Dark yellow	White	Cream
Motility	-	+	+	-	-	-	-
Production of PHA	+	NG	NG	+	-	+	+
Oxygen requirement	Strict aerobe	Strict aerobe	Strict aerobic	Aerobe	Facultative anaerobe	Facultative anaerobe	Strict aerobe
Oxidase	+	+	+	+	-	-	-
Catalase	W+	+	+	+	+	+	+
Reduction of nitrates to nitrites (NO_2)	-	+	+	+	-	+	-

Table 4.5 Optimum growth features of MARW_1-7-2^T and closely related species

Strains (data source in brackets): 1, MARW_1-7-2^T (this study); **2,** *H. taeanensis* BH539^T (Lee *et al.* 2005); **3,** *H. halophila* F5-7^T (Quesada *et al.* 1984); **4,** *H. salina* F8-11^T (Valderrama *et al.* 1991); **5,** *H. smyrnensis* AAD6^T (Poli *et al.* 2013); **6,** *H. elongata* 1H9^T (Vreeland *et al.* 1980); **7,** *Cobetia marina* ATCC 25374^T (Arahal *et al.* 2002a)
Abbreviations: T = Type strain; NG = Not given in novel species literature

Characteristics	1	2	3	4	5	6	7
Temperature (°C)							
Range	5 – 45	10 – 45	15 – 45	15 – 42	5 – 40	4 – 45	10 – 42
Optimum	35	35	30 – 37	32	37	37	37
NaCl (%, w/v)							
Range	0 – 20	1 – 25	2 – 30	2.5 – 20	3 – 25	0 – 32	0.5 – 20
Optimum	3	10 – 12	7.5	5	10	3.5 – 8	5
pH							
Range	5 – 9	7 – 10	5 – 10	6 – 10	5.5 – 8.5	5 – 9	5 – 10
Optimum	6.5 – 7	7.5 – 8.0	7	7.2	7	NG	7.5

Table 4.6 Biochemical test results of MARW_1-7-2^T and closely related species

Strains (data source in brackets): **1,** MARW_1-7-2^T (this study); **2,** *H. taeanensis* BH539^T (Lee *et al.* 2005); **3,** *H. halophila* F5-7^T (Quesada *et al.* 1984); **4,** *H. salina* F8-11^T (Valderrama *et al.* 1991); **5,** *H. smyrnensis* AAD6^T (Poli *et al.* 2013); **6,** *H. elongata* 1H9^T (Vreeland *et al.* 1980); **7,** *Cobetia marina* ATCC 25374^T (Arahal *et al.* 2002a)
Abbreviations: T = Type strain; + = Positive; - = Negative; W+ = Weak positive; NG = Not given in novel species literature; ND = Not done

Characteristics	1	2	3	4	5	6	7
Biochemicals - Acid production from							
D-Glucose	+	+	+	-	+	+	+
D-Mannose	-	W+	-	-	+	+	+
D-Sorbitol	-	+	+	-	NG	+	-
D-Xylose	-	-	+	-	NG	NG	-
D-Maltose	+	+	+	-	+	NG	-
D-Sucrose	+	+	-	-	+	+	+
D-Galactose	+	NG	+	-	NG	NG	-
L-Arabinose	-	+	-	-	-	NG	-
D-Trehalose	W+	+	-	-	NG	+	+
D-Lactose	-	+	-	-	-	+	-
D-Mannitol	+	+	-	-	NG	+	-
D-Adonitol	-	-	-	-	NG	NG	NG
Inositol	-	+	-	-	NG	-	-
Glycerol	-	+	-	-	NG	+	+
Hydrolysis of							
Gelatine	-	-	-	-	-	+	-
Urea	-	+	-	+	-	+	+
Starch	-	-	-	-	+	-	-
Casein	ND	-	-	-	+	-	+
Esculin	+	-	+	-	+	+	-
Gluconate	-	NG	NG	-	NG	+	+
Tween 80	ND	-	-	-	+	-	-
DNA	ND	-	NG	-	-	NG	+

4.3.2.4 Chemotaxonomic characterisation

Chromatogram analysis of ubiquinone showed two peaks in MARW_1-7-2^T, the major quinone was identified as Q-9 and traces of Q-8 was also detected (**Fig. 4.1**, **Table 4.7**).

This strain exhibited polar lipid profile of phosphatidylglycerol and phosphatidylethanolamine as major lipids, complemented with moderate amounts of phosphoaminolipid, one unknown phospholipid and a minor amount of diphosphatidylglycerol. The chromatogram of total lipids with polar lipid interpretations is shown in **Fig. 4.2**.

Analysis of the FAME profile of MARW_1-7-2^T showed presence of C18:1 ω7c (48.19%), C16:0 (17.9%), summed feature 3 (C16:1 ω7c/15 iso 2OH; 15.93%) and C12:0 3OH (7.11%) as major fatty acids (>5%). Detailed fatty acid profile is mentioned in **Table 4.8**.

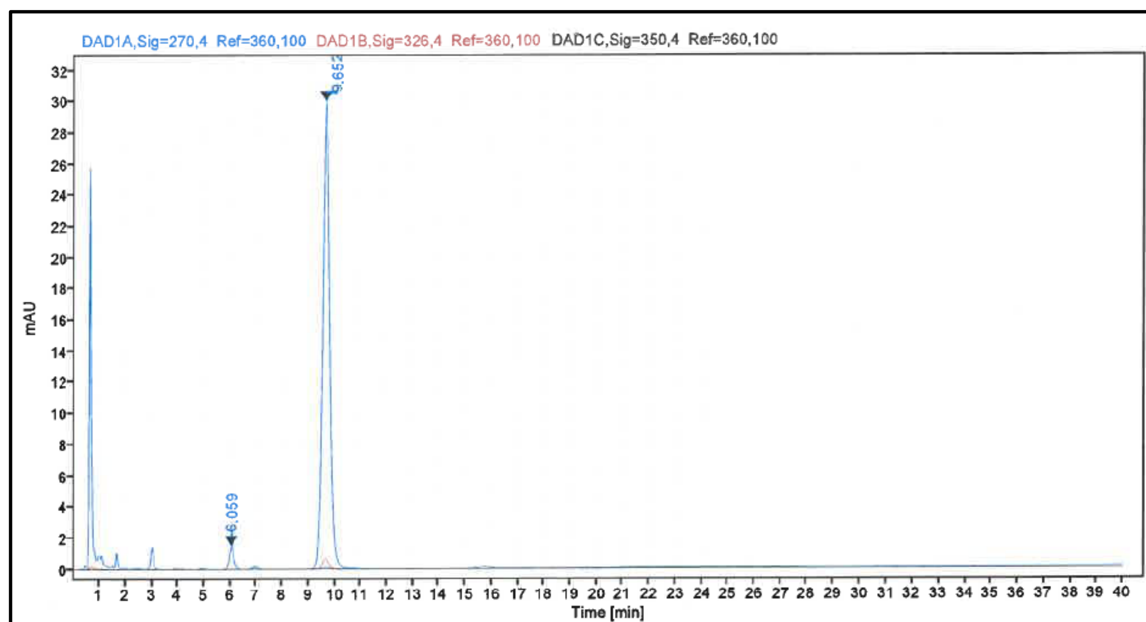

Figure 4.1 HPLC chromatogram showing the peaks for ubiquinone 8 and 9 extracted from the isolate MARW_1-7-2^T

Table 4.7 Interpretation of HPLC chromatogram peaks observed for ubiquinone extracted from MARW_1-7-2^T
Abbreviations: RT = Retention time

Signal: DAD1A, Sig=270,4 Ref=360,100						
RT [min]	**Type**	**Width [min]**	**Area**	**Height**	**Areas%**	**Name**
6.059	MM m	0.19	17.68	1.43	3.05	Q 8
9.652	BB	2.05	562.81	29.94	96.95	Q 9
		Sum	580.49			

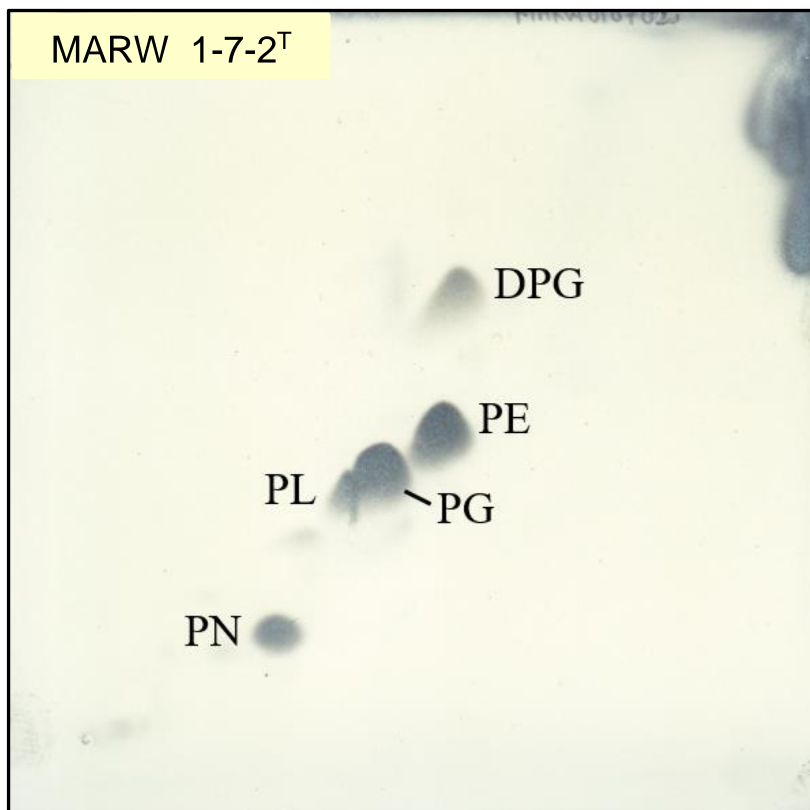

Figure 4.2 Polar lipid profile of MARW_1-7-2^T after separation by two-dimensional thin layer chromatography (TLC) and staining with molybdophosphoric acid. Polar lipid content quantified by observing the intensity and area covered by the individual spots that appeared on TLC plate

Abbreviations: PE = Phosphatidylethanolamine; PG = Phosphatidylglycerol; PN = Phosphoaminolipid; DPG = Diphosphatidylglycerol; PL = Phospholipid

Table 4.8 Fatty acid profiles of MARW_1-7-2^{T} and reference strains, all values in percentage

Strains (data source in brackets): **1,** MARW_1-7-2^{T} (this study); **2,** *H. taeanensis* BH539^{T} (Lee *et al.* 2005); **3,** *H. halophila* F5-7^{T} (Quesada *et al.* 1984); **4,** *H. salina* F8-11^{T} (Valderrama *et al.* 1991); **5,** H. smyrnensis AAD6^{T} (Poli *et al.* 2013); **6,** *H. salifodinae* BC7^{T} (Wang *et al.* 2008); **7,** *H. saccharevitans* AJ275^{T} (Xu *et al.* 2007); **8,** *H. elongata* 1H9^{T} (this study); **9,** *Cobetia marina* DSM 4741^{T} (Arahal *et al.* 2002a); **10,** *H. organivorans* G-16.1^{T} (García *et al.* 2004); **11,** *C. crustatorum* JO1^{T} (Kim *et al.* 2010c)
Abbreviations: T = Type strain; ND = Not detected in analysis; - = Not mentioned in novel species literature; NG = Not given in lnovel species literature; ¥ = Fatty acid percentages not mentioned in novel species literature; * = C16:1 ω7c/15 iso 2OH; © = Mixture of C16:1 ω7c and/or iso-C15:2-OH; $ = Mixture of C16:1 ω7c/iso-C15:0 2-OH; # = Less than 1%

Fatty Acid	1	2	3	4	5	6¥	7	8	9	10	11
C18:1 ω7c	48.19	29.10	-	-	22.6	Major	30.65	48.54	20.7	NG	3.8
C16:0	17.9	27.50			35	Major	20.6	21.76	26		24.6
Summed Feature 3	15.93*	13.7©			-	-	-	12.46©	16.7$		22.2$
C16:1 ω7c	ND	-			21.4	-	10.37	-	-		-
C16:0 ω7c	ND	-			-	Major	-	-	-		-
C12:0 3OH	7.11	6.9			10.7	-	-	6.16	9.5		25.3
C10:0	2.58	3			Trace	-	-	2.03	1.7		4.5
C19:0 CYCLO ω8c	1.67	9.40			5.6	-	4.42	2.69	4.9		ND
11 methyl 18:1 ω7c	1.66	-			-	-	-	-	-		-
C17:0 CYCLO	1.09	5.1			-	-	-	-	13.8		9.4
C18:0	0.83	0.5			-	-	14.78	-	Trace#		1
C12:0	ND	3.7			1.3	-	-	2.85	4		8
C17:0	1.07	-			-	-	-	-	Trace#		ND
Summed feature 8 (C18:1 ω7c/ω6c)	ND	-			-	-	-	-	-		-
Summed feature 3 (C16:1 ω7c/ω6c)	ND	-			-	-	-	-	-		-

4.3.2.5 Antibiotic susceptibility

Among tested antimicrobials MARW_1-7-2^{T} showed resistance to streptomycin (concentration per disc, 10 µg) and vancomycin (30 µg), and susceptibility to cefotaxime (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and cephalexin (30 µg). Its sensitivity to other antimicrobials in comparison to closest phylogenetic strains is shown in **Table 4.9.**

Table 4.9 Susceptibility to antimicrobials in MARW_1-7-2^{T} and closely related *Halomonas* species

Strains: **1,** MARW_1-7-2^{T}; **2,** *H. taeanensis* BH539^{T}; **3,** *H. halophila* F5-7^{T}; **4,** *H. salina* F8-11^{T}; **5,** *H. smyrnensis* AAD6^{T}; **6,** *H. elongata* 1H9^{T}; **7,** *Cobetia marina* ATCC 25374^{T}
Data source: MARW_1-7-2^{T} from this study, rest observations from Mata *et al.* 2002
Abbreviations: T = Type strain; + = Susceptible; - = Resistant; NG = Not given in novel species literature

Antimicrobials tested	1	2	3	4	5	6	7
Amoxicillin	+ (30 µg)	NG	+ (25 µg)	- (25 µg)	NG	- (25 µg)	+ (25 µg)
Nalidixic acid (30 µg)	+		-	+		+	+
Erythromycin (15 µg)	+		+	+		+	-
Carbenicillin disodium (100 µg)	+		+	+		-	+
Ampicillin (10 µg)	+		+	+		-	+
Chloramphenicol (30 µg)	+		+	+		+	-

4.3.2.6 Genetic studies

Phylogenetic analysis

BLAST analysis

The 1247 bp long 16S rRNA gene sequence of MARW_1-7-2^{T} that was obtained by the Sanger method and quality checked, was found to contain well-formed, distinctive, nucleotide peaks without any background noise. The preliminary BLAST analysis of this sequence phylogenetically placed MARW_1-7-2^{T} close to the genus *Halomonas.*

Further confirmation of this BLAST result and construction of phylogenetic tree was accomplished using the most complete 16S rRNA gene sequence of MARW_1-7-2^{T} retrieved from its genome sequence data. This 16S rRNA gene sequence was 1540 bp long which met the defined 16S rRNA gene sequence quality standard of

>1400 nucleotide suggested for new taxa characterisation (Arahal *et al.* 2007). This complete 16S rRNA gene sequence when BLAST on EzBioCloud and NCBI blastn web tool, the test isolate again showed phylogenetic relatedness to the genus *Halomonas*. Its first five closest phylogenetic (type strain) relatives found on EzBioCloud and NCBI blastn web search are as detailed in **Table 4.10**.

Table 4.10 Similarity based on 16S rRNA phylogeny between MARW_1-7-2^T and its closest type strain relatives, analysed using EzBioCloud and NCBI blastn tools

Closest relative	On EzBioCloud BLAST	On NCBI blastn tool
1st	*H. taeanensis* BH539^T (98.36%)	*H. smyrnensis* AAD6^T (96.36%),
2nd	*H. smyrnensis* AAD6^T (96.16%)	*H. halophila* CCM 3662^T (96.1%),
3rd	*H. salina* F8-11^T (95.89%)	*H. pacifica* NBRC 102220^T (96.04%)
4th	*H. salifodinae* BC7^T (95.87%)	*H. elongata* 1H9^T (95.65%)
5th	*H. halophila* CCM 3662^T (95.84%)	*H. caseinilytica* JCM 14802^T (95.58%)

Phylogeny tree

The phylogenetic position of MARW_1-7-2^T was further evaluated by comparing its complete 16S rRNA gene sequence to those of its closest relatives, which includes type species of the genus *Halomonas* and a few other closest genera of the family *Halomonadaceae* (**Fig. 4.3).** The derived maximum likelihood tree showed MARW_1-7-2^T falls within the cluster comprising *Halomonas* species. The new isolate was placed near to *H. taeanensis*, with a branch supported with the bootstrap value of 99%, that was also connected to two species of the genus *Cobetia* but with 81% bootstrap value. The 16S rRNA gene sequence similarity matrix values calculated from phylogenetic tree analysis further confirmed closest phylogenetic affinities of MARW_1-7-2^T to the type species of *H. taeanensis* (97.1%), followed by *H. halophila* (95.0%), *H. salina* (94.8%), *H. salifodinae* (94.6%), *H. saccharevitans* (94.6%), *H. elongata* (94.5%), *Cobetia marina* (94.3%), *H. organivorans* (94.3%), *Cobetia crustatorum* (93.6%), *H. smyrnensis* (93.4%) etc.

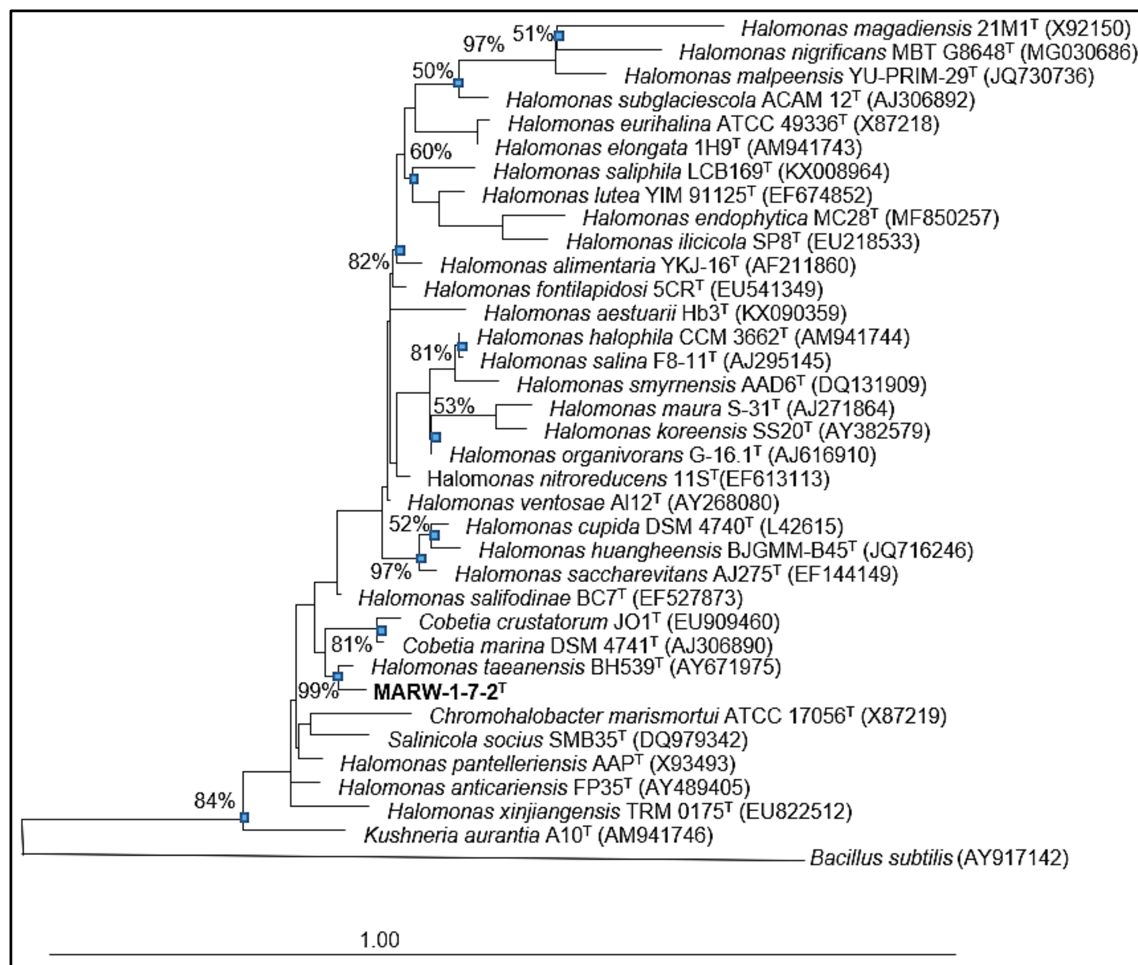

Figure 4.3 Maximum likelihood tree showing phylogenetic position of the isolate MARW_1-7-2^{T} and representatives of the related taxa. Affiliation of 16S rRNA gene sequences was done posteriori using the parsimony ARB tool (Ludwig *et al.* 2004) without changing the overall tree topology. Numbers at the branch nodes highlighted with blue squares, refer to bootstrap values >50% (1000 iterations). 16S rRNA gene sequence of *Bacillus subtilis* was used as an outgroup. GenBank accession numbers are indicated in parentheses.

Genome data analysis

Genome sequencing of MARW_1-7-2^{T} performed on Illumina HiSeq 2500 using 250 bp paired-end protocol and 30x coverage provided a total of 326,822 reads (≥ 0 bp). From these, a total of 79 contigs (≥ 0 bp) were obtained out of which 54 were ≥1000bp. The N50, indicating the shortest contig length at 50% of the entire assembly was 137,558 bp. Genome size was 3,544,736 bp. G+C content was of MARW_1-7-2^{T} and its closest relatives is as shown in **Table 4.11**.

In-silico ANI and DDH values obtained after comparing genomes of MARW_1-7-2^{T} with its closest phylogenetic strains of *Halomonas* and *Cobetia* species on EzBioCloud webtool, is shown in **Table 4.11**

Table 4.11 DNA G+C content, average nucleotide identity (ANI) and DNA-DNA hybridization (DDH) values of MARW_1-7-2^T and its closest relatives. Average nucleotide identity (ANI) and DNA-DNA hybridization (DDH) values calculated in-between MARW_1-7-2^T and closest relatives by their genome sequence comparison on ChunLab's Orthologous ANI and GGDC 2.1 webtool respectively

Data source of G+C contents: 1, MARW_1-7-2^T (this study, calculated from genome data); **2,** *H. taeanensis* BH539^T (Lee *et al.* 2005); **3,** *H. halophila* F5-7^T (Quesada *et al.* 1984); **4,** *H. salina* F8-11^T (Valderrama *et al.* 1991); **5,** *H. smyrnensis* AAD6^T (Poli *et al.* 2013); **6,** *H. saccharevitans* J275^T (Xu *et al.* 2007); **7,** *H. elongata* 1H9^T (Vreeland *et al.* 1980); **8,** *Cobetia marina* DSM 4741^T(Arahal *et al.* 2002a); **9,** *C. crustatorum* JO1^T (Kim *et al.* 2010c)
Abbreviations: T = Type strain; *Study results; NA = Genome sequence of type strains not available for ANI or DDH calculations

Genome Features	**Target DNA**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
G+C (mol%)	65.14	65.0	66.7	64.2	63.0	65.9	60.5± 0.5	63.0	61.4
ANI (%)*	100	84.8	NA	NA	79.3	79.1	77.1	74.8	73.0
DDH (%)*	100	29.2	NA	NA	23.5	22.8	21.7	21.5	20.2

4.3.3 *Pseudoalteromonas* Novel Species

4.3.3.1 Colony morphology and physiology

The colony morphology and physiology characteristics of MARW_1-2-27^T in comparison to its closest phylogenetic relatives are shown in **Table 4.12.**

MARW_1-2-27^T colonies produced a shiny orange coloured pigment which was diffusible in liquid growth medium. Colonies were circular, 2–3 mm in diameter, semi-transparent, convex with entire margin and smooth consistency after 24–48 h of incubation at 35 °C on marine agar. Orange pigment produced by culture was diffusible in the liquid media. When observed under light microscope, gas bubble like structures were observed in the colonies. Gram staining showed Gram-negative, 1 µm long, straight, thin, single rods. Cells were weak catalase positive and motile. Orange, 2-3 mm colonies were developed in aerobic conditions. Whereas in anaerobic conditions semi-transparent, 1-2 mm colonies were observed with scant growth that lacked orange pigmentation.

4.3.3.2 Optimum growth requirements

Results of growth curve tests of MARW_1-2-27^T performed at different temperature, NaCl and pH conditions are described in **Table 4.13**. This isolate displayed maximum growth at 35 °C, NaCl concentration of 1% (w/v) and pH 7. Its growth ceased when the pH was less than 6, as well as when temperature and NaCl concentration were raised above 40 °C and 13% (w/v) respectively.

4.3.3.3 Biochemical tests

Differential biochemical test results of MARW_1-2-27^T and closely related species are summarized in the comparative **Table 4.14.** The full description of these results for MARW_1-2-27^T are as mentioned ahead.

Carbohydrate metabolism when tested in the isolate MARW_1-2-27^T using an API® 50 CH test strip, it showed acid production from D-mannose, D-fructose, D-saccharose (sucrose) and esculin. Delayed acid production (6–7 days post-incubation) was observed in the presence of D-lyxose and potassium 5-ketogluconate. Acid was not produced from L-arabinose, D-ribose, D-glucose, L-rhamnose, D-cellobiose, lactose, D-melibiose, D-trehalose, D-raffinose, gentiobiose, turanose, D-fucose, L-fucose, amygdalin, salicin, amidon (starch), inositol, D-mannitol, D-sorbitol, and D-arabitol.

Breakdown of chemical compounds by MARW_1-2-27^T when tested with additional test strips e.g., API® 20 E and BIOLOG GEN III MicroPlate™, their cumulative tests results showed gelatine breakdown, positive test results for L-tryptophane, dextrin and weak growth in the presence of D-mannose. No hydrolysis was observed when incubated with L-arabinose, D-glucose, L-rhamnose, inositol, D-mannitol, D-sorbitol, amygdalin, D-melibiose, pyruvate, L-arginine, and urea.

Tests results of BIOLOG GEN III MicroPlate™, showed no microbial growth when incubated with D-fructose, myo-inositol, D-galactose, salicin, D-cellobiose, D-saccharose (sucrose), D-trehalose, D-raffinose, gentiobiose, turanose, D-fucose, L-fucose, D-arabitol, N-acetylglucosamine, inosine, glycerol, D-aspartic acid, D-serine, L-serine, L-glutamic acid, tween 40, L-lactic acid, alpha-hydroxy butyric acid, formic acid and lincomycin.

When analysed with API® ZYM test strips, positive enzymatic activity for alkaline phosphatase, esterase (C4), esterase lipase (C8), and naphthol-AS-BI-

phosphohydrolase, as well as weak positive results for lipase (C14) and leucine arylamidase was observed. Whereas, acid phosphatase, ß-galactosidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, ß-glucuronidase, α - and ß - glucosidase, N-acetyl-ß-glucosaminidase, α-mannosidase, α-fucosidase enzymatic activity not observed.

4.3.3.4 Chemotaxonomic characterisation

The composition of quinone as determined by HPLC is shown in **Fig. 4.4** and **Table 4.15**. Q-8 (ubiquinone with eight isoprene units) was the predominant quinone found in the isolate MARW_1-2-27^T.

Isolate MARW_1-2-27^T showed a polar lipid profile consisting of major lipids; phosphatidylethanolamine and phosphatidylglycerol. Moderate amount of aminophospholipid, trace amounts of amino lipid, unidentified lipid, and unidentified phospholipid were also found (**Fig. 4.5)**. Polar lipid profile of MARW_1-2-27^T in comparison to its closest phylogenetic relatives is as shown in **Table 4.16**.

Detailed fatty acid profile of MARW_1-2-27^T in comparison to its phylogenetic relatives is as described in **Table 4.17**. Analysis of its FAME profile revealed that its major fatty acids (>5%) included C12:0 3OH (24.75%), summed feature 3 (C16:1 ω7c/15 iso 2OH; 19.71%), C16:0 (16.73%), C12:0 (5.9%), C18:1 ω7c (5.6%) and C10:0 3OH (5.26%).

Table 4.12 Colony morphology and physiological characteristics of MARW_1-2-27^T and closely related species of genus *Pseudoalteromonas*
All taxa are Gram-negative rods, oxidase positive, reduce nitrates to nitrites, produce gelatinase

Strains (data source in brackets): **1,** MARW_1-2-27^T (this study); **2,** *P. piscicida* ATCC15057^T (Bein 1954; Buck *et al.* 1963; Hansen *et al.* 1965; Gauthier *et al.* 1995b; Gauthier and Breittmayer 1979; Ivanova *et al.* 2002c; Venkateswaran and Dohmoto 2000); **3,** *P. flavipulchra* 208^T (Ivanova *et al.* 2002c); **4,** *P. maricaloris* KMM 636^T (Ivanova *et al.* 2002c); **5,** *P. peptidolytica* F12-50-A1^T (Venkateswaran and Dohmoto 2000); **6,** *P. rubra* 18^T (Gauthier *et al.* 1995b); **7,** *P. spongiae* UST010723-006^T (Lau *et al.* 2005)
Abbreviations: T = Type strain; + = Positive; - = Negative; W+ = Weak positive; NG = Not given in novel species literature

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Isolation source	Salt marsh surface sea water, RSPB Marshside, UK	Red tide sea water, Florida, USA	Surface seawater off Nice, France	Sponge F*ascaplysinopsis reticulata*, Coral Sea	Sea water of Yamato Island, Sea of Japan	Mediterranean Sea near Nice, France	Sponge *Mycale adhaerens*, Hong Kong
Physiology							
Pigmentation	Orange	Yellow orange	Orange	Lemon-yellow	Yellow	Red	Pale orange
Gas bubble formation	+	NG	NG	NG	NG	NG	+
Motility	+	+	+	+	+	+	-
Oxygen requirement	Facultative anaerobe	Strict Aerobe	Strict aerobe	Strict aerobe	Aerobe	Strict aerobe	Strict aerobe
Catalase	W+	+	+	+	+	W+	+

Table 4.13 Optimum growth features of MARW_1-2-27^T and closely related species of genus *Pseudoalteromonas*

Strains (data source in brackets): **1,** MARW_1-2-27^T (this study); **2,** *P. piscicida* ATCC15057^T (Bein 1954; Buck *et al.* 1963; Hansen *et al.* 1965; Gauthier *et al.* 1995b; Gauthier and Breittmayer 1979; Ivanova *et al.* 2002c; Venkateswaran and Dohmoto 2000); **3,** *P. flavipulchra* 208^T (Ivanova *et al.* 2002c); **4,** *P. maricaloris* KMM 636^T (Ivanova *et al.* 2002c); **5,** *P. peptidolytica* F12-50-A1^T (Venkateswaran and Dohmoto 2000); **6,** *P. rubra* 18^T (Gauthier *et al.* 1995b); **7,** *P. spongiae* UST010723-006^T (Lau *et al.* 2005)
Abbreviations: T = Type strain; + = Positive; - = Negative; NG = Not given in novel species literature

Characteristics	1	2	3	4	5	6	7
Temperature (°C)							
Range	10 – 40	10 – 37	10 – 44	10 – 37	15 – 40	10 – 37	12 – 44
Optimum	35	NG	25 – 35	25 – 35	30	NG	NG
NaCl (%, w/v)							
Range	0 – 13	0.5 – 10	0.5 – 10	0.5 – 10	1 – 10	1 – 8.5	2 – 6
Optimum	1	NG	1 - 3	1 - 3	6	7	NG
pH							
Range	6 – 9.6 (or higher)	6 – 8	5 – 12	6 – 10	6 – 10	6 – 10	5 – 1 0
Optimum	7	NG	7.5 – 8	7.5 – 8	6 – 7	NG	NG
Growth in the absence of sodium chloride.	+	-	-	-	-	-	-
Growth in presence of NaCl >12% (w/v)	+	-	-	-	-	-	-
Growth at 40 °C	+	-	+	-	+	-	+

Table 4.14 Biochemical and enzyme activity results of MARW_1-2-27^T and closely related species of genus *Pseudoalteromonas*
The isolates from the present study and most of its closely related strains were negative for H_2S, Indole production, ß-galactosidase and urease activity.

Strains (data source in brackets): **1,** MARW_1-2-27^T (this study); **2,** *P. piscicida* ATCC 15057^T (Bein 1954; Buck *et al.* 1963; Hansen *et al.* 1965; Gauthier *et al.* 1995b; Gauthier and Breittmayer 1979; Ivanova *et al.* 2002c; Venkateswaran and Dohmoto 2000); **3,** *P. flavipulchra* 208^T (Ivanova *et al.* 2002c); **4,** *P. maricaloris* KMM 636^T (Ivanova *et al.* 2002c); **5,** *P. peptidolytica* F12-50-A1^T (Venkateswaran and Dohmoto 2000); **6,** *P. rubra* 18^T (Gauthier *et al.* 1995b); **7,** *P. spongiae* UST010723-006^T (Lau *et al.* 2005)
Abbreviations: T = Type strain; + = Positive; - = Negative; W+ = Weak positive; NG = Not given in novel species literature

Characteristics	1	2	3	4	5	6	7
Biochemical tests							
Voges Proskauer test	-	+	+	NG	NG	-	-
Utilization of:							
D-Galactose	-	+	-	+	-	NG	-
D-Maltose	-	+	+	+	+	-	+
D-Glucose	-	+	+	+	+	+	+
D-Fructose	-	+	+	+	-	NG	+
D-Mannose	W+	+	+	+	-	+	+
D-Melibiose	-	-	-	+	NG	NG	-
D-Saccharose (sucrose)	-	+	+	+	-	NG	-
D-Trehalose	-	+	+	+	NG	+	-
L- tryptophane (tryptophane deaminase)	+	NG	NG	NG	NG	+	-
Inositol	-	-	NG	NG	NG	NG	+
D-Mannitol	-	-	-	+	-	NG	-
D-Sorbitol	-	-	-	+	-	NG	-
Glycerol	-	-	-	+	-	-	-
N-acetylglucosamine	-	+	+	NG	+	NG	+
Amidon (starch)	-	+	+	+	+	+	+
Glycogen	-	+	+	+	NG	NG	-
Gelatine	+	+	+	+	+	+	+
Dextrin	+	NG	NG	+	NG	NG	NG
Citrate	-	+	+	+	-	-	-
Enzyme activity							
Catalase	W+	+	+	+	+	W	+
Lipase	W+	-	-	-	+	+	-

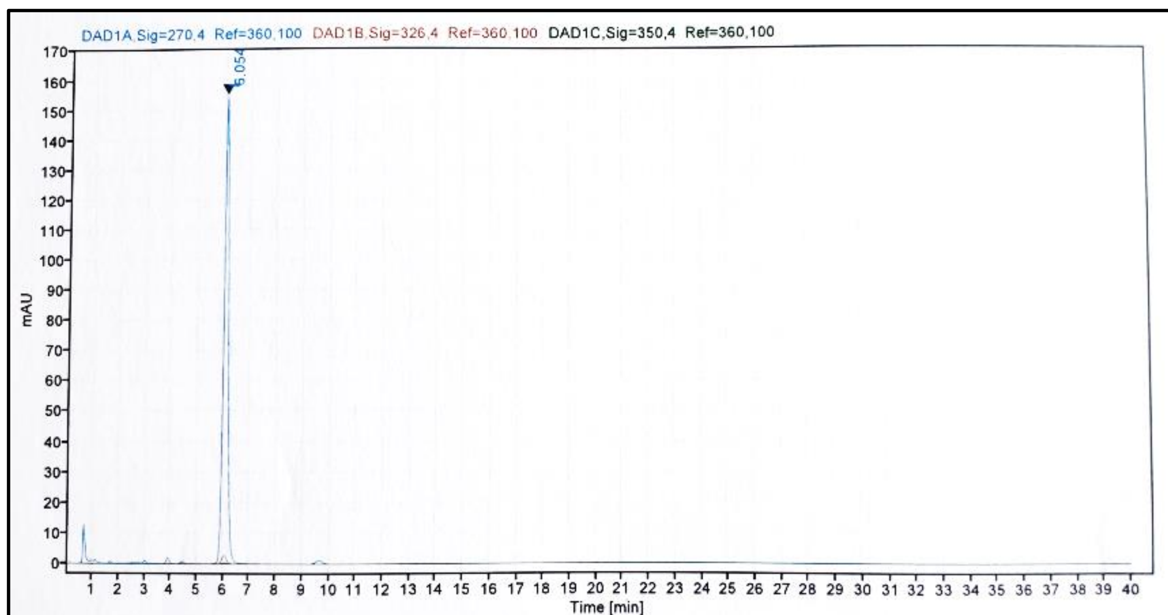

Figure 4.4 HPLC chromatogram showing the peak for ubiquinone 8 extracted from the isolate MARW_1-2-27^T

Table 4.15 Interpretation of HPLC chromatogram peak observed for ubiquinone extracted from MARW_1-2-27^T
Abbreviations: RT = Retention time

Signal:	**DAD1A, Sig=270,4 Ref=360,100**					
RT [min]	**Type**	**Width [min]**	**Area**	**Height**	**Areas%**	**Name**
6.054	MM m	0.19	1913.00	154.97	100	Q 8
		Sum	1913.00			

Table 4.16 Polar lipid profile of MARW_1-2-27^{T} and closest *Pseudoalteromonas* reference strains. Polar lipid content of MARW_1-2-27^{T} was quantified by observing the intensity and area covered by the individual spots that appeared on thin layer chromatography plates.

Strains: **1,** MARW_1-2-27^{T}; **2,** *P. piscicida* ATCC 15057^{T}; **3,** *P. flavipulchra* 208^{T}; **4,** *P. maricaloris* KMM 636^{T}; **5,** *P. peptidolytica* F12-50-A1^{T}; **6,** *P. rubra* 18^{T}; **7,** *P. spongiae* UST010723-006^{T}
Data source: [1]this study; [2-7]Ivanova *et al.* 2000b, Ivanova *et al.* 2002c and, Venkateswaran and Dohmoto 2000
Abbreviations: NG = Not given in novel species literature; ND = Not detected in analysis; Only for ref. strains, * = total content >80%; $ = total content 10–20%; ≠ = total content <10%

Polar lipid	1	2	3	4	5	6	7
Major*	phosphatidylethanolamine, phosphatidylglycerol	phosphatidylethanolamine	phosphatidylethanolamine	phosphatidylethanolamine	NG	phosphatidylethanolamine, phosphatidylglycerol	NG
Moderate$^{\$}$	aminophospholipid	phosphatidylglycerol	lyso-phosphatidylethanolamine, phosphatidylglycerol	phosphatidylglycerol and lyso-phosphatidylethanolamine		ND	
Traces$^{\neq}$	amino lipid, one unidentified lipid, one unidentified phospholipid	bisphosphatidic acid, lyso-phosphatidylethanolamine, phosphatidic acid	phosphatidic acid, bisphosphatidic acid	bisphosphatidic acid, phosphatidic acid, unidentified phospholipids		lyso-phosphatidylethanolamine, bisphosphatidic acid, and phosphatidic acid	

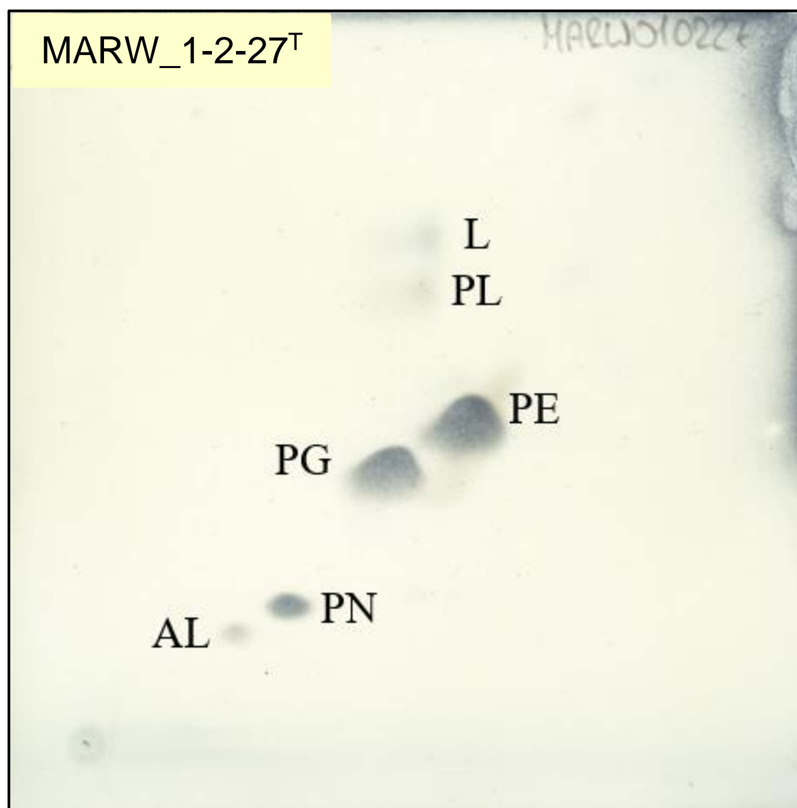

Figure 4.5 Polar lipid profile of MARW_1-2-27^T after separation by two-dimensional thin layer chromatography (TLC) and staining with molybdophosphoric acid. Polar lipid content quantified by observing the intensity and area covered by the individual spots that appeared on TLC plate. Polar lipid profile consisting of major lipids; phosphatidylethanolamine and phosphatidylglycerol, moderate amount of aminophospholipid and trace amounts of one amino lipid, one unidentified lipid, and one unidentified phospholipid

Abbreviations: PE = phosphatidylethanolamine; PG = Phosphatidylglycerol; PN = Aminophospholipid; AL = Amino lipid; L = Lipid; PL = Phospholipid

Table 4.17 Fatty acid profile of MARW_1-2-27^T and closest *Pseudoalteromonas* reference strains, all values in percentage

Strains (Data source in brackets): 1, MARW_1-2-27^T(this study); **2,** *P. piscicida* ATCC 15057^T (Ivanova *et al.* 2002c); **3,** *P. flavipulchra* 208^T (Ivanova *et al.* 2002c); **4,** *P. maricaloris* KMM 636^T (Ivanova *et al.* 2002c); **5,** *P. peptidolytica* F12-50-A1^T(Venkateswaran and Dohmoto 2000); **6,** *P. rubra* 18^T (Ivanova *et al.* 2002c); **7,** *P. spongiae* UST010723-006^T (Lau *et al.* 2005); **8,** P. haloplanktis strain 215^T (this study)

Abbreviations: T = Type species; * = Summed feature 3 (C16:1 ω7c/15 iso 2OH); ND = Not detected; NG = Not given in novel species literature

Fatty acid	1	2	3	4	5	6	7	8
C12:0 3OH	24.75	0.3	0.16	0.21	6.14	0.22	6.9±1.2	5.04
C16:0	16.73	27.4	17.58	16.3	17.46	16.5	18.4±2.9	15.95
C16:1 ω7c	19.71*	40.2	29.56	35.89	NG	34.44	29.1±5.9	40.95*
C12:0	5.9	0.6	0	0	1.85	0	NG	2.17
C18:1 ω7c	5.6	7.6	6.85	6.76	3.03	11.19	6.4±1.2	5.44
C10:0 3OH	5.26	NG	NG	NG	NG	NG	NG	0.83
C14:0	2.19	3.8	0.99	2.4	3.11	1.6	5.3±1.8	0.62
C18:0	2.13	1.4	0.65	0.44	0.93	0.61	NG	4.92
C12:0 ISO 3OH	1.93	0.3	0.16	0.21	6.14	0.22	NG	0.72
C10:00	1.84	NG	NG	NG	NG	NG	NG	0.15
C17:1 ω8c	1.6	5.5	18.95	15.98	0.19	14.23	9.8±5.3	5.81
C11:0 3OH	1.12	0.2	0.22	0.16	NG	0.2	NG	0.92
C16:0 ISO	1.08	0.7	1.96	0.95	0.12	1.71	NG	0.94
C17:0	1.04	2.15	6.72	4.04	NG	3.58	NG	4.36
C18:1 ω9c	0.76	0.3	0.37	0.38	0.39	0.48	NG	0.62
C16:1 ω9c	0.7	NG	NG	NG	NG	NG	NG	ND
C15:00	0.55	1.7	2.68	5.54	NG	3.78	NG	2.11
C15:1 ω8c	ND	1.7	2.35	2.8	0.35	1.68	NG	1.06
C18:0 ISO	0.28	0	0.35	0.12	NG	0.97	NG	0.57
C17:0 ANTEISO	ND	0	0.16	0.14	NG	0	NG	1.18
C17:0 ISO	ND	0.2	0.22	0.1	NG	0.26	NG	0.67
C13:0	ND	0.4	0.16	0.34	NG	0.51	NG	0.17
C19.0 10 methyl	ND	NG	NG	NG	NG	NG	NG	ND
C15 iso 2-OH	ND	NG	NG	NG	NG	NG	29.1±5.9	ND

4.3.3.5 Antibiotic susceptibility

The diffusion agar method (Mata *et al.* 2002) and GEN III MicroPlate test performed to check the susceptibility of MARW_1-2-27^T against different antimicrobial compounds, revealed that isolate was sensitive to cefotaxime (concentration per disc, 30μg), levofloxacin (5μg), ciprofloxacin (5μg), cephalexin (30μg), carbenicillin disodium (100μg), nalidixic acid (30μg) vancomycin (30μg), chloramphenicol (30μg), rifamycin (concentration not available), erythromycin (15μg), ampicillin (10 μg), lincomycin (concentration not available), streptomycin sulphate (10μg), but resistant to tetracycline (30μg). Weak resistance was exhibited to amoxicillin (or clavulanic acid, 30μg) with a 1 mm zone of inhibition. Comparison of these results to those of the closest phylogenetic relatives is as shown in **Table 4.18**.

Table 4.18 Differential susceptibility to antimicrobials by MARW -1-2-27^T and closely related species of the genus *Pseudoalteromonas*

Strains (Data source in brackets): 1, MARW_1-2-27^T (this study); **2,** P. piscicida ATCC 15057^T (Bein 1954; Buck *et al.* 1963; Hansen *et al.* 1965; Gauthier *et al.* 1995b; Gauthier and Breittmayer 1979; Ivanova *et al.* 2002c; Venkateswaran and Dohmoto 2000); **3,** P. flavipulchra 208^T (Ivanova *et al.* 2002c); **4,** P. maricaloris KMM 636^T (Ivanova *et al.* 2002c); **5,** P. peptidolytica F12-50-A1^T(Venkateswaran and Dohmoto 2000); **6,** P. rubra 18^T (Gauthier *et al.* 1995b;); **7,** P. spongiae UST010723-006^T (Lau *et al.* 2005)
Abbreviations: T = Type strain; + Positive; - = Negative; W+ = Weak positive; NG = Not given in novel species literature; * = Concentration not available

Characteristics	1	2	3	4	5	6	7
Tetracycline (30 μg)	-	-	-	-	NG	W+	+
Erythromycin (15 μg)	+	+	+	+	NG	+	NG
Ampicillin (10 μg)	+	-	-	-	NG	NG	+
Lincomycin*	+	-	-	-	NG	NG	NG
Streptomycin sulphate (10 μg)	+	+	-	+	NG	W+	-

4.3.3.6 Genetic studies

<u>Phylogenetic analysis</u>

BLAST analysis

Chromatogram obtained through Sanger sequencing when manually examined, it imparted well-formed, distinctive, evenly separated nucleotide peaks, without any or slight background noise that resulted in a good quality, unbiased, 633 bp long 16S rRNA gene sequence. Primary phylogenetic analysis of this sequence on EzBioCloud and NCBI blastn tools showed phylogenetic similarity to the genus *Pseudoalteromonas*.

Further confirmation of these BLAST results and assembly of a phylogenetic tree was performed using the most complete 16S rRNA gene sequence of MARW_1-2-27^T obtained from its genome. This 16S rRNA gene sequence was 1540 bp long. Analysis of this complete 16S rRNA gene sequence on EzBioCloud BLAST and NCBI blastn tool (performed on June 2020), confirmed phylogenetic assignment of MARW_1-2-27^T near to the genus *Pseudoalteromonas.* Furthermore, its closest type strain relatives observed on these two tools are as shown in **Table 4.19**.

Table 4.19 Similarity based on 16S rRNA phylogeny between MARW_1-2-27^T and its closest type strain relatives, analysed on EzBioCloud and NCBI blastn tools

Closest relative	On EzBioCloud BLAST	On NCBI blastn tool
1st	*P. piscicida* (99.86%)	*P. peptidolytica* (99.61 %)
2nd	*P. flavipulchra* (99.86%)	*P. issachenkonii* (96.95%)
3rd	*P. maricaloris* (99.79%)	*P. tetraodonis* (96.89%)
4th	*P. peptidolytica* (99.59%)	*P. spongiae* (96.89%)
5th	*P. rubra* (98.52%)	*P. phenolica* (96.82%)

Phylogenetic tree

The phylogenetic position of MARW_1-2-27^T with its closest relatives from genus *Pseudoalteromonas* and few other closest genera of the family Alteromonadaceae was evaluated by comparing their complete 16S rRNA gene sequences, as shown in the phylogeny tree **Fig. 4.6.** The derived maximum likelihood tree showed that MARW_1-2-27^T falls within the cluster comprising of *Pseudoalteromonas* species.

From the tree analysis, it showed closest phylogenetic affinities to *P. piscicida* (99.61% 16S rRNA gene sequence similarity matrix values from phylogenetic tree analysis), *P. flavipulchra* (99.58%), *P. maricaloris* (99.45%), *P. peptidolytica* (99.09%), *P. rubra* (98.22%), *P. byunsanensis* (96.34%), and *P.* sp*ongiae* (96.27%).

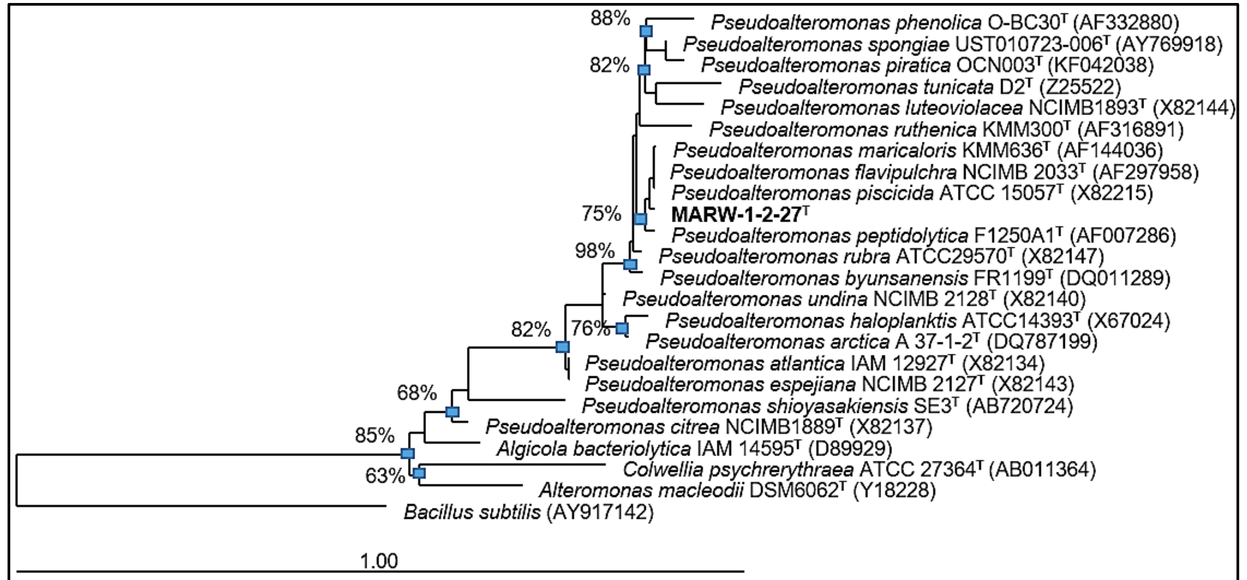

Figure 4.6 Maximum likelihood tree showing phylogenetic position of the isolate MARW_1-2-27^{T} and representatives of the selected related taxa. Affiliation of 16S rRNA gene sequences was done posteriori using the parsimony ARB tool (Ludwig *et al.* 2004) without changing the overall tree topology. Numbers at the branch nodes highlighted with the blue squares, refer to bootstrap values >50% (1000 iterations). 16S rRNA gene sequence of *Bacillus subtilis* was used as an outgroup. GenBank accession numbers are indicated in parentheses.

Genome data analysis

Genome sequencing of MARW_1-2-27^{T} performed on Illumina HiSeq 2500 using 250 bp paired-end protocol and 30x coverage provided 1,956,858 reads. Total of 220 (≥ 0 bp) contigs were obtained out of which 95 were ≥ 1000bp. The N50, indicating the shortest contig length at 50% of the entire assembly was 127,268 bp. Genome size was 5,427,456 bp. The G+C content of this isolate was derived from whole genome and its comparison to its closest phylogenetic relatives is as shown in **Table 4.20**.

The resultant in-silico ANI and DDH similarities between MARW_1-2-27^T and its closest phylogenetic relatives calculated by comparing their genomes are shown in **Table 4.20**.

Table 4.20 DNA G+C content, average nucleotide identity (ANI) and DNA-DNA hybridization (DDH) values of MARW_1-2-27^T and closest relatives. ANI and DDH values calculated by genome sequence comparison on ChunLab's Orthologous ANI and GGDC 2.1 webtool respectively.

Strains and G+C data sources in brackets: **1,** MARW_1-2-27^T (this study); **2,** *P. piscicida* ATCC15057^T (Ivanova *et al.* 2002c) **3,** *P. flavipulchra* 208^T (Ivanova *et al.* 2002c); **4,** *P. maricaloris* KMM 636^T (Ivanova *et al.* 2002c); **5,** *P. peptidolytica* F12-50-A1^T (Venkateswaran and Dohmoto 2000); **6,** *P. rubra* 18^T (Gauthier 1976); **7,** *P. spongiae* UST010723-006^T (Lau *et al.*2005); **8,** *P. haloplanktis* 215^T (Baumann *et al.* 1972)
Abbreviations: T = Type strain; * = Study results; NA = Genome sequence of type strains not available for ANI or DDH calculations

Characteristics	1	2	3	4	5	6	7	8
G+C (mol%)	43.21	42.7	41.7	38.9±0.4	42.0	47.1	40.6	42.9
ANI (%)*	100	95.72	71.16	NA	80.65	71.52	70.66	71.24
DDH (%)*	100	64.6	20.3	NA	23.9	19.6	23.1	19.3

4.4 DISCUSSION

The interpretations of the polyphasic analysis results of MARW_1-7-2^T and MARW_1-2-27^T are discussed as follows.

4.4.1 *Halomonas* Novel Species

4.4.1.1 Colony morphology, physiology and biochemical characters

Colony morphology and physico-chemical characteristics of MARW_1-7-2^T showed its similarities as well as distinctiveness to its closely related strains.

Cream white pigmentation, Gram-negative character, halo-tolerance, oxidase production, and absence of intracellular granules, as well as negative results for Ortho nitrophenyl-ßD-galactopyranosidase (ONPG) activity, indole production, and Voges-Proskauer test; were some of the common morphological, physiochemical characteristics of genus *Halomonas* also exhibited by the potential novel isolate (Whitman 2015).

On the other hand, lack of motility, weak catalase production showed by MARW_1-7-2^T, as well as its NaCl requirement of <5% (w/v) for optimum growth, and ability

to grow at temperature below 10 °C separated it from most of its closest phylogenetic relatives (**Tables 4.4 and 4.5**). Salt requirement studies of this isolate performed in medium A showed its ability to grow in the absence of NaCl which is distinctive not only from its closest reference strains but also from most of the species belonging to the genus *Halomonas* (Arahal *et al.* 2007). MARW_1-7-2^T could be further differentiated from recognized *Halomonas* and *Cobetia* species based on additional unique properties like production of acids from sugars and hydrolysis of various substrates (**Table 4.6**). In contrast to the closest phylogenetic relative *H. taeanensis,* isolate MARW_1-7-2^T could not reduce nitrates to nitrites (NO_2), didn't produce acid from D- mannose, D-sorbitol, D- lactose, glycerol etc. and failed to hydrolyse urea but could hydrolyse esculin (**Table 4.6**).

These and another morphological and physiological characteristics of MARW_1-7-2^T as mentioned in **Tables 4.4–4.6** have shown its biochemical status with respect to species of the genus *Halomonas* and furthermore strengthened its placement as a new species of this genus.

Furthermore, positive test results for the production of hydrolytic enzymes such as alkaline phosphatase, naphthol-AS-BI-phosphohydrolase esterase, and lipase, as well as diverse growth features exhibited by MARW_1-7-2^T exhibit its potential ecological role in hydrolysing organic phosphorus compounds (Annis and Cook 2002) and its application as a control measure in polluted extreme environments (Gangola *et al.* 2018; Rao *et al.* 2010; Sánchez-Porro *et al.* 2003; Sanchez-Hernandez 2011).

In addition to the salt tolerance tests performed, it would be useful to confirm the result of NB medium with 0% NaCl for incubation longer than 70 h, which was not possible during this study due to time limitation. This would further explore the NaCl or other marine salt requirements of the isolate MARW_1-7-2^T.

4.4.1.2 Chemotaxonomic characters

Cellular components such as fatty acids, respiratory quinones and polar lipids composition are considered as useful chemotaxonomic markers for novel microbe species description and were therefore assessed in the present study (Collins and Jones 1981; Franzmann and Tindall 1990; Komagata and Suzuki 1988). Chemotaxonomic features of MARW_1-7-2^T such as identification of Q-9 as a major quinone, a lipid profile containing phosphatidylethanolamine, phosphatidylglycerol

and phospholipid as well as the fatty acid composition characterised by the presence of C18:1 ω7c, C16:0, C16:1 ω7c/15 iso 2OH and C12:0 3OH in major amounts (>5%) (**Table 4.7, 4.8; Fig. 4.1, 4.2)** were aligned with the typical chemotaxonomic pattern of the genus *Halomonas* (Franzmann and Tindall 1990; Whitman 2015). However, insignificant proportions of C19:0 CYCLO ω8c and C17:0 CYCLO in MARW_1-7-2^T, contrasted with their reported values in its closest relative *H. taeanensis* (**Table 4.8,** Whitman 2015).

4.4.1.3 Antibiotic susceptibility

The test results of MARW_1-7-2^T against different antimicrobial compounds (**Table 4.9**) were mostly comparable to the antibiotic sensitivity characteristics of its phylogenetic relatives *H. halophila* and *H. salina* (5 out of available 6 results matched) than to *H. elongata* and *C. marina.* This possibly indicate the cellular and functional similarity of MARW_1-7-2^T to the *H. halophila* and *H. salina* than the latter two. However, no further sensitivity data was available for detailed comparison of the isolate with the rest of the closest relatives. Studying antimicrobial susceptibility patterns in comparison to the phylogenetic relatives is considered as one of the required microbial features in novel species description (Arahal *et al.* 2007), hence further exploration of this information for the isolate MARW_1-7-2^T remains necessary.

4.4.1.4 Genetic studies

In general, species within the genus *Halomonas* have a remarkably wide range of G+C content, 51.4–74.3 mol% as reported in the several *Halomonas* novel species literatures (**Table 4.11,** Gerhardt *et al.* 1981). In this study, DNA G+C content of MARW_1-7-2^T derived from its genome sequence data was 65.14 mol%, which was within this range (**Table 4.11**).

The 16S rRNA gene sequence of MARW_1-7-2^T derived from Sanger sequencing and draft genome sequence were 1247 bp and 1540 bp long respectively. As the genome sequence provided full length 16S rRNA gene sequence (>1400 nucleotide) suggested for new taxa characterisation (Arahal *et al.* 2007), it was employed further for phylogenetic identification and tree construction of MARW_1-7-2^T. EzBioCloud BLAST analysis and phylogenetic tree construction performed using this sequence showed closest affinity of the isolate to *H. taeanensis*. While performing its BLAST analysis, though variations were observed in between NCBI

blastn and EzBioCloud tool results with respect to species level identification and 16S rRNA gene similarity ratios (**Table 4.10**), EzBioCloud results were acknowledged in this study for it is more commonly referred in *Halomonas* novel species literature and contains extensively curated sequences as compared to the NCBI blastn tool (Park *et al.* 2012).

The phylogenetic tree constructed with the 16S rRNA gene sequence retrieved from draft genome showed closest affinity of MARW_1-7-2^T to *H. taeanensis*. Both these isolates were clustered together to form a clade with 99% bootstrap support and 16S rRNA gene sequence similarity matrix value of 97.1%. According to the recent recommendations, 98.7–99% sequence similarity value has been proposed as a cut-off for identification of novel bacterial isolates (Stackebrandt and Ebers 2006; Kim *et al.* 2014), which is acknowledged in this study as a first indication to recognize MARW_1-7-2^T as a potential novel species of the genus *Halomonas*. The separate branch of MARW_1-7-2^T from *H. taeanensis* also suggested the distinct phylogeny between them at species level (**Fig. 4.3**).

Besides 16S rRNA gene, other phylogenetic markers e.g., 23S rRNA (Kämpfer *et al.* 2018; Lee *et al.* 2005), gyrB and rpoD genes (Gan *et al.* 2018; Okamoto *et al.* 2004) have also been reported for comparative phylogenetic analysis of *Halomonas* species. But so far these markers are shown to have a little impact on the taxonomy of the family *Halomonadaceae* (Diéguez *et al.* 2020; Ludwig and Klenk 2001). Also, there is a scarce availability of these reference sequences in public gene databases as compared to the 16S rRNA gene data. Thus, 16S rRNA gene remains a foundation of current taxonomic classification (Ludwig and Schleifer 1994; Ramasamy *et al.* 2014; Ludwig and Klenk 2001; Arahal *et al.* 2007). Concurrent complementary genome comparison is also offered by phylogenetic web tools e.g., ANI and DDH (Goris *et al.* 2007) which makes them useful for species level description (Arahal *et al.* 2007; Varghese 2015).

ANI and DDH tests were conducted in this study, as it remains essential to determine genetic differences of the potential novel bacterium to its closely related species. ANI measurement is a recommended tool for the evaluation of similarity between two genomes, which involves the fragmentation of these genome sequences into fragments of approximately 1000 bp, followed by nucleotide sequence search, alignment, and identity calculation (Goris *et al.* 2007; Jain *et al.* 2018). A standalone ANI web calculator available at ChunLab's Orthologous ANI

server, applied in the present study for genome comparison uses OrthoANIu; the improved version of OrthoANI algorithm. According to Yoon *et al.* (2017), OrthoANIu exhibits good correlation, greater accuracy and runs faster than other commonly used ANI computational algorithms such as ANIb (ANI algorithm using BLAST) and ANIm (ANI using MUMmer). Both ANI and OrthoANI share similar species demarcation cut-off at 95–96% (Konstantinidis and Tiedje 2005; Lee *et al.* 2015; Richter and Rosselló-Móra 2009). ANI values calculated between MARW_1-7-2^T and its closest phylogenetic relatives, were concluded according to this revised threshold of 95–96%; stated for the novel prokaryotic species definition. Since all ANI values calculated between MARW_1-7-2^T and its phylogenetic relatives fall within 73–85% (**Table 4.11**), this further confirmed the novel status of MARW_1-7-2^T.

DDH tests were also conducted for taxonomic purpose (Arahal *et al.* 2007; Keswani and Whitman 2001; Stackebrandt and Ebers 2006) as an important trait in species discrimination when 16S rRNA gene sequence similarities are ≥97% (Meier-Kolthoff *et al.* 2013b). This method for inferring whole-genome distances has been considered as a robust and more effective method for genome-based species delineation (Auch *et al.* 2010) and has a cut-off value of ≤70% to identify a potential new microbial species (Tindall *et al.* 2010; Wayne *et al.* 1987). The in-silico DDH similarity values between MARW_1-7-2^T and its closest phylogenetic relatives were 20–30%, (**Table 4.11**), far below the aforementioned DDH threshold of species boundary. Thus, it supported the placement of MARW_1-7-2^T as a new species within the genus *Halomonas.*

Phylogeny with Cobetia

The phylogenetic tree derived in this study showed that MARW_1-7-2^T and its closest relative *H. taeanensis* formed a close branch with species of the genus *Cobetia* (**Fig. 4.3**). Such proximity between these two genera has also been previously observed by Lee *et al.* (2005) and could have hampered the assignment of MARW_1-7-2^T to the correct genus. However, in the present study this branch formed with *Cobetia* has a bootstrap value of 81%, which is less significant when compared to the robust branches of MARW_1-7-2^T made with other species of *Halomonas.* Furthermore, higher 16S rRNA gene sequence similarity matrix values were observed between the MARW_1-7-2^T and species of the genus *Halomonas* (up to 97%) than with Cobetia (up to 94.3%). ANI and DDH values calculated

between MARW_1-7-2^T and reference type species of the genus *Cobetia* were also less than that between MARW_1-7-2^T and type species of the genus *Halomonas* (**Table 4.11**). Additional biochemical differences observed in **Table 4.5** and **4.6** further confirms that the new isolate should be classified within the genus *Halomonas* rather than *Cobetia*.

4.4.1.5 Overall conclusion

Based on physiological, biochemical and phylogenetic properties it is suggested that strain MARW_1-7-2^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas aestuarensis* sp. nov. is proposed (*ae.stu.ar.en'sis*. L. neut. n. *aestuarensis*, of an estuary).

4.4.2 Pseudoalteromonas Novel Species

4.4.2.1 Colony morphology, physiology and biochemical characters

Morphological and physiological characteristics of MARW_1-2-27^T (**Tables 4.12–4.14**) such as orange coloured pigment production, Gram-negative, rod shape, motile cells, production of oxidase and gelatinase, ability to tolerate high NaCl concentration, resembles its similarity to the genus *Pseudoalteromonas* (Dworkin 2006).

MARW_1-2-27^T was observed to be a facultative anaerobe as it grew both in presence and absence of oxygen, as opposed to closely related species within the genus *Pseudoalteromonas* which are reported to be strict aerobes. Interestingly, when incubated in an anaerobic environment a scant growth of MARW_1-2-27^T was observed as semi-transparent colonies of 1–2 mm diameter, that lacked orange pigmentation. This might be the indication of oxygen requirement for the optimum microbial growth and for pigment production (**Table 4.12**).

The genus *Pseudoalteromonas* could be regarded as obligately marine microorganisms as it mainly consists of species isolated from marine environments and most of them require sea water or sodium ions for their growth (MacLeod 1968; Dworkin 2006). They are halotolerant (Dworkin 2006) and can tolerate up-to 15% (w/v) NaCl concentration (Bowman 1998). The tests performed in this study to check halotolerance ability of MARW_1-2-27^T showed its unique features with respect to its closest phylogenetic relatives. MARW_1-2-27^T grew in absence of NaCl and could tolerate NaCl concentration up-to 13% (w/v). Ability of this isolate

to grow at 40 °C was an uncommon trait amongst its compared closest relative strains, e.g., *P. piscicida*, *P. maricaloris*, and *P. rubra* as they can grow only up to 37 °C (**Table 4.13**).

Distinctive characteristics of MARW_1-2-27^T such as weak catalase activity, inability to utilize D-glucose, amidon (starch), D-maltose, N-acetylglucosamine, glycogen and D-trehalose for growth contrast with most of its closest known *Pseudoalteromonas* strains which are catalase positive and could use these substrates for growth (**Table 4.12 and 4.14**).

This morphological and physiological comparison of MARW_1-2-27^T to its phylogenetically closest strains have featured its resemblance to some of the general characteristics of the genus *Pseudoalteromonas* as well as unique characters to support its inclusion as a new species of this genus.

Furthermore, it would be useful to conduct an optimum growth experiment at pH higher than 9.6, and in a medium devoid of NaCl and other marine salts (e.g.in NB medium) that would allow further comparison of pH and salt requirements of MARW_1-2-27^T to those of its closest relatives.

These biochemical test results of MARW_1-2-27^T such as ability to survive at a diverse range of growth conditions (up-to 13% (w/v) NaCl and temperature 40 °C) and hydrolytic enzyme production have been reported in microbial studies before to have potential ecological applications such as in remediation of polluted environments (Sanchez-Hernandez 2011; Gangola *et al.* 2018; Rao *et al.* 2010; Sánchez-Porro *et al.* 2003) and plant growth promotion (Annis and Cook 2002).

4.4.2.2 Chemotaxonomic characters

Quinone composition of MARW_1-2-27^T resembled the results obtained by Akagawa-Matsushita *et al.* (1992) for the type species of *P.* piscicida, *P. haloplanktis*, *P. rubra* and *P. espejiana* as well as to *P. shioyasakiensis* (Matsuyama *et al.* 2014), where it was found to be 100% Q8 (**Fig. 4.4, Table 4.15**).

The phospholipids pattern specific to *Pseudoalteromonas* was also noted amongst MARW_1-2-27^T and its closest phylogenetic relatives, such as major-moderate amount of phosphatidyl ethanolamine, and phosphatidyl glycerol as well as absence of diphosphatidyl glycerol and glycophospholipids (**Table 4.16**, Ivanova *et al.* 2000b). However, lyso-phosphatidylethanolamine which was either present in

moderate (10–20 %) or trace amounts (<10 %) in closest relatives was not detected in MARW_1-2-27^T **(Table 4.16, Fig 4.5**).

Though the characteristic absence of cyclopropionioic fatty acids and a noticeable degree of similarity among fatty acid components (e.g., C16:0 and C18:1 ω7c) of MARW_1-2-27^T was in line with the fatty acid profile of genus *Pseudoalteromonas* (Ivanova *et al.* 2000b, **Table 4.17)**, some of its fatty acid components were quite divergent when compared with to its closest phylogenetic relatives. A greater proportion (>5%) of C12:0 3OH and C12:0 as well as lesser proportions of C16:1 ω7c and C17:1ω8c in MARW_1-2-27^T found to be unique as compared to most of its closest relatives (**Table 4.17).**

4.4.2.3 Antibiotic susceptibility

Resistance to ampicillin and lincomycin has been reported before for the closest and far phylogenetic relatives of the MARW_1-2-27^T (Dworkin 2006). In contrast, MARW_1-2-27^T showed its unique susceptibility to ampicillin and lincomycin; antibiotics known to affect bacterial cell wall and protein synthesis respectively (**Table 4.18**).

4.4.2.4 Genetic studies

G+C content of MARW_1-2-27^T derived from its genome data was 43.21 mol% which fall within the G+C range 37–48.5 % of the *Pseudoalteromonas* species and its closest relatives, as mentioned in the previous novel species literature of *Pseudoalteromonas* (**Table 4.20**).

On EzBioCloud, MARW_1-2-27^T shared closest phylogenetic similarity with *P. piscicida*, whereas its closest relative on NCBI was *P. peptidolytica*. 16S rRNA gene sequence of *P. piscicida* type strain found to be missing on NCBI blastn results. Though great variations were observed on EzBioCloud and NCB tool regarding the phylogenetic identification (**Table 4.19**), it was decided to continue with EzBioCloud results as most of the recent *Pseudoalteromonas* novel species literature refers to this webtool, and EzBioCloud seems to be more accurately curated tool (Park *et al.* 2012).

Though 16S rRNA gene sequence similarity value of 98.7–99% has been proposed as a cut-off for the identification of new bacterial species as per recent recommendations (Stackebrandt and Ebers 2006; Kim *et al.* 2014); this criterion is not conclusive for MARW_1-2-27^T in the present study. This proposed novel

species indicated sequence similarity of >99% with some of its closest relatives *P. piscicida*, *P. flavipulchra, P. maricaloris* and *P. peptidolytica* (**Table 4.19**).

As evolutionary position of MARW_1-2-27^T was compared with its closest relatives based on their 16S rRNA gene sequences on phylogenetic tree (**Fig. 4.6**), *P. flavipulchra*, *P. maricaloris* and *P. piscicida* were found to be clustered together on a single clade and closely related to MARW_1-2-27^T. From this clade, an independent sub-cluster is formed with MARW_1-2-27^T and *P. peptidolytica.* Identical to these current observations, a description of novel species; *P. peptidolytica* (Venkateswaran and Dohmoto 2000) has reported high sequence similarity (99.1%) with *P. piscicida*. In this case, to confirm its novelty DDH and an additional gyrase B gene (gyrB) were analysed that resulted in much lower likeness between these two species (Venkateswaran and Dohmoto 2000). Similar phylogenetic results were also observed by Vanova *et al.* (2002c) between *P. piscicida*, *P. flavipulchra*, and *P. maricaloris* as their 16S rDNA sequence sequences always grouped them on a single clade and do not allow a distinction between them as a different species, except when analysed with DDH ratios instead (Ivanova *et al.* 2002c). It seems for some of these *Pseudoalteromonas* species 16S rRNA gene at times lacks the specificity required for the differentiation of close relatives and they cannot be distinguished based on their 16S rRNA gene threshold level (Venkateswaran and Dohmoto 2000). But when other supportive traits like DDH (Ivanova *et al.* 2002c), ANI (Huang *et al.* 2017b) or housekeeping genes like gyrB (Venkateswaran and Dohmoto 2000), recA, gapA and ftsZ (Beurmann *et al.* 2017) were applied, differences were observed amongst such closely related *Pseudoalteromonas* species. Thus, from these literature studies, it was observed that DDH and ANI could be a faithful solution to define new *Pseudoalteromonas* species even in absence of 16S rRNA gene differentiation. So, in the present study to surpass the limitation of having lower 16S rRNA gene differences between MARW_1-2-27^T and its reference strains, these genomic traits of DDH, and ANI were tested.

The ANI value between isolate MARW_1-2-27^T and its closest relative *P. piscicida* was 95.72%, (**Table 4.20**) which is on the borderline of new species threshold of 95–96% (Konstantinidis and Tiedje 2005; Richter and Rosselló-Móra 2009) but is still applicable for novel species description of MARW_1-2-27^T. ANI values of ≤80%

with the remaining reference isolates further indicates the distinctiveness of MARW_1-2-27^{T}.

The recommended DDH cut-off value that confirms the novel characteristic of the query isolate is ≤70% (Tindall *et al.* 2010; Wayne *et al.* 1987). As shown in **Table 4.20**, since all DDH values in-between MARW_1-2-27^{T} and its reference strains were <70%. this is a supplementary indication that MARW_1-2-27^{T} could be a novel species.

4.4.2.5 Overall conclusion

Based on these polyphasic characterisation results, this study presents MARW_1-2-27^{T} as a novel species of the genus *Pseudoalteromonas* for which the name *Pseudoalteromonas belisamaea* sp. nov. is proposed (be.li.sa.maea. L. masc./fem. adj., of Belisama estuary, the Latin name for the Ribble estuary UK where the type strain was isolated, named after a Gallo-Brythonic goddess.

Salt marsh has been sampled before for the isolation of a novel *Pseudoalteromonas* species from a tidal flat sediment (Park *et al.* 2005). However, the characterisation of *Pseudoalteromonas belisamaea* in my study could be the first evidence of isolating the species of this genus from a salt marsh pond sea water.

Chapter 5

Draft genome analysis of two novel species *Pseudoalteromonas belisamaea* and *Halomonas aestuarensis*, isolated from a salt marsh in the UK

5.1 INTRODUCTION

Developments in DNA sequencing and bioinformatics tools have promoted revolutionary wider applications of these techniques in several areas of biological research, including in microbial genomics. After the development of the first-generation sequencing method in 1977 by Sanger *et al.*, advances in the second and third generation sequencing technologies have improved performance by providing longer read sequences, reconstructing larger portions of genomes with the higher coverage, and assembly of more complex genomes (Kchouk *et al.* 2017). Though some of these techniques are laborious and highly expensive per sequence run (Watson 2014; Miyamoto *et al.* 2014), ongoing progresses offer easier sample preparation (Bashir *et al.* 2012), faster sequencing and lower running costs (Koren *et al.* 2012; Goldberg *et al.* 2006), in combination with well-adapted computational tools that manage and analyse the biological sequence data (Kchouk *et al.* 2017; Mestan *et al.* 2011).

Advances in genome sequencing technologies have resulted in a large amount of microbial data being generated that is now amassed in various online databases such as NCBI GenBank, the European Nucleotide Archive, and the International Nucleotide Sequence Database Collaboration. This helps researchers to quickly find and access reference genomes and analyse them for the features of interest (Karp *et al.* 2019). Useful information can be derived from these resources by analysing and visualising it in suitable microbial genome web portals such as PATRIC (Brettin *et al.* 2015), The Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa *et al.* 2007), KBase (Arkin *et al.* 2018), Ensembl Bacteria (Howe 2020), BioCyc (Karp *et al.* 2002) and Rapid Annotations using Subsystems Technology (RAST, Aziz *et al.* 2008). Each of these web portals have a set of tools such as genomic and metabolic analysis tools, search engines and analytical web-services.

Genome sequencing and analysis techniques are widely applied in microbial studies to explore the microbiome population structure (Sunagawa *et al.* 2015), identify novel microbes difficult to cultivate in the lab (Caputo *et al.* 2019; Eisen 2007), fulfil the taxonomic requirements of novel species description (Stackebrandt *et al.* 1994;Tindal *et al.* 2010), map and identify gene mutations (Zuryn *et al.* 2010), and understand the transmission of pathogens in healthcare systems (Quainoo *et al.* 2017). Additional applications of genome studies also include describing

microbial evolution and physiology (Fraser *et al.* 2000; Fraser-Liggett 2005), linking with proteomics (Walhout and Vidal 2001) and identifying gene targets of bio-prospective potential (Gerdes *et al.* 2002; Roumpeka *et al.* 2017).

Annotation is an effective way to reconstruct the metabolic features hidden inside the genome of an organism (Stothard and Wishart 2006). It is a process of identifying genes of the sequenced genome and labelling these genes with the useful information obtained by matching it with information on genome databases of known organisms. This process aids in assessing genome quality, and predicting assembly structure, gene locations and functions. The evaluation of biochemical pathways and the discovery of genes reflecting beneficial functions can be also achieved by this approach (Reed *et al.* 2006; Karp *et al.* 1999; Xie et. al 2012; Steward *et al.* 2017).

Like sequencing technologies, rapid advances in annotation tools and improvements in their performance were also addressed (Zhou and Miller 2002; Overbeek *et al.* 2007; Galperin *et al.* 2015; Brettin *et al.* 2015). Vast numbers of bacterial, viral, and archaeal genomes can be predicted in a short period of time to produce standard-compliant and user-friendly output files using automated annotation tools, including those offered by NCBI (Tatusova *et al.* 2016), RAST (Aziz *et al.* 2008), IMG (Chen *et al.* 2019; Mukherjee *et al.* 2019), DFAST (Tanizawa *et al.* 2018) and COG (Galperin *et al.* 2015), to name but a few.

The present work describes the genome sequencing and analysis of two novel species, MARW_1-2-27^T and MARW_1-7-2^T, respectively named *Pseudoalteromonas belisamaea* and *Halomonas aestuarensis* for the prediction of gene structure and gene function. Their draft genomes, sequenced on the second-generation 'Illumina HiSeq' platform and annotated on the RAST tool were used to map the quality, structure, and metabolic features of their genomes.

RAST is a web-based, fully automated annotation pipeline that predicts the gene assembly, protein coding and non-coding genes, reconstructs the metabolic network, and can also compare the newly annotated genome to the annotated genome datasets maintained in its SEED Viewer framework (Aziz *et al.* 2008). The RAST server works on the subsystem-based assertions (a set of abstract functional roles) and predicts which subsystems are present in the query genome. Metabolic reconstruction is made automatically once the genes of the query genome are

connected to the RAST subsystems and functional roles of these systems are assigned to these genes. It produces a detailed estimation of the gene contents of both bacterial and archaeal genomes (Aziz *et al.* 2008; Overbeek *et al.* 2014). In the present study the RAST annotation tool was applied to explore the genome structures and interpret the gene functions of the novel isolates.

5.1.1 Objectives

One of the taxonomic requirements of novel species characterisation was fulfilled in this chapter by sequencing and describing the genomes of *Pseudoalteromonas belisamaea* and *Halomonas aestuarensis*. For novel strains of bacterial taxa having 16S rRNA gene sequence similarity value >97%, additional tests of genome comparison such as DDH and ANI are recommended. They can be derived by in-silico analysis of the microbial genome as described in detail in chapter 4 of this thesis (Tindal *et al.* 2010; Stackebrandt and Ebers 2006). The genome description of the novel taxa is also considered mandatory to provide functional characteristics and general understanding of taxonomic groups (Thompson *et al.* 2013).

To receive further biological insights into these two isolates, this study was also focused on their genes related to the adaptation, ecological and biotechnological potential, and virulence. The gene expression related to these features studied in novel microbes isolated from gradient-rich environments like salt marshes would be valuable for understanding of these novel taxa and it would also fulfil the demands of the microbial taxonomy (Thompson *et al.* 2015; Didelot *et al.* 2012; Van Belkum *et al*. 2001).

5.2 METHODS

5.2.1 Genome Sequencing

The draft genomes of the novel species *P. belisamaea* and *H. aestuarensis* were sequenced on the Illumina HiSeq 2500 platform in March 2019 using the sequencing service provided by MicrobesNG (UK). The detailed sequencing procedure was as follows.

At the Edge Hill University lab, one individual colony was picked from freshly grown pure cultures of each isolate and emulsified in a barcoded bead tube provided by

MicrobesNG. These tubes contain beads and a cryo-preservative solution in sterile condition. The tubes were immediately transported to MicrobesNG at room temperature for further processing. As per the operating protocol of MicrobesNG, three beads from each sample tube were washed with extraction buffer (containing lysozyme and RNase A) and incubated at 37 °C for 25 min. Further to this, proteinase K and RNase A were added to each tube and incubated for 5 min at 65 °C. Using an equal volume of SPRI (Solid Phase Reversible Immobilization) beads, the genomic DNA was purified and resuspended in an elution buffer (10 mM Tris-HCl at pH 8.5) with no EDTA. SPRI beads are paramagnetic beads, that selectively bind to the nucleic acid by its type and size, facilitate high-performance isolation, purification and clean-up of DNA (Zhong *et al.* 2011). The extracted DNA was then fluorometrically quantified in triplicate with the Quant-iT dsDNA HS (high sensitivity) assay in an Eppendorf AF2200 plate reader.

Next, genomic DNA libraries were prepared using a Nextera XT Library Prep Kit (Illumina, San Diego, USA) as per the manufacturer's protocol with the following modifications: an input of two nanograms of DNA was used instead of one nanogram, and the PCR elongation time was increased from 0.5 to 1 min.

DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using a Kapa Biosystems library quantification kit for Illumina platform on a Roche light cycler 96 qPCR instrument. Libraries were sequenced on a short-read sequencing Illumina HiSeq platform using a 250 bp paired end protocol. The sequencing reads were then adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger *et al.* 2014). The quality of reads was assessed using the bwa-mem software. De novo assembly was performed on trimmed reads using SPAdes version 3.7 (Bankevich *et al.* 2012).

The sequence files of genome assembly were further assessed for its quality, gene structure and gene functions at Edge Hill University using RAST tool (Aziz *et al.* 2008).

5.2.2. Quality Check (QC) of the Genomes

The approaches taken while accessing the quality of the final genome assembly of *P. belisamaea* and *H. aestuarensis* were as follows (Schmieder and Edwards 2011).

A quality assessment tool (QUAST 5.0.2) was used at MicrobesNG to evaluate the genome assembly by computing various metrics (Gurevich *et al.* 2013). Most of the assembly metrics were computed for the trimmed contigs larger than 500 bp. Operational parameters such as total depth of the sequencing coverage, trimming of sequencing reads at the beginning of the pipeline, and average number of uncalled bases (N's) per 100,000 assembly bases were observed for the assessment of study genomes.

Additional quality checks were performed at Edge Hill University. Both sequenced genomes were evaluated for completeness and contamination on KBase Predictive Biology software that runs the application CheckM- v1.0.18 lineage workflow (KBase Predictive Biology, n.d., viewed November 2020; Parks *et al.* 2015). CheckM, is an automated method for assessing the quality of a genome using a broader sets of marker genes (Parks *et al.* 2015). Furthermore, to confirm if the full-length genomes have been sequenced, their resultant sequence sizes (length sum of all contigs) were compared to the genome size of their closest phylogenetic relatives. The genome data of these phylogenetic relatives was taken from the NCBI and EzBioCloud database (National Center for Biotechnology Information, n.d., viewed June 2020; EzBioCloud, Chunlab, May 2020, viewed June 2020). Additionally, the 16S rRNA gene sequences retrieved from the isolate genomes were employed to confirm the authenticity of the final genome assembly (Chun *et al.* 2018). This 16S rRNA gene sequences were BLAST on the EzBioCloud tool (EzBioCloud, Chunlab, May 2020, viewed June 2020), and their results were compared with the BLAST results of the 16S rRNA genes of the isolates originally obtained from the Sanger sequencing method. The standards suggested for the prokaryotic genome annotation, such as requirements of specific sets of tRNAs, rRNAs and protein coding genes, were also observed in annotated genomes to confirm the annotation quality (Klimke *et al.* 2011).

5.2.3. Genome Analysis

Gene components and their functions in the microbial isolates *P. belisamaea* and *H. aestuarensis* were predicted by annotating their genomes on the RAST server (Aziz *et al.* 2008, RAST (n.d.), viewed November 2020). For this purpose, the Classic RAST tool with 'RAST' gene caller, and FIGfam of version 'Release70' was used to process the genome. Remaining working parameters like automatic fix errors, frameshift correction, the backfilling of gaps were set to 'Yes'. The annotated

genomes and their metabolic reconstructions were then analysed using the SEED-Viewer environment which exists within the RAST (Aziz *et al.* 2008; Overbeek *et al.* 2014). Annotated genomes were observed for the identification of protein-encoding genes (PEGs), tRNA and rRNA genes, the allocation of gene functions, and the reconstruction of the metabolic model. The subsystem data and associated PEGs of the annotated genomes were explored to find the genes related to the adaptation, ecological and biotechnological potential, and virulence of these microbes.

5.3 RESULTS

5.3.1 General Features of the Novel Isolates

A summary of some general physico-chemical characteristics of the novel isolates of the present study analysed through wet-lab experiments (detailed description in **chapter 3**) can be found in **Table 5.1**. These characteristics were in accord with the related genome annotation results of the novel isolates of this study.

Table 5.1 General physico-chemical features of the novel isolates
Abbreviations: + = Positive; - = Negative; W+ = Weak positive

General features	*P. belisamaea*	*H. aestuarensis*
Oxidase	+	+
Catalase	W+	W+
Motility	+	-
Carbon metabolism	Diverse metabolism	Diverse metabolism
Growth ranges, optimum conditions in brackets		
Salinity (% w/v)	0–13 (1)	0–20 (3)
pH	6–9.6 (7)	5–9 (6.5–7)
Temperature (°C)	10–40 (35)	5–45 (35)

5.3.2 Genome Sequencing

The draft genomes of *P. belisamaea* and *H. aestuarensis* obtained from Illumina HiSeq sequencing platform using a 250 bp paired end protocol exhibited the following characteristics.

5.3.2.1 Assembly and replicon information of the sequenced genomes

The genome reads and assembly of the novel isolates sequenced with 30x total depth of sequencing coverage can be described as mentioned in **Table 5.2**. This data is extracted from the genome reports provided by MicrobesNG.

Table 5.2 The genome reads and assembly features predicted for the novel isolates of the present study

Genome reads and assembly	*P. belisamaea*	*H. aestuarensis*
Number of reads (≥ 0 bp size)	1,956,858	326,822
Number of reads (≥ 500 bp size)	602,734	203,000
Number of Contigs (≥ 0 bp size)	220	79
Number of Contigs (≥ 500 bp)	116	57
Number of Contigs (≥ 1000 bp)	95	54
G+C (mol%)	43.21	65.14

5.3.2.2 Genome QC

As MicrobesNG filtered out all short reads in the beginning of the pipeline, most of the assembly metrics computed were of the contigs larger than 500 bp. The list of assembly metrics analysed to assess the quality of the draft genomes of the isolates is mentioned in **Table 5.3**.

Observations of the genome sizes of *P. belisamaea* and *H. aestuarensis* in comparison to their phylogenetic relatives, were as shown in **Tables 5.4 and 5.5** respectively.

Table 5.3 Genome features predicted for the isolates, studied for the genome QC
Abbreviations: N50 = Size of contig for which 50% of assembled reads are in a contig of that size or larger; N’s = Average number of uncalled bases per 100,000 assembly bases

Genome features for QC	*P. belisamaea*	*H. aestuarensis*
Total depth of sequencing coverage	30x	30x
Total length (≥ 0 bp)	5,427,456	3,544,736
N50 (base pair, bp)	127,268	137,558
N's	0	0
Number of tRNAs	115	62
Number of rRNAs	6	7

Table 5.4 Genomic sizes of the isolate *P. belisamaea* and its closest phylogenetic relatives. The genomes of closest relatives were obtained from the NCBI and EzBioCloud genome database.

Target species	*P. belisamaea*	*P. piscicida* ATCC15057^T	*P. flavipulchra* NCIMB 2033^T	*P. peptidolytica* F12^T
Genome Size (bp)	5,427,456	5,488,279	4,501,528	5,135,196

Table 5.5 Genomic sizes of the isolate *H. aestuarensis* and its closest phylogenetic relatives. The genomes of closest relatives were obtained from the NCBI and EzBioCloud genome database.

Target species	*H. aestuarensis*	*H. taeanensis* BH539^T	*H. smyrnensis* AAD6^T	*H. salina* B6^T
Genome Size (bp)	3,544,736	3,756,722	3,561,919	4,259,015

The 16S rRNA genes extracted from the genomes of *P. belisamaea* and *H. aestuarensis* both showed BLAST similarity to the results obtained from the same genes sourced by Sanger sequencing- *P. piscicida* and *H. taeanensis,* respectively.

When isolate genomes were evaluated on CheckM software, both genomes exhibited ≥99.9% completeness and ≤0.7% contamination.

5.3.2.3 Genome annotation

The genome annotation features of *P. belisamaea* (RAST ID 6666666.707877) and *H. aestuarensis* (RAST ID 6666666.711011) obtained by annotating their genomes on the RAST tool are mentioned below (**Table 5.6**). Annotation results were available within 6–12 h of the genomes being submitted on the RAST tool.

Table 5.6 Genome annotation features predicted for *P. belisamaea* and *H. aestuarensis* using the RAST server
Abbreviations: * = Number of amino acids for which at least one tRNA is annotated in the genome

Genome features	*P. belisamaea*	*H. aestuarensis*
Number of annotated features	4940	3199
Number of Coding sequences	4819	3130
No. of amino acids with tRNA*	20	20

When analysed on the RAST, the distribution of the coding sequences of the genomes of *P. belisamaea* and *H. aestuarensis*, into subsystems categories representing different functions, were observed as shown in **Fig. 5.1 and 5.2** respectively**.** For each genome, a bar chart and a pie chart displayed on the SEED Viewer elaborated the distribution of genes connected to the various RAST subsystem categories. Each of these categories were expandable down to the specific PEGs found in each subsystem (**Fig. 5.2)**. The numbers in brackets represent the number of PEGs found in that subsystem.

In *P. belisamaea*, 44% of genes were found to be corelated to the RAST subsystems; in the case of *H. aestuarensis* this portion was 60%. In both annotated genomes the largest number of sequences were observed in 'Amino acids and Derivatives' and 'Carbohydrates' categories. The subsystem distribution of the coding sequences of the new species genomes on RAST were additionally observed for those PEG categories specifically predicting functional genes for adaptations to environmental stress, ecological and biotechnological potential, and virulence. These predicted genes and their functions for the isolates *P. belisamaea* and *H. aestuarensis* are cumulated in **Tables 5.7 and 5.8** respectively. The numbers in brackets represent the number of PEGs found in that subsystem.

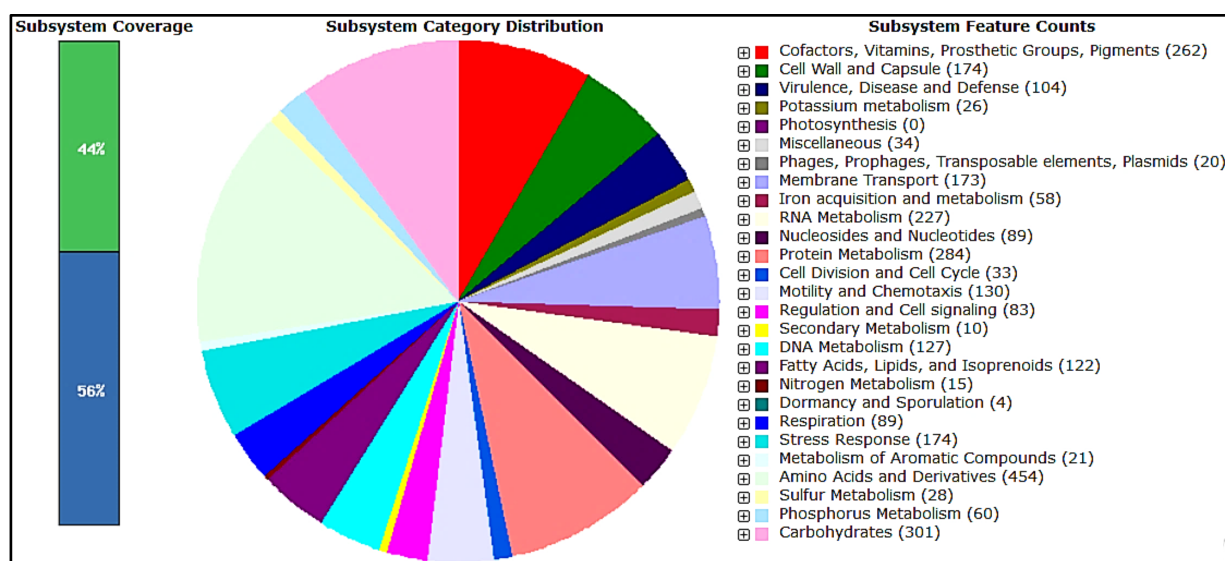

Figure 5.1 The functions assigned to the new genome *P. belisamaea* by the RAST subsystems. The leftmost bar chart (subsystem coverage) depicts the percentage of features from the selected organism that are in RAST subsystems i.e., 44%. A pie chart shows the distribution of genes from the selected organism in different RAST subsystem categories.

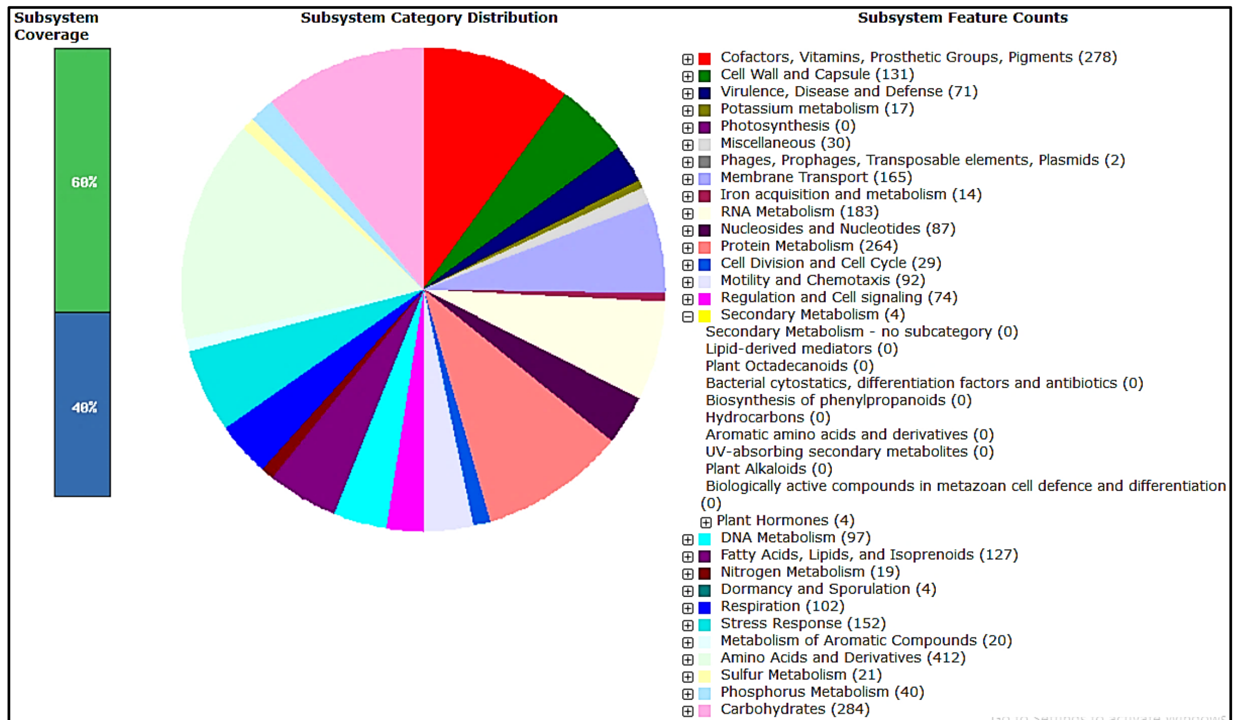

Figure 5.2 The functions assigned to the new genome *H. aestuarensis* by the RAST subsystems. The leftmost bar chart (subsystem coverage) depicts the percentage of features from the selected organism that are in RAST subsystems i.e., 60%. A pie chart shows the distribution of genes from the selected organism in different RAST subsystem categories. Each of these categories were expandable down to the specific genes found in each subsystem, e.g., secondary metabolism.

Table 5.7 RAST subsystems and categories of protein encoding genes (PEGs) representing specific functions predicted in the *P. belisamaea* genome. The numbers in brackets represent PEGs found in that subsystem

(A) Adaptive features		
Subsystems	PEG categories	PEG categories in detail
Stress Response (174)	Oxidative stress (74)	Oxidative stress (30) Protection from Reactive Oxygen Species (11)
	Stress Response - no subcategory (64)	Bacterial haemoglobins (44)
	Detoxification (15)	Glutathione-dependent pathway of formaldehyde detoxification (3)
	Osmotic stress (7)	-
	Heat shock (16)	Heat shock dnaK gene cluster extended (16)
(B) Ecological potential		
Subsystems	PEG categories	PEG categories in detail
Phosphorus Metabolism (60)	Phosphorus Metabolism - no subcategory (60)	High affinity phosphate transporter and control of PHO regulon (14) Phosphate metabolism (32)
Nitrogen Metabolism (15)	Nitrogen Metabolism - no subcategory (15)	Ammonia assimilation (14) Nitrosative stress (1)
Sulphur Metabolism (28)	Inorganic Sulphur assimilation (16) Organic Sulphur assimilation (3)	-
Iron acquisition and metabolism (58)	Iron acquisition and metabolism - no subcategory (55) Siderophores (3)	-
Secondary Metabolism (10)	Plant Hormones (5)	Auxin biosynthesis (5)

Table 5.7 continued…

(C) Biotechnological potential		
Subsystems	PEG categories	PEG categories in detail
Metabolism of Aromatic Compounds (21)	Metabolism of central aromatic intermediates (14)	Catechol branch of beta-ketoadipate pathway (4) Salicylate and gentisate catabolism (5) Homogentisate pathway of aromatic compound degradation (5)
	Metabolism of Aromatic Compounds - no subcategory (6)	Aromatic Amin Catabolism (2) Gentisate degradation (4)
	Peripheral pathways for catabolism of aromatic compounds (1)	Quinate degradation (1)
Fatty Acids, Lipids, and Isoprenoids (122)	Isoprenoids (35)	Isoprenoid Biosynthesis (14) Polyprenyl Diphosphate Biosynthesis (5) Nonmevalonate Branch of Isoprenoid Biosynthesis (7)
Amino Acids and Derivatives (454)	Aromatic amino acids and derivatives (60)	Chorismate Synthesis (12) Chorismate: Intermediate for synthesis of Tryptophan, PAPA antibiotics, PABA, 3-hydroxyanthranilate and more. (16) Common Pathway for Synthesis of Aromatic Compounds (DAHP synthase to chorismate) (8)
Virulence, Disease and defence (104)	Bacteriocins, ribosomally synthesized antibacterial peptides (12)	Marinocine, a broad-spectrum antibacterial protein (4)
Secondary Metabolism (10)	Secondary Metabolism - no subcategory (3)	Lanthionine Synthetases (3)
	Bacterial cytostatics, differentiation factors and antibiotics (2)	Paerucumarin Biosynthesis (2)

Table 5.7 continued…

(D) Virulence		
Subsystems	**PEG categories**	**PEG categories in detail**
Protein Metabolism (284)	Protein degradation (64)	Protein degradation (8) Proteolysis in bacteria, ATP-dependent (16) Proteasome bacterial (9)
Motility and Chemotaxis (130)	Motility and Chemotaxis - no subcategory (41)	Bacterial Chemotaxis (41)
	Flagellar motility in prokaryotes (89)	Additional flagellar genes in Vibrionales (4) Flagellum in Campylobacter (8)
Virulence, Disease and Defence (104)	Resistance to antibiotics and toxic compounds (78)	Fosfomycin resistance (1) Resistance to fluoroquinolones (4) Tetracycline resistance, ribosome protection type (2) Beta-lactamase (9) Multidrug Resistance Efflux Pumps (27)
	Bacteriocins, ribosomally synthesized antibacterial peptides (12)	Tolerance to colicin E2 (1) Colicin V and Bacteriocin Production Cluster (7)
	Invasion and intracellular resistance (14)	-
DNA Metabolism (127)	DNA Metabolism - (30)	External and Outer Membrane Nucleases (1)
	CRISPRs (7)	-
Regulation and Cell signalling (83)	Regulation of virulence (9)	-
Cell Wall and Capsule (174)	Capsular and extracellular polysaccharides (47)	
	Gram-Negative cell wall components (62)	KDO2-Lipid A biosynthesis (17)

Table 5.8 RAST subsystems and categories of protein encoding genes (PEG) representing specific functions predicted in the *H. aestuarensis* genome. The numbers in brackets represent PEGs found in that subsystem

(A) Adaptive features		
Subsystems	PEG category	PEG category in detail
Stress Response (152)	Oxidative stress (41)	Oxidative stress (17) Protection from Reactive Oxygen Species (3) Rubrerythrin (3)
	Stress Response - no subcategory (43)	Bacterial haemoglobins (27)
	Detoxification (23)	Uptake of selenate and selenite (5) Glutathione-dependent pathway of formaldehyde detoxification (3)
	Osmotic stress (33)	Choline and Betaine Uptake and Betaine Biosynthesis (28) Ectoine biosynthesis and regulation (4) Osmoregulation (1)
(B) Ecological potential		
Subsystems	PEG category	PEG category in detail
Sulphur Metabolism (21)	Organic Sulphur assimilation (6) Inorganic Sulphur assimilation (5) Sulphur Metabolism - no subcategory (10)	-
Phosphorus Metabolism (40)	Phosphorus Metabolism - no subcategory (40)	High affinity phosphate transporter and control of PHO regulon (8) Phosphate metabolism (20) Polyphosphate (2) Phosphonate metabolism (10)
Nitrogen Metabolism (19)	Nitrogen Metabolism - no subcategory (19)	Nitrate and nitrite ammonification (7) Ammonia assimilation (12)
Iron acquisition and Metabolism (14)	-	-
Secondary Metabolism (4)	Plant Hormones (4)	Auxin biosynthesis (4)
Cell Wall and Capsule (131)	Capsular and extracellular polysaccharides (36)	dTDP-rhamnose synthesis (8) Capsular Polysaccharides Biosynthesis and Assembly (7) Rhamnose containing glycans (11) Sialic Acid Metabolism (10)

Table 5.8, continued…

(C) Biotechnological potential		
Subsystems	PEG category	PEG category in detail
Metabolism of Aromatic Compounds (20)	Metabolism of central aromatic intermediates (15)	Homogentisate pathway of aromatic compound degradation (5) Catechol branch of beta-ketoadipate pathway (5) Salicylate and gentisate catabolism (3) N-heterocyclic aromatic compound degradation (2)
	Peripheral pathways for catabolism of aromatic compounds (2)	Quinate degradation (1) Biphenyl Degradation (1)
	Metabolism of Aromatic Compounds - no subcategory (3)	Gentisate degradation (3)
(D) Virulence		
Subsystems	PEG category	PEG category in detail
Protein Metabolism (264)	Protein degradation (33)	Protein degradation (6) Proteasome bacterial (6) Proteolysis in bacteria, ATP-dependent (12)
Regulation and Cell signalling (74)	Regulation of virulence (7)	-
Cell Wall and Capsule (131)	Gram-Negative cell wall components (38)	Lipopolysaccharide assembly (25) Peptidoglycan lipid II lipase (1) LOS core oligosaccharide biosynthesis (8) Lipoprotein sorting system (4)
DNA Metabolism (97)	CRISPRs (0)	-

5.4 DISCUSSION

The draft genomes of the two novel species *P. belisamaea* and *H. aestuarensis* sequenced in the present study are discussed with respect to their quality, annotated gene structure and gene functions as follows.

5.4.1 Genome QC

In the present study, QC checks to estimate the quality of the final assembly and the genome annotations were performed according to the standards given by Chun *et al.* (2018) and Klimke *et al.* (2011). Although the annotation guidelines were formulated for complete genomes, they are applicable to draft genomes as well (Klimke *et al.* 2011).

According to these guidelines, the genome sequencing on an Illumina platform and the filtration of the raw reads before the genome's assembly, as performed in the present study, are known to generate sequences of the standard required for taxonomic description (Chun *et al.* 2018). Trimming and filtering the low-quality raw reads with lengths of less than 500 bp is known to increase the quality of the data and improve the downstream analysis (Del Fabbro *et al.* 2013).

The absence of uncalled bases also fulfilled one of the criteria of the quality sequenced genomes (Chun *et al.* 2018). The genome sizes of *P. belisamaea* and *H. aestuarensis* were within the ranges of the genome lengths of their closest phylogenetic relatives (**Tables 5.4 and 5.5)**, and the completeness observed on CheckM tool was ≥99.9%, which together indicate that the query genome sequence has been nearly complete.

According to Chun *et al.* (2018), one way of evaluating the contamination or mislabelling of a genome which can occur during sequencing is to observe the authenticity of the genome assembly. This authenticity of the genome assembly was also confirmed for the currently sequenced genomes of *P. belisamaea* and *H. aestuarensis*. The 16S rRNA gene sequence retrieved from their genome assemblies showed the expected phylogenetic similarity to the genera *Pseudoalteromonas piscicida* and *Halomonas taeanensis* respectively, as the isolates had already shown under investigation performed in **chapter 3, Table 3.9**. As observed in CheckM analysis, ≤0.7% contamination of both study genomes further confirms the purity of the genomes.

According to the standards provided for the prokaryotic genome annotations (Klimke *et al.* 2011), the annotated genome is required to have a set of ribosomal RNAs (at least one each of 5S, 16S, 23S), a set of tRNAs (at least one for each amino acid) and protein coding genes at expected density (all core proteins annotated and not all named hypothetical). The fulfilment of these standards observed while analysing the annotated genomes of the isolates of the present study; thus, has predicted the quality of this information required to include for preliminary draft genome analysis (**Table 5.6**).

Furthermore, the depth of sequencing coverage is defined as the average number of reads of each base in the final assembly and is usually expressed in folds. e.g., the total depth of sequencing coverage provided by MicrobesNG on Illumina HiSeq was 30x. It means each base in the final assembly was read in 30 times on average. The sequencing coverage differs for all next generation sequencing (NGS) platforms due to their different accuracies and read lengths. Therefore, the value of coverage could vary for different sequencing platforms, and its requirement as per sequencing purposes. For the currently available NGS platforms, the coverage value of ≥50x has been recommended (Chun *et al.* 2018). This value was unfortunately not covered in the present genome sequencing study despite being analysed on Illumina HiSeq (reasons could be the genome production at a very competitive rate). However, since the 30x depth of coverage applied in this study fulfilled the minimal quality standards of the draft genome assembly and annotations, it was decided to proceed further with the sequence data analysis.

5.4.2 The RAST Tool

The genome annotation computed in this study on the RAST tool was based on mapping the genes to the RAST subsystems, followed by metabolic reconstruction. This tool offered high quality gene calling and functional annotation for both the complete and draft query genomes (RAST, n.d.; Aziz *et al.* 2008). It is one of the most rapid and user-friendly annotation servers and is fully automated. The software tools are already built in the RAST server for querying, visualising and analysing the data. Performing the advanced analysis in this server does not require additional packages or advanced user knowledge. It also effectively displays the sequences and functional annotation results in accessible formats that speed up the understanding of the genomic information (Bakke *et al.* 2009). RAST was also mentioned as one of the referred automatic annotation pipelines for the microbial

genomes (Klimke *et al.* 2011). For these reasons, to infer the genomes of the isolates, RAST is considered as a suitable annotation tool in the present study. However, one of the limitations of the RAST tool is the lack of software to identify pseudogenes in the query genomes. Pseudogenes possess high sequence similarity to their parental gene and their annotation can be problematic in function assignments (Aziz *et al.* 2008; Tutar *et al.* 2012).

5.4.3 Genome Annotation Features of new species *P. belisamaea* and *H. aestuarensis*

The genomes of *P. belisamaea* and *H. aestuarensis*, studied in this chapter, fulfilled one of the requirements of obtaining the genomic outlook for new taxa description of these isolates (Stackebrandt *et al.* 1994; Tindal *et al.* 2010; Thompson *et al.* 2013). Simultaneously, these genomes allowed the assessment of revolutionary relationship and the rational taxonomic classification of these isolates into genetically and phenotypically coherent microbial taxa as described in **chapter 4**. In the absence of full-fledged wet lab resources required to explore the additional facets of these microbes, this information was extracted from their genome sequences. The genomic information increased our in-depth understanding of these microorganisms through their metabolic reconstruction and additionally satisfied the demands of the microbial taxonomy to provide adequate descriptions of these microbes in terms of their roles in ecological, industrial, and clinical environments (Thompson *et al.* 2015; Didelot *et al.* 2012; Van Belkum *et al.* 2001).

When the genomes of *P. belisamaea* and *H. aestuarensis* were annotated on RAST it allowed the distribution of their genes into different subsystems of PEG categories. Predicted genes and their gene functions were then further analysed to describe these microbes for their adaptations to environmental stress, ecological and biotechnological potential, and virulence (**Tables 5.7 and 5.8**). These subsystems and PEGs of *P. belisamaea* and *H. aestuarensis* predicted in the RAST are discussed as follows.

5.4.3.1 Adaptations to environmental stress

P. belisamaea and *H. aestuarensis*, analysed for genome description in this chapter, were isolated from an upper intertidal marshy ecosystem called a salt marsh which is characterised by alternative cycles of high and low tides that bring alternative phases of flooding and drying of its marshy zones (Hemminga *et al.*

1993). Physico-chemical gradients of e.g., salinity, pH, oxygen saturation, sulphates, nitrates, phosphates (Joye *et al.* 1995; Kim *et al.* 2010a; Sauret *et al.* 2016; Cook *et al.* 2004; Gallagher *et al.* 1974) created across the salt marsh sites due to the tidal cycles are also affected by different seasons (Bolhuis *et al.* 2011) and geographical locations (Smith *et al.* 2002; Marton *et al.* 2015). Developing adaptations to the stress created by these highly dynamic conditions of salt marsh environments is therefore a key factor for the endurance of various organisms thriving across such regions, and hence for the overall sustenance of the ecosystem. The assessment of the genomes of *P. belisamaea* and *H. aestuarensis* for such adaptive features also described the interactions of these microorganisms with their native environment (**Tables 5.7 and 5.8**).

In coastal wetlands, elemental recycling of nitrogen consists of nitrification followed by denitrification, resulting in the eventual return of nitrogen to the environment. In tidal wetlands the denitrification rates are greater (Valiela and Teal 1979; Seitzinger *et al.* 1988; Howarth 1988), which is the stepwise reduction of nitrate (NO_3^-) to a nitrogen gas (N_2). The last two steps of complete denitrification are the reduction of nitric oxide (NO) to nitrous oxide (N_2O), followed by the reduction of N_2O to N_2. In stressful conditions, an excess of reactive nitrogen species such as NO which are liberated during denitrification can cause nitrosative stress in higher plants (Corpas and Barroso 2013) and in microbes (Farr and Kogoma 1991). An excess of such reactive nitrogen species as well as oxygen radicals in the indigenous environment can cause damage to the bacterial proteins, cellular membranes, and nucleic acids (Farr and Kogoma 1991). An adaptive response to such stressful conditions however is predicted in *P. belisamaea* and *H. aestuarensis*. Their annotated genomes projected the production of bacterial haemoglobins (**Tables 5.7 and 5.8**) which are widely known to detoxify reactive nitrogen and oxygen species, probably by direct consumption of these radicals, that in turn offers protection to the microbes (Poole 2020; Frey *et al.* 2002).

Formaldehyde, which is produced at significant levels in the environment by both non-biological and biological sources (e.g., as an intermediate of methylotrophic metabolism in salt marshes), is known to induce inactivation of various cellular components (Yurimoto *et al.* 2005). Detoxification of formaldehyde in a glutathione-dependent pathway has been predicted in some of the detoxifying PEGs of *P. belisamaea* and *H. aestuarensis* (**Tables 5.7 and 5.8**). This capacity to metabolise

formaldehyde through entrapment by cofactors such as glutathione has been reported before as a stress relief to the organisms from this toxic substance (Achkor *et al.* 2003). Additional genes which encode detoxification of naturally occurring toxic substances such as selenate and selenite were also projected in the annotated genome *of H. aestuarensis*. These substances otherwise would be mutagenic at high concentrations (Achkor *et al.* 2003; Bébien *et al.* 2002; Bebien *et al*. 2001).

Genes encoding a nonheme iron protein called rubrerythrin, which has an antioxidant property, were also called in the *H. aestuarensis* genome (**Table 5.8**). Rubrerythrin has previously been reported to protect microbes against oxidative stress caused due to oxygen and organic radicals, starvation, adverse pH and temperature (Zhao *et al* 2007; Baatout *et al.* 2006), and reactive nitrogen species (Mydel *et al.* 2006). Additionally, osmoadaptation was predicted in *H. aestuarensis* as its 33 genes mapped for accumulation of osmoprotectants called betaines and ectoine (**Table 5.8**). Betaines and ectoine are taken directly from the environment, and betaines can also be synthesised from choline. These osmoprotectants are known to efficiently accumulate inside the microbial cells at high cytoplasmic concentrations, which could prove beneficial to the *H. aestuarensis* in response to the osmotic stress conditions of salt marshes (Kappes *et al.* 1999). These osmolytes are also known as a source of nitrogen, carbon, and energy in some bacterial species (Barra *et al.* 2006). These predicted osmoregulatory properties of *H. aestuarensis* seems to create suitable conditions for its survival in high salinity gradient environments such as salt marshes.

As such large numbers of genes encoding stress responses were predicted in the genomes of *P. belisamaea* and *H. aestuarensis* (**Tables 5.7 and 5.8**), it provides evidence for the possible adaptive nature of these strains to the continuously changing salt marsh environments and their ubiquitous nature.

4.4.3.2 Ecological potential

Salt marshes are known to promote biogeochemical cycles and plant growth, protect upward land from floods, maintain marine water quality, reduce global warming, and display aesthetic value (Shepard *et al.* 2011; Van 1985). Microbial interactions with various salt marsh components are the driving force for most of these functions and one of the contributing factors towards maintenance of these ecosystems (Chmura *et al.* 2003; Martins 2011; Gayathri *et al.* 2010; McKew *et al.*

2013; Buchan *et al.* 2003). The annotated genomes of salt marsh microbes *P. belisamaea* and *H. aestuarensis* have displayed several genes predicted to metabolise phosphorus, sulphur, and nitrogen (**Tables 5.7 and 5.8**). This indicates the beneficial role of these novel microbes in enriching the salt marsh sediments with organic and inorganic nutrients via different elemental cycles (Parkes *et al.* 2012; Bernhard *et al.* 2010; Senior *et al.* 1982; Banat *et al.* 1981). Additionally, these microbes have exhibited genes involved in the synthesis of the plant growth promoting hormone auxin (Costacurta *et al.* 1995).

There are 58 genes in *P. belisamaea* which predict microbe mediated iron capturing and highlight its possible ecological role in iron deficient marine environments (Butler 2005). Such microbe mediated enrichment of ecological sediments additionally protects salt marsh morphodynamics and functionality and forms the basis of food chains existing among these areas (Chmura *et al.* 2003; Yakimov *et al.* 2007).

The large number of genes required for the secretion of extracellular substances, exopolysaccharide, as previously reported in *Halomonas* species (Diken *et al.* 2015; Schwibbert *et al.* 2011; Park *et al.* 2020), were also found in the annotated genome of *H. aestuarensis*. These extracellular substances are known to promote plant growth in saline environments by imparting plants with a salt tolerance by limiting the content of sodium ions available for the plants' uptake, stabilise the salt marsh soil sediments, and restrict the soil erosion (Stal *et al.* 2010; Van Gemerden 1993; Upadhyay *et al.* 2011; Bergmann *et al.* 2009; Ruiz-Lozano *et al.* 2000).

These roles of elemental recycling, the production of phytohormone and exopolysaccharides predicted in *P. belisamaea* and *H. aestuarensis* can promote the growth of diverse flora (Gayathri *et al.* 2010). This could in turn create dense halophytic zones of salt marshes and protect the upper land from flooding by slowing and absorbing floodwater, filtering the toxic waste, and maintaining the water quality (Shepard *et al.* 2011; Van 1985). Adaptive features predicted by the genes of *P. belisamaea* and *H. aestuarensis* to neutralise the naturally occurring toxic substances such as nitric oxide, formaldehyde, selenate, and selenite, which are lethal to the living organisms at high concentrations, could also prove a valuable bio-remedy for these and other organisms living in the vicinity of such contaminated environments (Achkor *et al.* 2003, Bébien *et al.* 2002; Bebien *et al.* 2001). These genes and their ecological function defined for *P. belisamaea* and *H. aestuarensis*

could thus promote the creation of distinct niches and house different plants, animals, and microbes in the salt marshes (Wilson and Whittaker 1995; Gray 1992; Dini-Andreote *et al.* 2014).

4.4.3.3 Biotechnological potential

Biotechnologically relevant genes annotated in the genomes of *P. belisamaea* and *H. aestuarensis* for the metabolism of aromatic compounds and their intermediates such as catechol, salicylate, gentisate, quinate, homogentisate, biphenyl, xylenols and cresols indicated the possible role of these microbes in bioremediation of such problematic environmental pollutants (Robertson and Hansen 2001; Xiang 2020; Díaz 2004; García *et al.* 2004). Additional genes encoding the metabolism of isoprenoids and antibiotics in *P. belisamaea*, and the secretion of exopolysaccharides in *H. aestuarensis*, showcase the commercial significance of these organisms (**Tables 5.7 and 5.8**).

Isoprenoids predicted to be metabolised in *P. belisamaea* are the largest family of natural products and have wider applications in health, nutraceuticals, biofuels, cosmetics, and the food industry (Heuston *et al.* 2012; Li and Wang 2016). Additionally, some 36 genes of chorismate subsystems have been anticipated in an annotated genome of this organism. Chorismate is an intermediate for the synthesis of p-aminophenylalanine, which is a precursor for three antibiotics - chloramphenicol, pristinamycin and obafluorin (Blanc *et al.* 1997; Herbert and Knaggs 1992). Genes involved in the synthesis of the broad-spectrum antibiotic marinocine, and the secondary metabolism of paerucumarin and lanthionine synthetases were also predicted in an annotated genome of *P. belisamaea* (**Table 5.7**). Lanthionine synthetases is a known key constituent in lantibiotics synthesis, a prevalent class of peptide antibiotics (Denoël *et al.* 2018). The presence of these genes predicting the synthesis of multiple antibiotics, and of the additional 58 genes predicting iron acquisition (**Table 5.7**), impart possible antifouling property to *P. belisamaea* which will probably be beneficial in pathogens biocontrol e.g., in plants and fish farming (Ahmed and Holmström 2014; Fernandes and Kerkar 2019; Imada *et al* 1985; Kobayashi *et al.* 2003; Maeda *et al.* 1997; Wang *et al.* 2018; Gorospe *et al.* 1996; Uchida *et al.* 1997) and as an eco-friendly alternative to toxic marine anti-foulant chemical paints (Holmström and Kjelleberg 1999; Yee *et al.* 2007; Dobretsov *et al.* 2007).

Extracellular polysaccharides, predicted to be secreted by the *H. aestuarensis* (**Table 5.8**), are reported before to have multiple commercial applications, such as bioremediation of heavy metal contaminated environments, crude oil emulsification, viscosity enhancements in food products, and immunomodulation (Quesada *et al.* 2004; Amjres *et al.* 2015; Dworkin 2006).

As these favourable adaptive features predicted in the genomes of *P. belisamaea* and *H. aestuarensis* are discussed for their survival in diverse conditions of salt marshes, the aforementioned biological compounds can be expected to have vital commercial applications even in extreme environmental conditions (Sánchez-Porro *et al.* 2003).

5.4.3.4 Virulence

Several *Pseudoalteromonas* and *Halomonas* species have been recognised as causative agents of infections in humans, plants, and animals (Sawabe et al. 1998; Choudhury et al. 2015; Bein 1954; Holmström and Kjelleberg 1999; Kim *et al.* 2013; Yeo *et al.* 2016; Kim *et al.* 2010b). As the ecological and biotechnological potential of *P. belisamaea* and *H. aestuarensis* has been suggested through their genome annotations in this study, applications of these strains for future usage therefore demands further analysis of their pathogenicity, and safety for users and their native environments.

An annotated genome of P. belisamaea predicted a number of genes producing biologically active agents such as proteolytic compounds, bacteriocins, and nucleases (**Table 5.7**), which are known to impart antifouling properties to their producers and make them competitive, particularly in nutritionally poor environments (Gordon et al. 2007; Palanichamy *et al*. 2017; Raetz and Whitfield 2002; Wang and Quinn 2010; Sawabe *et al*. 1998). This genome of P. belisamaea has also predicted its ability to invade diverse host environments through annotation of its large number of genes related to bacterial chemotaxis and motility, resistance to antimicrobial and toxic compounds, and iron acquisition (Josenhans and Suerbaum 2002; Erhardt 2016; Brown *et al*. 2001). Additionally, the CRISPRs sequences detected in the annotated genome of P. belisamaea are an indication of the microbe’s immunity against phage invasion (Barrangou *et al*. 2007).

The genes of H. aestuarensis have revealed multiple genes potentially involved in protein degradation, chemotaxis, regulation of virulence, and production of capsule

and extracellular polysaccharides (**Table 5.8**). These features are known to impart pathogenicity, antifouling activity, and protection to microbes from the host defence (Rojas *et al.* 2009; Erhardt 2016; Joly-Guillou 2005). However, the genome lacked CRISPRs subunit genes, known for the bacterial immunity against phages (Barrangou *et al.* 2007).

5.4.4 Inference of This Study Findings

The present study has fulfilled the minimal genome and annotation standards for the use of draft genome data in the taxonomy of identified novel isolates (Thompson *et al.* 2013). Evaluation of genes related to the adaptation, ecological and biotechnological potential, and virulence was also accomplished through the genome annotation. However, a few pitfalls of these methods have also been noticed.

The genome-based interpretation of microbial functions requires a high-quality complete genome and accurate analysis of their sequences that would infer their biochemical, physiological and ecological meaning (Klimke *et al.* 2011). Draft genome sequencing performed with sufficient redundancy and annotation quality is known to provide sufficient information at primary studies e.g., for taxonomic purposes (Chun *et al.* 2018). But fragmented, short reads of the draft genomes can decrease the annotation efficacy that requires further mitigations to minimize false positive or false negative genome analysis results (Klassen and Currie 2012). Multiple gene fragments of same open reading frames (ORFs) being annotated separately (false positive) or shorter ORFs remained unannotated (false negative) could be detrimental for genome studies like description of evolution and adaptation (Klassen and Currie 2012; Ricker *et al.* 2012). The high-quality assemblies and completion of genome without any gaps and unidentified bases minimise such gene calling errors and can be more informative than the draft genome. However, given the challenges and comparatively higher cost related to completing the sequence by filling the gaps in the original genome assembly, for now draft genomes appear to be widely used in microbial genomics, which is essential for creating adequate information of the vast microbial diversity (Klassen and Currie 2012).

Furthermore, defining genes for their physiological features in a cultivation-independent way without lab experiment data to verify the computational genomic results could limit the in-depth understanding of the actual microbial processes

(Vaattovaara *et al.* 2019). Predicted functional genes of an annotated genome may or may not be expressed in their native environments under certain conditions and there are possibilities of missing or vague gene assignments, that is also varied with the quality of the available genome (Brenner 1999; Iliopoulos *et al.* 2003; Vaattovaara *et al.* 2019). The predicted gene function(s) can also be part of a biochemical cascade that would only activate when the remaining components of the cascade are available in the surrounding environment. Thus, the draft genomes described in the present study provides just a glimpse of the actual physiology of the novel isolates.

Conversely, at primary study stages, in the absence of wet lab facilities or pure cultures, metabolic reconstructions of the query genomes derived by projecting the characteristics and biochemical capabilities of other known studied organisms could produce an estimate of a metabolic portrait of the organism of interest (Zhou and Miller 2002), which is undoubtedly useful to improve the general understanding of the biology of microorganisms. However, after utilising the genome data to narrow down to the specific features, the authentication of these gene results through wet lab-based studies would remain necessary.

In the present PhD study, few physico-chemical parameters of both the novel species *P. belisamaea* and *H. aestuarensis* (**Table 5.1**) were indeed obtained via wet-lab experiments (more details in **chapter 4**) that could be related to the observations of their genome annotations. These wet-lab results of both the novel isolates showed diverse metabolism, oxidase and catalytic activity, wider growth range of salinity, pH and temperature that features their adaptability to diverse gradient-rich environment of the salt marshes (**Table 5.1**). These features could also be related to the large number of stress response, chemotaxis and bioremediation related genes inferred in their annotated genomes (**Tables 5.7 and 5.8**). Further lab-testing of this isolates in conjugation with genomic data exploration would be useful to provide additional microbial insights.

Chapter 6

Gel-stabilised Gradient Plates: A New Cultivation Approach for The Laboratorial Recreation of Naturally Occurring Interfaces

A more concise version of this chapter has been submitted to the Microbiology journal for the publication:

More-Mutch, P., Huber, R., and Antunes, A. (2020) Gel-stabilized gradient plates: a new cultivation approach for the laboratorial recreation of naturally occurring interfaces. Microbiology (Submitted)

6.1 INTRODUCTION

The advent and widespread use of 16S rRNA gene cloning and sequencing methods, together with other culture-independent studies employed to identify microorganisms in natural environments have revealed phylogenetically novel, uncultivated diversity among these habitats, some of which are extraordinarily microbe rich (Tang *et al.* 2018; Bolhuis and Stal 2011; Dojka *et al.* 2000). High proportions of microorganisms in most environments are still uncultured and frequently cited as approximately 99% (Fournier *et al.* 2017; Steen *et al.* 2019; Oren 2004; Schleifer 2004; Harris *et al.* 2004; Rappé and Giovannoni 2003). However, precise identification of these proportions is unclear as the underlying data are hard to identify (Fournier *et al.* 2017; Oren 2004; Schleifer 2004). Such results highlight the inadequacy of standard cultivation methods to provide a clear and faithful picture of the existing microbial biodiversity.

“Candidate” phyla (phyla having no representatives in culture), such as the bacterial groups; TM6, TM7, WS6, and Marine Group A, are widely distributed, abundant in many environments and are probably important in bio-geo-chemical processes (Cottrell *et al.* 1999; Dong *et al.* 2019; Dojka *et al.* 2000; Rappé and Giovannoni 2003; Olapade 2020). Via sequence-based techniques, clone libraries or phylotypes of cultivation-resistant bacteria were also found to be associated with a variety of pathological conditions (Behera *et al.* 2021; Kuehbacher *et al.* 2008; Fredricks *et al.* 2005; Brinig *et al.* 2003). Molecular biology thus has supported the investigation of yet uncultivable bacteria in various environmental settings.

Function-based metagenomics is increasingly applied to study gene functions of such uncultured bacteria (Wang *et al.* 2012; He *et al.* 2015; Verberkmoes *et al.* 2009), but further efforts to define their culture-specific physiology, stable taxonomic classification and species description still require their pure cultures in the lab (Rosselló-Móra and Amann 2015). Despite the frequent availability of genomic information, evolutionary processes of such candidate phyla in large populations are not well understood. Additionally, their microbial diversity, genome, biochemical and metabolic novelty remains largely unexplored (Rappé and Giovannoni 2003). The inability to bring many of these organisms into cultured state, also severely limits our ability to elucidate important aspects of these microbes such as their environmental role (e.g., in ecology and nutrient cycling) and potential applications (Stewart 2012; Bodor *et al.* 2020). A prospective gain of isolating previously

uncultivable microbes for example could be the exploration of valuable catalysts and enzyme molecules for industrial applications (Lewis *et al.* 2010; Galvão *et al.* 2005; Lorenz and Eck 2005) as well as the detection of novel antimicrobial producers that would address the 'discovery void'; a term coined for the ongoing collapse in disclosure of new pharmaceutical compounds (Silver 2011; Leeds *et al.* 2006).

The well-recorded discrepancy between the microbial numbers observed under a microscope and the much lower number of microbes that grow on media plates is commonly known as 'the great plate count anomaly' (Staley and Konopka 1985). This could be due to either the existence of non-viable microbial cells or the failure of living microbes to form visible growth on general lab media (Harwani 2013).

In classical lab cultivation methods, the over-reliance on homogeneous, eutrophic media that share little resemblance with the complexity of the natural environments is likely to be one of the major reasons why this enormous untapped biodiversity still eludes us. The natural habitats of most microbes are extremely dynamic and include spatial gradients of growth substrates, electron acceptors, pH, salts, and inhibitory compounds (Emerson *et al.* 1994; Brune *et al.* 2000). Though wide-range gradient-rich environments are perceived as hotspots of microbial diversity, targeting the diverse nutritional requirements of these microbes for their growth in the lab could be a challenging task. Most of the uncultivated bacteria exhibit slow growth rates (Chaudhary *et al.* 2019; Song *et al.* 2009; Vartoukian *et al.* 2010). The use of homogenous, eutrophic cultivation media might result in overgrowth of fast-growing microbes; obscuring the growth of these slow-growing or rare microbial species (Bodor *et al.* 2020; Poindexter 1981). Substances already present in original media components or generated during media preparation could also curtail the microbial growth (Kawasaki and Kamagata 2017; Tanaka *et al.* 2014; Finkelstein and Lankford 1957).

Other environmental factors that restrict the growth of uncultured microbes *in vitro* can be listed as: their rare occurrence or dormant phase that remained unbroken in general lab culture conditions (Deming and Baross 2000; Kell and Young 2000), symbiotic microbial association (Dunny and Winans 1999; Kim *et al.* 2011) and quorum sensing mechanism (Abisado *et al.* 2018; Li and Tian 2012; Romero *et al.* 2011) that exists in environments but are not created in the lab, secondary metabolite dependence (Kell *et al.* 1995), fastidious or oligotrophic growth

requirements (Cartwright *et al.* 1994; Bakshi *et al.* 2019; Hata 2017; Connon and Giovannoni 2002), competition (e.g, Hibbing *et al.* 2010; Bell *et al.* 2013; Grover *et al.* 2000) or inhibition (Stewart 2012; Watsuji *et al.* 2007) e.g. due to bacteriocins produced by other microbes in mixed cultures (Shioya and Shimizu 2001; Subramanian and Smith 2015).

To address the cultivability issue of microbes, the last few decades have brought some progresses with the development of a range of new lab approaches including: a) recognising and alleviating the pitfalls in the media preparations by adding substances that scavenge the toxic compounds produced while preparing media (Alvarez and Guijarro 2007; Bruns *et al.* 2002), altering the solidifying agents (e.g. Tamaki *et al.* 2009; Kawasaki and Kamagata 2017) and media preparation steps (e.g. Tanaka *et al.* 2014; Kato *et al.* 2018), b) designing growth media composition according to the molecular-based data of the members of the microbiome, that utilizes highly expressed metabolic genes of the microbiome to design a culture medium (e.g. Bomar *et al.* 2011), c) high-throughput extinction culturing for the isolation of oligotrophic prokaryotes from marine ecosystem that also involved microarray method for growth detection (Zengler *et al.* 2005; Connon and Giovannoni 2002); d) mimicking in situ environmental conditions of symbiotic microorganisms by cultivating them in a semi-permeable diffusion growth chambers (Kaeberlein *et al.* 2002), as well as e) enclosing single bacterial cells in microdroplets of solidified agarose followed by individual colony sorting into microtiter plates (Zengler *et al.* 2005).

These new or modified lab techniques have brought interesting insights on cultivation strategies and have promoted growth of some previously undescribed, uncultured, or fastidious microbes (e.g., MSC1, Kaeberlein *et al.* 2002; *Flavobacterium psychrophilum*, Alvarez and Guijarro 2007). Some of these new methods are straightforward to implement e.g., modifications in media preparations and longer incubations (Kato *et al.* 2018; Alvarez and Guijarro 2007). Despite their promising results, most of them have not yet been widely adopted. That could be due to number of reasons such as preference for homogenous growth media that fail to meet the diverse growth requirements of many environmental microbes; their complex, time-consuming (Kaeberlein *et al.* 2002), and expensive (Connon and Giovannoni 2002) methodological protocols which lack applicability for large scale cultivation studies and inability to propagate or maintain the isolated pure novel

cultures in the lab (e.g. Kaeberlein *et al.* 2002); as well as preferable cultivation of only dominant microbes of the study environment (e.g. Bomar *et al.* 2011). Thus, cultivation attempts still remain heavily dependent on classical cultivation methods.

The present study attempts to partially address the current gap in cultivation techniques by focusing on ameliorating and testing a plate diffusion technique that can be easily applied to replicate gradients of a wide-range biotopes as diverse as fresh water and coastal ecosystems (e.g. rivers, ponds, lagoons, estuaries, salt marshes; Bernhard *et al.* 2010; Cao *et al.* 2008), coniferous forests, tropical forests (Baldeck *et al.* 2016), grasslands (Tardella *et al.* 2016), fire-dependent ecosystems (Kirkman *et al.* 2001), and even more extreme environments such as deep-sea brines (Antunes *et al.* 2011; Antunes *et al.* 2019) or geothermal lakes (Weltzer *et al.* 2013).

Plate diffusion methods have come a long way since the pioneer works of Beijerinck (1888). Later works resulted in the development of the ingenious wedge plate technique (Szybalski 1952; Szybalski and Bryson 1952) and the steady-state two-dimensional gradient plate (Caldwell and Hirsch 1973), which constituted two major breakthroughs in this field. Further studies by Wimpenny and colleagues (Wimpenny *et al.* 1981; Wimpenny and Waters 1984; Wimpenny and Waters 1987; Peters *et al.* 1991; Thomas and Wimpenny 1993; Thomas and Wimpenny 1996a; Thomas and Wimpenny 1996b) improved the wedge plate technique and even permitted the establishment of multidimensional systems (i.e., systems where multiple parameters are varied simultaneously). Plate diffusion technique has been used before for studying microbial strain behaviour and growth patterns when exposed to varying concentrations of different substrates (Wimpenny and Waters 1984; Panagou *et al.* 2005), for testing antibiotic susceptibility (Liu *et al.* 2011), and for surveying the antibacterial effects of food preservatives (Thomas *et al.* 1993). Despite some advances, the full potential of plate diffusion methods in the several fields of microbiology has not been truly explored, with its applications remaining almost exclusively restricted to auxanographic studies. The limited use of these techniques may be due to the bulky structure of diffusion plates and multi-reservoir systems (Caldwell and Hirsch 1973; Caldwell *et al.* 1973), expensive experimental setting (Hill 1991) or narrow range salinity and pH gradients that lack stability and replicability confirmations (Thomas and Wimpenny 1993; Thomas and Wimpenny 1996b; Panagou *et al.* 2005).

A gel-stabilised gradient plate diffusion method was developed in the present study to extend its application as a new technical approach for the cultivation and isolation of microbes from gradient-rich environments. This method was specifically adapted to replicate the salinity and/or pH gradients encountered in marine environments. Extremely dynamic and heterogeneous gradient-rich environments at marine ecosystem like the one formed at the salt marshes, and brine and seawater interface of the deep-sea brines were areas of research interest in this study.

Salt marshes are very diverse and extreme, compared to other marine ecosystems in terms of frequent environmental changes. Alternating wet and desiccated phases create unique horizontal and vertical spatial physico-chemical gradients across these environments. Physico-chemical gradients of e.g., salinity, pH (Nelson *et al.* 2009; Rey 1992), redox potential (Rand 2001) and moisture content (Leeuw *et al.* 1990) created at different flooding cycles and seasons across salt marshes are known to shape the microbial community structure and abundance (Bolhuis *et al.* 2013; Webster *et al.* 2015; Nelson *et al.* 2009). Further details on salt marsh structure and their functions are given in **chapter 1**. Deep sea brine pools are formed by a process of re-dissolution of sub-bottom miocene evaporites followed by evaporites' migration to the seafloor and later deposition in topological depressions of the deeps (Craig 1969; Cita 2006). As sea water circulates through these deposits, gradient areas of extremely varying salinity (4–28% w/v) and pH (5.2–8) are formed (Antunes *et al.* 2011; Shanks and Bischoff 1977). Characteristically steep gradients of temperature, salinity, pH and oxygen formed at these interfaces also trap organic and inorganic materials coming from seawater (Shanks and Bischoff 1977; Antunes *et al.* 2011; Cita 2006). Physico-chemical and nutrient gradients generated at this ecosystem supports microbial diversity which also forms the basis of food webs populating among these areas (Eder *et al.* 2001; Eder *et al.* 2002; Yakimov *et al.* 2007).

By focusing on gradients formed at these heterogeneous high salinity environments like the one in deep-sea brines (ca 4–28% NaCl, Eder *et al.* 2001; Antunes *et al.* 2011; Kaartvedt *et al.* 2016) and co-varying gradients present in salt marshes (salinity 1.2–3.6 % and pH 5.3–7.6, Piceno *et al.* 1999; Nelson *et al.* 2009), some of the promising features of gel-stabilised plates developed in this study were to have wide-ranged, stable, improved gradients, with high salinity values. Furthermore, the present gradient plate approach was aimed to develop as an easy,

robust, low-tech, and low-cost tool for the artificial recreation of naturally occurring interfaces and hence has a novel potential of direct and wider application in the isolation of microorganisms from these ecosystems, which is distinguishable to the previously reported gradient diffusion methods.

6.2 METHODS

6.2.1 Media Preparation

Appropriate volumes of media, adjusted to different NaCl concentrations and supplemented with agar (Oxoid, LP0012) were prepared and autoclaved, followed by pH adjustment in sterile conditions. Gradient plates mimicking salt marsh ecosystem and brine-sea water interface were prepared with marine agar (BD Difco™, 2216; media composition at **chapter 2, Table 2.4**) and Halobacteria medium (HBM medium, composition in g/L, yeast extract 5.0, casamino acids 5.0, sodium glutamate 1.0, potassium chloride 2.0, trisodium citrate 3.0, magnesium sulphate 20.0, ferric chloride 0.036, manganese chloride 0.00036, agar 15.0, sodium chloride 200.0) respectively. These media were selected for gradient experiments as their contents nearly replicated the major mineral composition of sea water and other nutrients required for the growth of heterotrophic marine bacteria (Lyman and Fleming 1940) and as later medium supported the growth of *Haloarcula marismortui*; the strain used in mixed-culture inoculation of gradient plates as described later in this chapter at **section 6.2.6**.

NaCl and pH values of media preparations required to develop a gradient mimicking salt marsh ecosystem were adjusted in marine agar (**Table 6.1)**. The required NaCl concentrations were achieved with addition of NaCl powder and measured using a hand refractometer (Atago, S-28E, Japan). The pH of autoclaved media was adjusted by adding freshly prepared 1M NaOH or 1M HCl under aseptic conditions. Final pH was confirmed by testing aseptically aliquoted 1 ml media with pH indicator strips (non-bleeding, MColorpHast™, Merck, 1.09584.0001). All media flasks were partitioned in 30 ml aliquots and dispensed into sterile 50 ml screw-cap tubes and were kept at 70–80 °C until immediately before pouring.

Table 6.1 NaCl and pH concentrations adjusted in marine agar media for gradient development mimicking salt marsh ecosystem

Media layer	NaCl % (w/v)	pH
1st layer	5.5	8.5–9
2nd layer	1	5

Salt marsh physico-chemical parameters can vary from site to site, so values reported by Piceno *et al.* (1999) and Nelson *et al.* (2009) (salinity 1.2–3.6% (w/v) and pH 5.3–7.6) were adopted as a reference for media preparation. These values were used in our experimental setup to prepare two versions of plates; single-gradient plates (either pH or salinity) and combined-gradient plates having both pH and salinity gradients co-occurring in the same plate.

Additionally, high salinity plates were developed based on the results obtained in gradient optimization experiment (**section 6.2.4**). They mimicked the salinity gradient observed in brine-sea water interfaces, for which a salinity range of 4–28% (w/v) was taken as a reference value (Antunes *et al*. 2011; Kaartvedt *et al*. 2016). While developing these gradients, the four parallel plate approach was employed. The NaCl concentrations of first and second media layers of these four plates were adjusted as 6, 10, 14 and 18% (w/v) and 13, 17, 21 and 25% (w/v) respectively, to get slightly overlapping salinity values among these plates, cumulatively in the range of 6–24% (w/v). Four parallel plate approach was developed as a feasible alternative for covering the required salinity without suffering from faster gradient dissipation that would occur with single plate prepared with highly divergent salinity value.

6.2.2 Gradient Plate Preparation

All gradient plate pouring steps were performed on a levelling board, carefully adjusted by the four screws attached to its corners and confirmed with a spirit level. The use of a levelling board was essential to guarantee that uneven lab benches would not interfere with the process and assured replicability of conditions throughout all experiments.

The levelling board consisted of a 45 cm x 25 cm acrylic rectangular sheet, firmly mounted with two 45 cm x 5 cm acrylic narrow boards, placed parallel to each other

with a 9–10 cm gap in-between them to hold the agar plate in a slanted position (**Fig. 6.1A**).

Gradient plates were established by consecutively pouring two 30 ml layers of medium having different conditions (of salinity and/or pH) into 10 cm x 10 cm x 2 cm wettable surface squared petri dishes (Sarstedt, 82.9923.422). To get the required media wedge height, the edge of each petri dish was raised using the levelling board (**Fig. 6.2A**) prior to pouring the first media layer, with higher substrate concentration. Plates were allowed to cool and set in this tilted position, thus allowing the medium to solidify as a wedge shape (**Fig. 6.2B**). After returning plates to their horizontal position (**Fig. 6.2C)**, the second layer, with lower substrate concentration, was then poured over the first layer (**Fig. 6.2D**). The plates were allowed to set and dry so that the layers could equilibrate vertically, and gradients could be established. This was immediately followed by incubation of these plates at the desired temperature.

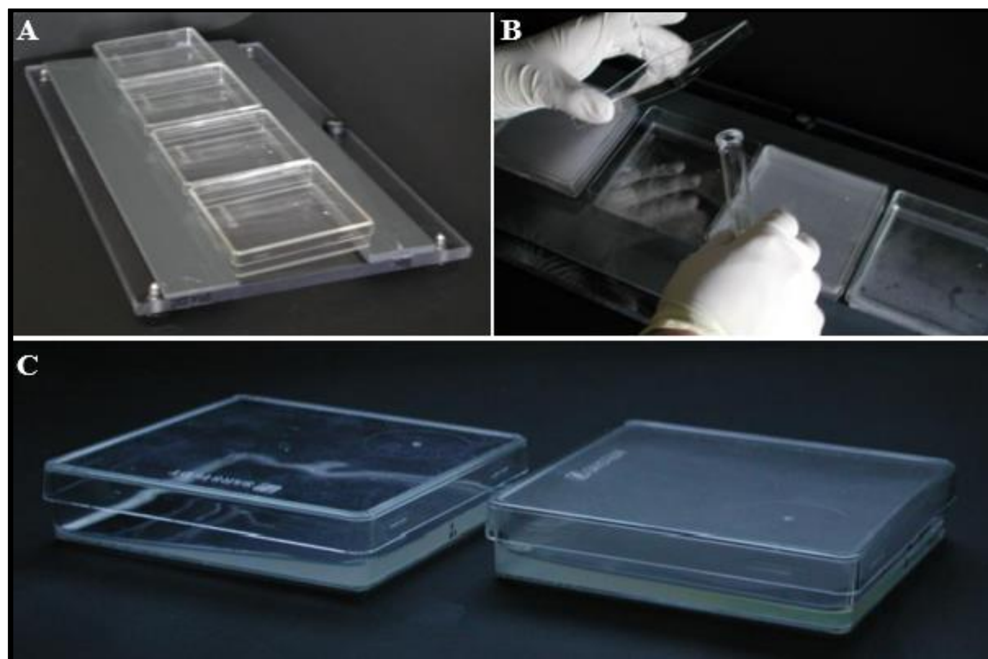

Figure 6.1 Overview of the gradient plating system and plating process used for media pouring. A- Plating system setup; B- Media pouring; C- Plates after pouring of 1st (left) and 2nd media layer (right)

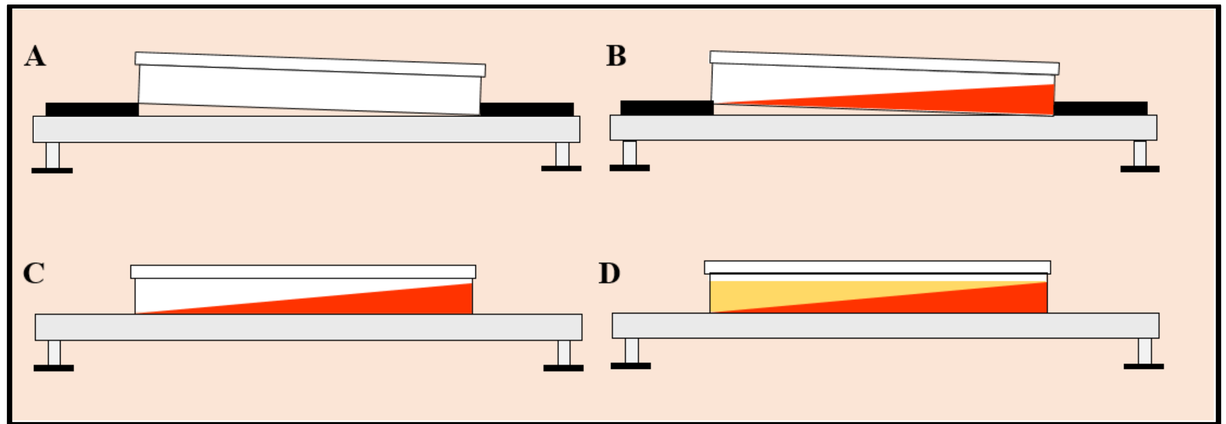

Figure 6.2 Schematic representation of the various steps of gradient plating. A- Original setting of plate in tilted position prior to pouring 1st media layer; B- Pouring of 1st media layer with high substrate concentration; C- Placement of plate in horizontal position after agar setting and prior to pouring 2nd media layer; D- Pouring of 2nd media layer on top of the 1st one

6.2.3 Gradient Measurement

After starting their incubation, gradients were measured every 24 h in 4–5 different sections of the agar plates along the direction of the gradient (as shown in **Fig. 6.3**). pH gradients were measured semi-quantitatively by placing the reaction zone of pH indicator strips directly over the different sections of the agar plates. After 2 min of contact time with the media surface, these strips were read by evaluating the colour change of its reaction zone. For salinity measurements, gradient plates were sectioned with a clean spatula to retrieve square agar core blocks of ca. 5 mm^2. The salinity of each agar block was assessed with a hand refractometer after placing them over the prism and gently squashing them by closing and pressing the covering lid.

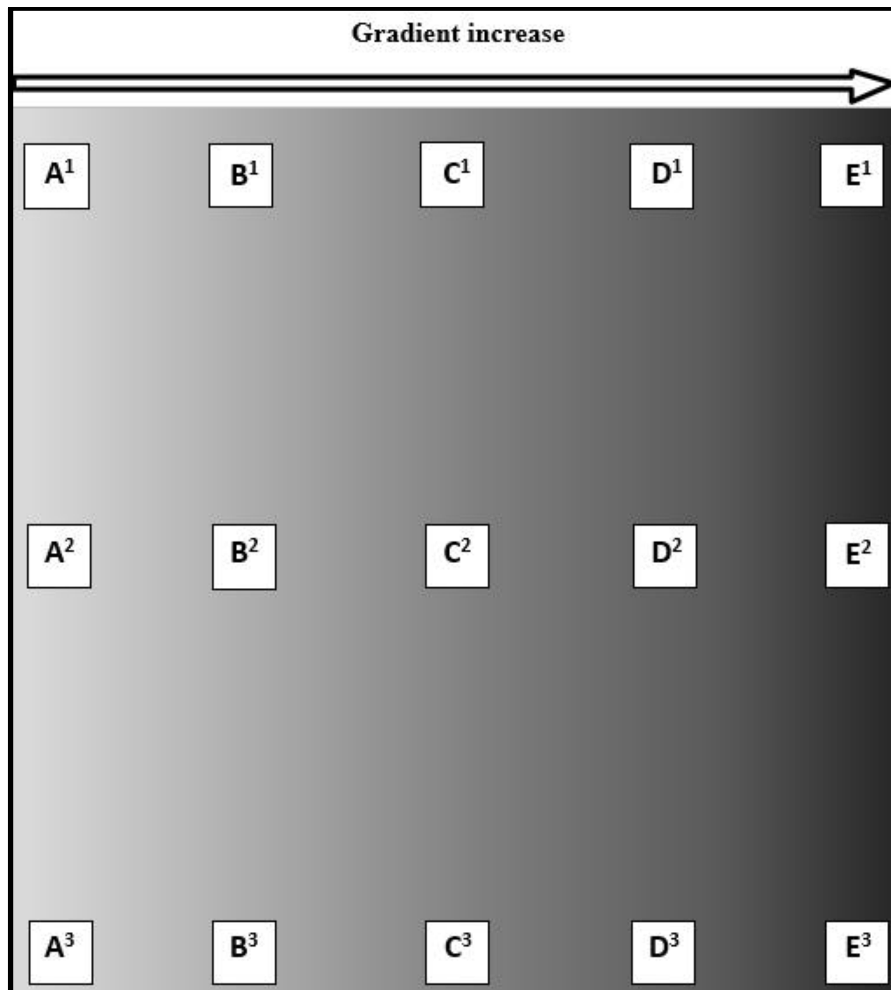

Figure 6.3 Schematic representation of agar core block retrieval from five sections (A, B, C, D, E) in triplicate to assess gradient stability throughout incubation time

6.2.4 Gradient Optimization

Different parameters were tested to confirm optimal conditions of gradient establishment and long-term stability. This testing included two sets of each parameter conditions of media wedge height (6 and 9 mm), agar concentration (1.2 and 1.5% (w/v)), incubation temperature (25 and 37 °C) as well as combinations of media layers with varying pH**.** These parameters conditions were decided so as to make the gradients applicable for the present study requirements of microbial cultivation and incubation.

While developing wider salinity gradients, the variation in their dissipation rates was confirmed by preparing triplicate plates in HBM medium with different divergent salinity values between both the media layers (a. 5% divergence- salinity of two

layers; 2.5% and 7.5%; b. 15% divergence- 0% and 15%; and c. 25% divergence- 2.5% and 27.5%) and testing these plates for salinity over 7–8 days of incubation at 30 °C.

6.2.5 Gradient Evolution and Stability

Considering the results obtained during the optimization study, suitable gradient plates mimicking salt marsh and brine-sea water interface ecosystem were prepared (**section 6.2.1**). For all gradient batches, multiple identical plates were produced and incubated together. Gradients were measured 24 h after pouring, with further measurements performed using one of the gradient plates from the same batch. The stability and evolution of gradients was assessed by repeating this procedure at regular time intervals for a period of at least 8–10 days. Gradient measurements for each plate were performed in triplicate (**Fig. 6.3**). During incubation, to avoid excessive media drying and its potential disruptive effect on gradients, plates were kept in sealed bags containing moistened tissue paper.

6.2.6 Mixed-Culture Inoculation of Gradient Plates

For proof of concept, parallel plates covering an extended salinity gradient of 6–24% (w/v) were prepared for use in mixed-culture inoculation. To this effect, a defined mixed culture containing *Salinisphaera shabanensis* (DSM 14853^T) and *Haloarcula marismortui* (DSM 3752^T), grown in HBM medium with 15% (w/v) NaCl was used for testing purposes. Salinity gradient plates were prepared using the same method as described above in **section 6.2.1**. These plates were inoculated with 0.1 ml of appropriate dilutions of freshly grown pure broth cultures, which were mixed, and the inoculum was spread evenly across the surface of the agar using sterile Q-tips. The dilution of the original cultures allowed both microbes to be brought to equivalent cell densities and lowered inoculum density to a value that resulted in discrete colony growth. Parallel plates were prepared and incubated uninoculated, under the same conditions to confirm the range of the obtained salinity gradient.

6.3 RESULTS

6.3.1 Gradient Optimization

The observed pH of six sets of gradient optimization plates, prepared and incubated at different combinations of parameter conditions, are mentioned in the result **Table 6.2**.

As a result of the first set of experiments testing different temperatures, agar concentrations, and media wedge heights in this study, it became possible to optimise conditions to get the desired pH range for salt marsh gradient of 5.3–7.6. The best setting was the use of 1.5% (w/v) agar and 9 mm media wedge height (set III and VI of **Table 6.2)**. Both tested mesophilic temperatures 25 and 37 °C showed favourable wider gradient establishment at these settings. The gradient obtained with these optimized conditions remained fairly stable for 7–8 days (same as **Fig. 6.5**).

For the same temperature gradient set-up, among tested parameters media wedge height is found to have a greater effect on gradient than different incubation temperatures and agar concentrations (**Table 6.2)**. A wider pH range was obtained with 9 mm media wedge height (set III and VI) than that of 6 mm wedge height. After initial experiments, the use of 1.2% (w/v) agar was discontinued and was not tested further with media wedge height 9 mm, because at tested temperatures 1.5% (w/v) agar conc. was found to work better than 1.2% (w/v) to reduce drying of the media during longer incubations.

Table 6.2 pH gradient optimization at different incubation temperatures, agar concentrations and media wedge heights. pH measurements performed in triplicate at 5 different sections (A–E) across the plate. Desired pH gradient of 5.3–7.6 and stability of 7–8 days obtained in set III and VI

Set	**Incubation temp. (°C)**	**Agar % (w/v)**	**Media wedge height (mm)**	**pH across media sections**				
				A	**B**	**C**	**D**	**E**
I	25	1.2	6	6	6.5	6.8	7.7–7.9	7.9–8.1
II	25	1.5	6	6.5	6.5	6.5	7.7	7.9
III	**25**	**1.5**	**9**	**5**	**5–5.5**	**6**	**7.5**	**8**
IV	37	1.2	6	6.5	6.5–6.8	7.4	8.1	8.1
V	37	1.5	6	6.5	6.5–7	7.1–7.4	7.9	8.1
VI	**37**	**1.5**	**9**	**5.5–6**	**6.5–7**	**7.5**	**8–8.5**	**8.5**

The results of preliminary studies on wider salinity gradient development are shown in **Fig. 6.4.** Media layers prepared with high divergent salinity values (15 and 25%) yielded wider gradients, but with more pronounced dissipation rates.

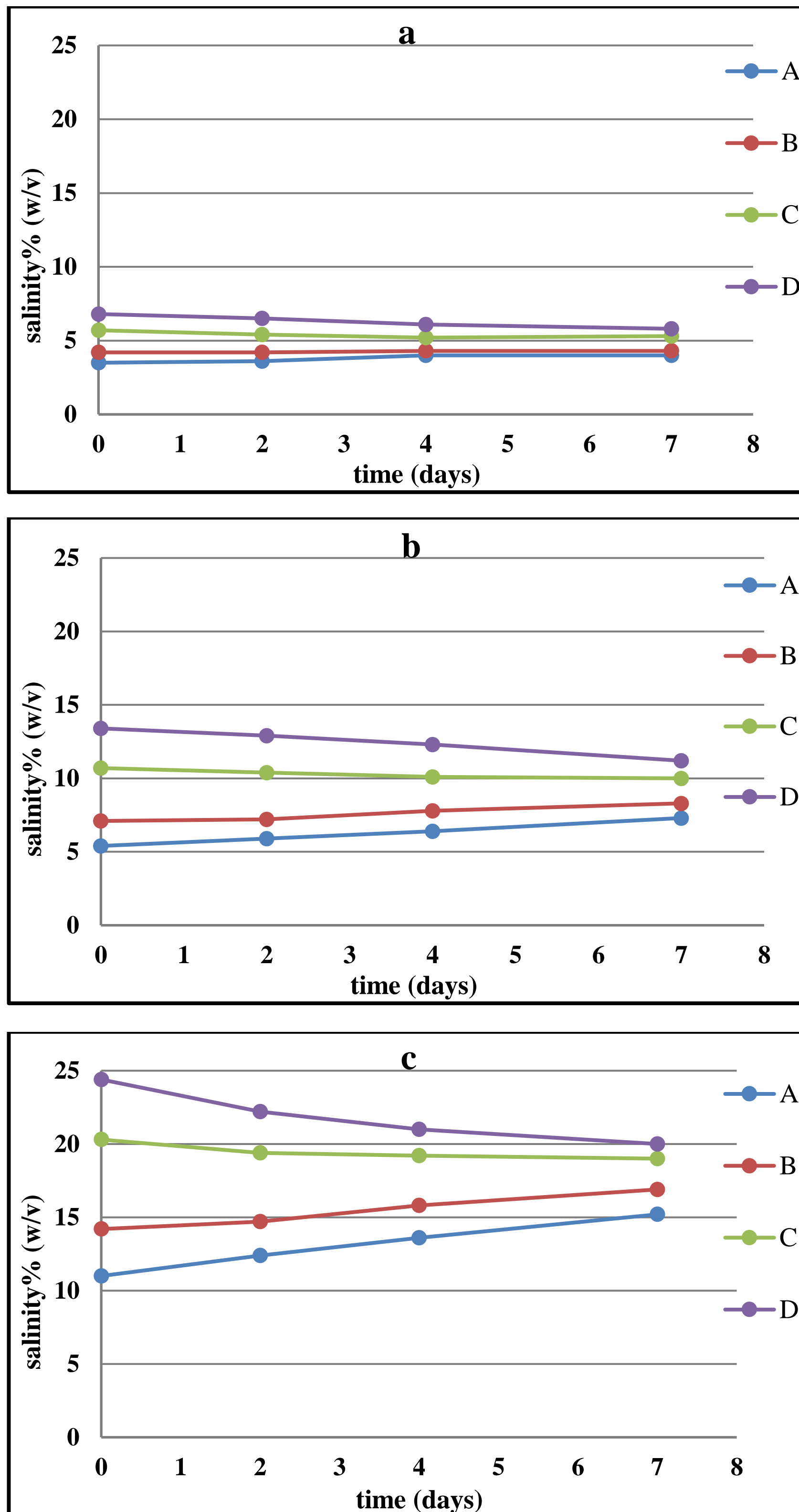

Figure 6.4 Salinity gradient dissipation rate relationship with the salinity divergence of the 1st and 2nd media layer (a = 5% NaCl divergence; b = 15% NaCl divergence; c = 25% NaCl divergence). Agar block retrieved from four sections (A, B, C, D) from each plate to test gradient stability. Media layers with high divergent salinity values resulted in wider gradients, but with more pronounced dissipation rates

6.3.2 Gradient Evolution and Stability

Results of single-gradient plates (**Fig. 6.5** and **6.6**) prepared under optimized conditions for either pH or salinity reproduced salt marsh conditions, confirmed gradient stability for a period of 7–8 days. After 10–11 days of incubation, these gradients were greatly dissipated, so their measurement was discontinued. A mixed gradient was obtained with pH 5.5–8.5 and salinity of ca. 1.8–5.4% (w/v) (**Fig. 6.5** and **6.6**). Measurements for these combined-gradients were equivalent to single-gradient versions of the plates, with little variation in obtained values for pH and salinity and similar results regarding their dissipation.

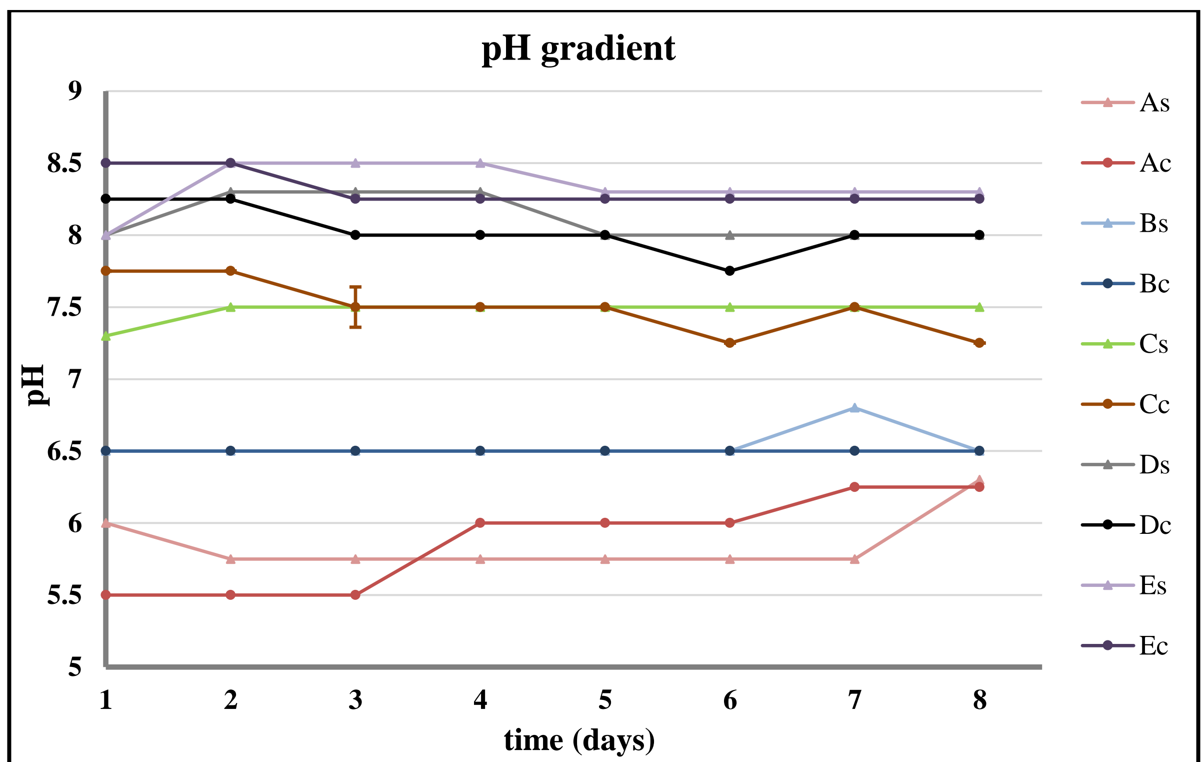

Figure 6.5 Temporal comparison of pH values in single-gradient versus combined-gradient plates mimicking salt marsh pH gradient. Plot of average pH values with error bars calculated using their standard deviation. As, Bs, Cs, Ds, Es- Measurements across the single-gradient plates; Ac, Bc, Cc, Dc, Ec- Measurements across the combined-gradient plates. Required pH gradient covered in both single and combined-gradient plates with minimal variations in values but similar dissipation

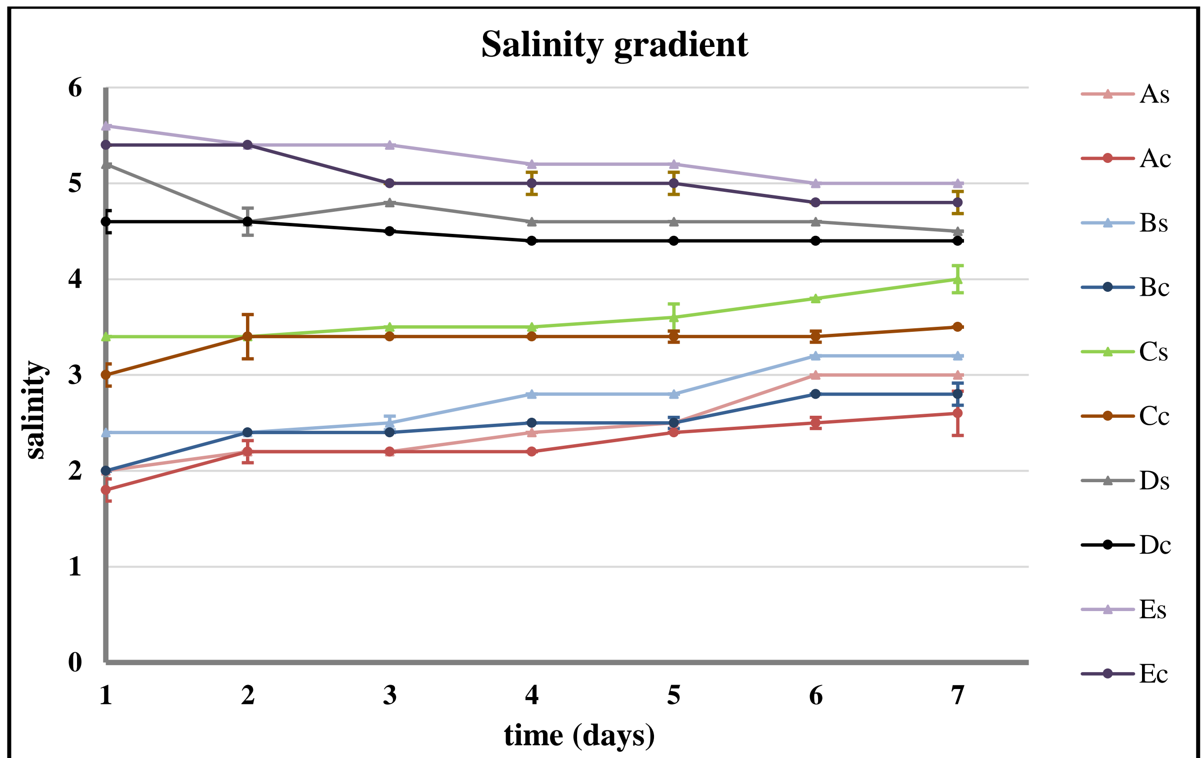

Figure 6.6 Temporal comparison of salinity values in single-gradient versus combined-gradient plates mimicking salt marsh salinity gradient. Plot of average salinity values with error bars calculated using their standard deviation. As, Bs, Cs, Ds, Es- Measurements across the single-gradient plates; Ac, Bc, Cc, Dc, Ec- Measurements across the combined-gradient plates. Required salinity gradient covered in both single and combined-gradient plates with minimal variations in values but similar dissipation

The parallel four plates approach developed in this study allowed the establishment of a total salinity gradient range of 6.5–24.5% (w/v) to mirror the wide range of salinities encountered in brine-seawater interfaces. The resultant salinity ranges for these four plates were (w/v) 6.5–12.5%, 10.5–16%, 15–20% and 19–24.5% (**Fig. 6.7)**.

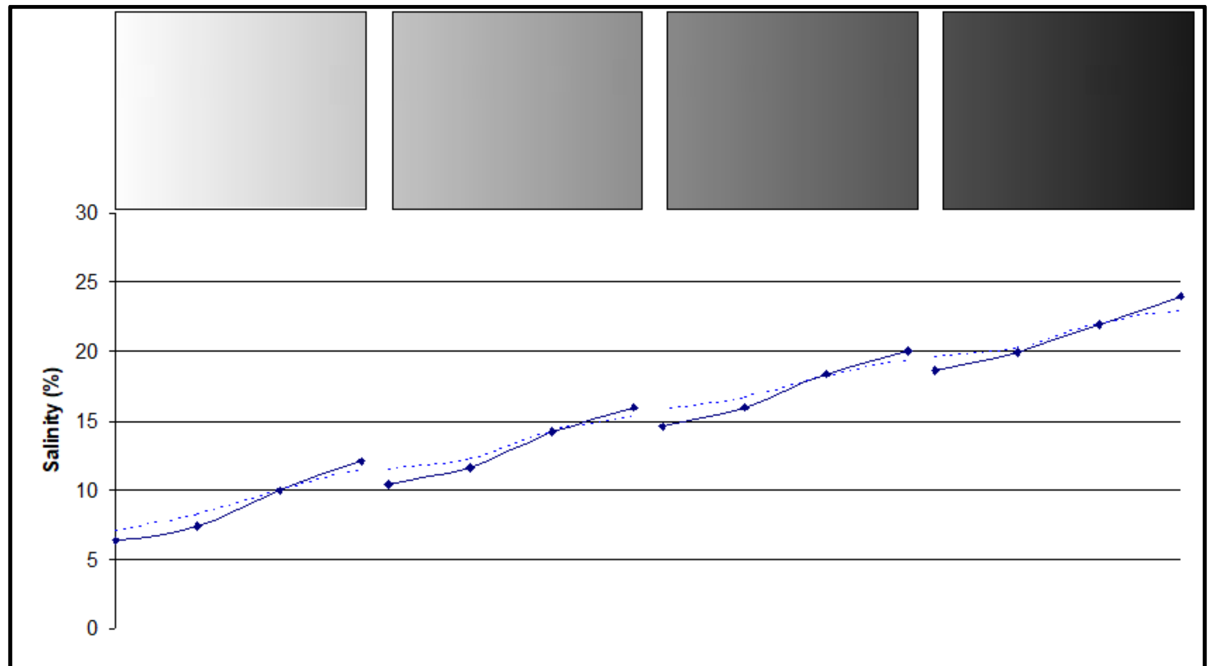

Figure 6.7 Wide salinity gradient developed using parallel four-plate approach. Slightly overlapping salinity values 6.5–24.5% (w/v) were obtained; mimicking brine-sea water interfaces. (◆, initial salinity values; --, salinity values after four days)

6.3.3. Mixed-culture Inoculation of Gradient Plates

The pilot experiment performed to check the applicability of gel-stabilised gradient plates by mixed culture inoculation with *Salinisphaera shabanensis* (white colonies) and *Haloarcula marismortui* (red colonies) showed the successful and clear separation of both these strains by selective growth along a salinity gradient (6.5–24.5% w/v) as observed in **Fig. 6.8**. The white colonies of *Salinisphaera shabanensis* could be observed at the lower to middle salinity end of the gradient, which agrees with its moderately halophilic character (optimal growth at 10% (w/v) NaCl, Antunes *et al.* 2003). While the red colonies of the extremely halophilic archaea *Haloarcula marismortui* occupied the upper salinity range, with some overlap occurring at middle-high salinity (optimal growth at 20–23% NaCl (w/v), Oren *et al.* 1990).

Figure 6.8 Differential growth pattern of extremely halophilic archaea *Haloarcula marismortui* (red colonies) and the moderately halophilic bacteria *Salinisphaera shabanensis* (white colonies) along three of the parallel salinity gradient plates covering NaCl concentration 6–24.5% (w/v). The red colonies of the extremely halophilic archaea *Haloarcula marismortui* occupied the upper salinity range, while the white colonies of *Salinisphaera shabanensis* occupied the middle to lower salinity end of the gradient, with some overlap occurring at mid-high salinity. The successful and clear separation of both these strains by selective growth along a salinity gradient was observed.

6.4 DISCUSSION

As an attempt to partially address the current gap in cultivation techniques the present study developed the gradient diffusion plate approach to extend its application as a new method for cultivation and isolation of microbes from gradient-rich environments. It was specifically adapted to replicate the salinity and/or pH gradients encountered in marine environments. The attempts were also made to keep it robust and low-tech for its direct and wider application in microbial culturing.

6.4.1 Gradient Optimization

During optimization tests, the gradient development using 1.5% (w/v) agar concentration was continued instead of 1.2% (w/v) to minimize the drying of growth media caused due to long incubation period at 25 and 37 °C. However, the gradient that would support the growth of microbes which grow at temperatures less than 25 °C; could be prepared with 1.2% (w/v) or lower agar concentration as the degree of media drying would be less at these low temperatures. Furthermore, gradient evolution at optimised conditions (9 mm media wedge height and 1.5% (w/v) agar), but at temperatures in between 25–30 °C would be required to test, as most of the microbial cultivations of environmental samples are conducted at these temperatures. Satisfyingly, development of gradient with favourable stability at 30 °C could be predicted for it is the intermediate temperature value to that of used in the gradient sets III and VI (**Table 6.2)** which showed optimum results.

The preliminary studies on wider salinity gradient development confirmed the temporal progression of the gradient dissipation process, which results from lateral diffusion. This study's results showed that the use of media layers with highly divergent salinity values (e.g., 15% and 25% (w/v) NaCl) resulted in wider gradients originally, but also in much more pronounced dissipation rates, which would limit their applicability (**Fig. 6.4**). These results compelled us to use layers with less divergent salinities, while increasing the number of plates used to compensate the decrease in salinity ranges obtained (when necessary). In this regard, a multiple parallel plate approach, which consisted in using several plates with slightly overlapping salinity values at their borders, was deemed the most convenient for very wide salinity ranges. This approach offered the best compromise between medium-term gradient stability and number of plates necessary; and allowed to reach a total salinity gradient range of 6.5–24.5% (w/v) using four plates **(Fig. 6.7)**.

6.4.2 Gradient Evolution and Stability

The four parallel plates approach developed in this study (**Fig. 6.7**) mirrored the wide range of salinities encountered in brine-seawater interfaces (4–28% (w/v), Eder *et al.* 2001; Antunes *et al.* 2011; Kaartvedt *et al.* 2016) and confirms the robustness of this method and applicability to higher salinity settings. To our knowledge, the establishment of gel-stabilised plates of such wide-ranged salinity gradients (6.5–24.5% (w/v) has never been previously attempted. This provides an appropriate approach to partly replicate the extremely dynamic saline environment of brine-sea water interface and its physico-chemical gradients (Eder *et al.* 2001; Eder *et al.* 2002; Yakimov *et al.* 2007). Although some growth tests with gradient plates were previously described using salinity as a parameter, only relatively low NaCl concentrations (2–9%) were tested, some of which lack replicability data and their applications restricted exclusively to auxanographic studies (Panagou *et al.* 2005; Thomas and Wimpenny 1993; Wimpenny *et al.* 1986).

Equivalent experiments performed in this study assessing pH gradient plates showed that the effects of divergence in pH between both media layers is much less pronounced than for salinity as the total range for pH was much narrower (5.5–8.5). Therefore, this was found not to be a limiting issue for the pH range used in this study (**Fig. 6.5**).

The gradient optimization experiments performed in this study were vital in obtaining the required gradient range, as well as confirming their dissipation rate and long-term stability. Cultivable microbes from different environments generally take 24–72 h to grow *in-vitro* (Abbas *et al.* 2018; Fidalgo *et al.* 2016; Mata *et al.* 2002; Pandey *et al.* 2019). However, cultivation and isolation of yet-to-cultivate environmental microbes frequently requires extended incubation of several days and they can have specific growth requirements (Davis *et al.* 2005). Many environmental microbial strains including oligotrophic ones, are very slow-growing and need longer incubation periods (Vartoukian *et al.* 2010); that could be one to two weeks or more (Chaudhary *et al.* 2019).

Previous diffusion plate studies have traditionally reported gradient stability for no more than 48-96 h (Liu *et al.* 2011; Thomas *et al.* 1993; Venables *et al.* 1995; Wimpenny *et al.* 1986). Furthermore, there were few reports on the long-term stability of their gradients, which could limit its applicability, especially for slow-

growing microbes (Panagou *et al.* 2005). However, in the present study gradient plates prepared under optimized conditions showed gradient stability for a period of 7-8 days, longer than previously reported studies (Liu *et al.* 2011; Thomas *et al.* 1993; Venables *et al.* 1995; Wimpenny *et al.* 1986). This allows the applicability of gel-stabilised gradient plates for extended incubation period compared to the previous diffusion plates.

Additionally, previous experiments with multiple gradients on a single plate mostly relied on the use of four media layers, poured in different directions which increased the complexity of the technique and narrowed the range of the gradient obtained in each plate (Thomas and Wimpenny 1996a; 1996b; 1993). The easy to produce, low-tech, two layered gradient plate approach of this study is ideal for environments with co-occurring gradients and can be easily adjusted to the unique settings of each site and requirements of different studies.

6.4.3 Mixed-culture Inoculation of Gradient Plates

While testing the concept of gel-stabilised gradient plates for microbial culturing, *Salinisphaera shabanensis* and *Haloarcula* marismortui were selected as examples of halophilic microbes having preference for different salinities and clearly distinct colony pigmentation. Accordingly, the different salinity growth ranges displayed by both strains resulted in clear differences in colony distribution patterns along the wider salinity gradient of 6–24% (w/v). Thus, plate diffusion methods, namely the ingenious wedge plate technique has potential to replicate conditions encountered in gradient-rich environments and for the clear separation of different environmental strains by their selective growth along a gradient. With this pilot experiment, the applicability of gel-stabilised gradient plates in separating different microbial populations is confirmed.

6.4.4 Strengths and Limitations of the Proposed Gel-stabilised Gradient Plate Study

The recent developments of alternative cultivation techniques are commendable, and we acknowledge the efforts of these previous works in providing cultivation conditions closer to their natural counterparts. However, their technical complexity, high costs and inability to maintain the viability of preserved pure isolate have limited their adoption and curtailed their impact. The approach of present study builds upon the strengths of classic cultivation methods by making use of an easy,

low-tech, and low-cost approach which we expect will facilitate its wide-spread dissemination and use.

This study has been conducted as a proof of concept and it requires further evaluation through cultivation of ecological samples and optimization with additional growth substrates. Nevertheless, it confirms the suitability of gradient plate approach for mimicking natural gradients in a replicable manner, and for the separation of different microbial populations. It readily fulfils the environmental microbes' requirements of diverse salinity and pH concentrations that could differentially cultivate them from brine, sea water, salt marsh samples etc. The microbes' growth distribution across the gradient plate according to their favourable growth conditions would also keep diverse microbial colonies distant, resulting into reduction of the possible inter-microbial competition or inhibition. Thus, the application of such gel-stabilised gradient plates with gradient stability of 7–8 days could favour the growth and cultivation chances of some of the uncultured novel microbes which are missed during the traditional cultivation attempts. Furthermore, growth management of fast-growing cultures outcompeting slow growing novel microbes could be achieved by further incorporation or dilution of specific nutrients or substrates into media. This could favour isolation and abundant growth of the desired slow growing microbial species from rest of the microbial population.

Chapter 7

General Discussion

The dynamic nature of salt marshes (Kim *et al.* 2012; Nelson *et al.* 2009; Rand 2001; Leeuw *et al.* 1990), their ability to support vast microbial diversity (Bolhuis *et al.* 2013; Webster *et al.* 2015; Nelson *et al.* 2009), and the need to explore uncultured microbes from these habitats has inspired the present study to investigate salt marshes in the UK. Cultivation- and molecular-based microbial investigations of these environments were the focus of this study. These investigations were conducted on two UK-based salt marshes - RSPB Marshside and Fingringhoe Wick Nature Reserve. The original contributions of the present study to the existing knowledge can be summarised as (a) the exploration of culturable microbial diversity of gradient-rich, geographically and climatically distinct, previously unstudied salt marshes in the UK; (b) insights into the biotechnological potential of the cultivated salt marsh microbes; (c) the identification and full characterisation of two novel bacteria isolated from the RSPB Marshside; (d) the development of a methodological approach that mimics natural gradients and has direct and wider application in microbial culturing.

Salt marsh zones of approx. 45,500 ha surrounding the UK are providing coastal and inland protection and maintaining ecosystems (JNCC 2019, viewed November 2020). However, the ecological and scientific potential of most of these salt marshes, including study sites, have remained unexplored and undocumented. There is also a large pool of uncultured microbes hidden in these environments (Mußmann *et al.* 2005; Gray *et al.* 2014). This gap in knowledge has been partly addressed through the present work by evaluating microbes of UK salt marshes for cultivation, phylogenetic identification, cytology and physiology observations, salt tolerance, and antimicrobial production. To the best of my knowledge, this is the first study that has described the in-depth cultivable microbial diversity of these salt marshes. Though a moderate fraction of the isolated microbes were analysed in this study, the resultant substantial phylogenetic diversity and the discovery of four antimicrobial producing isolates and seven potential novel species have highlighted the ecological and biotechnological importance of UK salt marsh environments.

To further address the novel status of two isolates, MARW_1-7-2^{T} and MARW_1-2-27^{T}, a recommended polyphasic approach comprised of a genus specific set of biochemical and genomic tests was applied (Ramasamy *et al.* 2014; Arahal *et al.* 2007; Chun *et al.* 2018; Stackebrandt and Ebers 2006; Richter and Rosselló-Móra 2009). These test results cumulatively suggested that the isolates MARW_1-7-2^{T} and MARW_1-2-27^{T} represent two novel species, for which the names *Halomonas aestuarensis* and *Pseudoalteromonas belisamaea* were proposed, respectively. Recently, the addition of novel species *Halomonas niordiana* ATF 5.4^{T} to the bacterial taxonomy has been observed (Diéguez *et al.* 2020). It is the second closest relative of MARW_1-7-2^{T} with 16S rRNA gene sequence similarity, DDH and ANI of 97.54%, 27% and 83.5% respectively. These in-silico genome analyses are confirming the novel status of MARW_1-7-2^{T}. Further comparative analysis with *Halomonas niordiana* for physico-chemical tests (e.g. oxidase, catalase, motility, Gram-staining, API® biochemical tests) and chemotaxonomic analysis (such as cellular fatty acid analysis), and the representation of this isolate in a phylogenetic tree would be beneficial for the description of MARW_1-7-2^{T}.

The addition of a good quality genome and its annotation results are the absolute requirement of today's microbial taxonomy world for novel species description (Ramasamy *et al.* 2014; Klimke *et al.* 2011). These analyses are also important to predict the cellular functions, pathogenesis, microbial significance, novel applications etc. (Sunagawa *et al.* 2015; Fraser *et al.* 2000; Quainoo *et al.* 2017; Gerdes *et al.* 2002). A good quality draft genome sequence and its annotation (Aziz *et al.* 2008) obtained in the present study have accomplished these requirements and have predicted the representative features of the novel isolates *Halomonas aestuarensis* and *Pseudoalteromonas belisamaea* (Tatusova *et al.* 2013). Though genome sequencing and annotation services are continually improving, the possibility of missing or vaguely annotated genes (Brenner 1999; Iliopoulos *et al.* 2003) and variations of actual gene expression in microbes' native environments still exist (Brenner 1999; Iliopoulos *et al.* 2003). Hence, after utilising the genome data of *Halomonas aestuarensis* and *Pseudoalteromonas belisamaea* to narrow down to the specific features, it is still necessary to validate these results through lab experiments.

The advanced molecular analysis of environmental samples has shown that a high proportion of microbes are yet uncultured. This dictates the inadequacy of standard

lab cultivation techniques to recreate natural growth environments (Fournier *et al.* 2017; Steen *et al.* 2019; Oren 2004; Schleifer 2004; Harris *et al.* 2004; Rappé and Giovannoni 2003). The resultant absence of pure cultures in the lab severely restricts our understanding of this uncultured microbial diversity. Hence, the present study aimed to address this issue by developing a gel-stabilised gradient plate diffusion technique that could mimic the physico-chemical gradients of natural environments and can be applied as a new technique for the cultivation and isolation of microbes from these ecosystems. Compared to previously reported plate diffusion methods (Panagou *et al.* 2005; Thomas and Wimpenny 1993; Wimpenny *et al.* 1986), the present gel-stabilised gradient plate offers a robust, low-tech, and low-cost tool that has a capacity for the recreation of wider and stable natural gradients.

Recommendations for Future Studies

1. The cultivation and isolation of salt marsh microbes conducted in the present study has analysed the moderate fraction of the isolated microbes. Considering the depths of observed microbial diversity among studied isolates, it would be beneficial to analyse the remaining culture collection for physiological and phylogenetic identification, halotolerance, and extending this identification to archaeal population as well. There could be ample opportunities for recovering significant novel microbes that could further boost the scientific and economic value of the UK salt marshes.

2. Temporal variations in the physical structure of marsh soil, pH, salinity and other physico-chemical factors that affect microbial niches (Marton *et al.* 2015; Sahan and Sahan 2008; Piceno *et al.* 1999), and the strong influence of seasonal patterns on nitrification rates (Dini-Andreote *et al.* 2014) have been described in salt marsh literature. Phylogenetic analysis of the remaining isolates from the present study would also offer an advantage of observing seasonal changes in the microbial diversity at RSPB Marshside. Out of three sampling visits to RSPB Marshside, samples collected in June and October 2017 (called MARW_1 and MARW_2 respectively) were cultured for microbial diversity in the present study. Thorough analysis of isolates from MARW_1 visit was performed in this study. Further comprehensive microbial analysis of cryopreserved samples of

MARW_2 to compare with the microbial data of MARW_1 could reveal any effects of seasonal variations on bacterial diversity at this marsh.

3. Manmade structures are employed worldwide at seaside regions (e.g. sea walls, coastal jetties etc.) for mosquito control, to protect coastal population and assets, to utilize marine resources and as an alternative energy supply (Dugan *et al.* 2011; Hannaford 2006; Rey *et al.* 1992). Coastal marshes have been affected by such human interference, leading to their squeezing and habitat loss. Approximately 50% of salt marsh worldwide has already been lost or degraded (Adam 2002; Barbier *et al.* 2011). Rising sea levels due to global warming and climate variations have worsened the salt marsh situation leading to its further abrasion. To counteract these detrimental effects, the restoration and creation of artificial salt marshes are performed worldwide (Sinicrope *et al.* 1990). The main aim of this manmade revival activity is to recreate lost habitat while maintaining equivalent biological characteristics to their natural counterparts. Studies have been conducted to observe the effects of such artificial recreation on salt marsh physico-chemical properties, vegetation, birds and marine invertebrates (e.g. Spencer *et al.* 2008; Mossman *et al.* 2012; Overton *et al.* 2015; Browne *et al.* 2011), though very little attention has been paid to the effects of restorations on native microbial populations and their interactions. Considering the major role of microbial elemental recycling in natural habitats, it is necessary to check the microbial diversity and physico-chemical parameters among various restored salt marshes. UK salt marshes, including the study sites, have also experienced significant restoration but were never studied for their innate microbial diversity. From an ecological health status point of view, it would be advantageous to study the microbial diversity from such restoring environments and compare them with the equivalent healthy sites to reveal the salubrious status of the restored marshes. e.g. sulphate reducing bacteria can be checked to evaluate restoration effects on salt marshes. Sulphate reduction is the dominating microbial metabolism in salt marshes (Valiela *et al.* 1995). Sulphide concentration also controls the nitrification-denitrification and methanogenesis rates in costal environments (Joye and Hollibaugh 1995; Ortiz *et al.* 2013; Caffrey *et al.* 2010; Kemp *et al.* 1990; Jensen *et al.* 1988). An increased sulphide concentration, with the subsequent development of anoxic and acidic environments, was noted due to

altered tidal flow restrictions (Rev *et al.* 1992) and improper halophytic vegetation growth during coastal restoration activity (Zhou *et al.* 2009). This resulted in detrimental effects on aquatic lives including fish and native vegetation. The above literature data suggest that restored salt marshes, if compared with the healthy reference marsh sites for the microbial composition of sulphate reducing microbes and physico-chemical parameters, may provide important insights on the impact of salt marsh restoration activities on native microbial communities.

4. Furthermore, isolated microbes, especially halotolerant from salt marsh sites, could be screened for plant promoting features such as exopolysaccharide secretion and phosphate solubilisation. Microbially secreted exopolysaccharides in salt marsh sediments are known to impart salt tolerance and boost dinitrogen fixation to halophytes and enhance biomineralization (Upadhyay *et al.* 2011; Bergmann *et al.* 2009; Ruiz-Lozano *et al.* 2000; Braissant *et al.* 2007; Dupraz and Visscher 2005). Phosphate solubilizers release insoluble and fixed forms of phosphorous, which are useful for plant growth (Vessey 2003). However, the establishment and performance of phosphate-solubilizing microorganisms are known to be severely affected by environmental factors, especially under stressful conditions (Mehta *et al.* 2001). Screening the isolates for exopolysaccharide secretion and phosphate solubilisation in high salt stressed environments like salt marshes would add an insight on the plant-microbes' interactions in salt marsh environments, including for those marshes under restoration. Exopolysaccharide secreting and phosphate solubilising halotolerant isolates screened through this study could also act as valuable resources for plant production systems where soils or irrigation water contain high salt concentrations (Ruiz-Lozano *et al.* 2000). The procedures of qualitative screening of isolates for exopolysaccharide secretion and phosphate-solubilization as described by Heulin *et al.* (1987) and Mehta *et al.* (2001) respectively, can be adapted for testing purposes.

5. Marine microbes are the source of many abundant but underexploited natural products (Lam 2006; Lee *et al.* 2011). Microbial studies combined with biotechnological screening can discover such natural bioactive compounds in salt marsh environments that could be novel pharmaceuticals. The present

study has also retrieved four bacterial isolates having anti-microbial activity from salt marsh sites. The therapeutic potential of these isolates can be further confirmed by extracting their antimicrobial compounds, and then testing the structure, novelty, safety, efficacy and yield of those compounds. For downstream processing, this study would also need to target the metabolites responsible for antibiotic production in these isolates. A non-targeted, standard metabolomic approach was indeed conducted in the present study via ultra-high-performance liquid chromatography-tandem mass spectrometry (LC-MS) that detected almost all general metabolites of these microbes (data not shown). Preliminary analysis of this LC-MS data has shown promising features, including the production of prodigiosin by one of the antimicrobials producing isolate MARW_01-02-21. Prodigiosin is a bioactive secondary metabolite and a potential therapeutic drug, and has wider antimicrobial, antimalarial and anticancer properties (Darshan and Manonmani 2015; Montaner and Prez-Toms 2003). These observations feature the importance of salt marsh isolates in therapeutics and have also opened a new research window of further analysis of these compounds for their microbe-specific chemical structure and ecological significance in gradient-rich environments.

6. In the present study, the preliminary inoculation of gel-stabilised gradient plates with salt marsh sea water showed gradual variation in microbial load with increasing salinity and pH (data not shown). It would be beneficial to further evaluate the performance of these plates for inoculation of environmental samples and observe microbes' colony patterns and phylogeny for their novelty. These gradient plates can further accommodate other types of substrates and conditions as per experimental needs. The future integration with the multi-dimensional concepts and experiments of Wimpenny and colleagues (Peters *et al.* 1991; Thomas and Wimpenny 1993; 1996a; 1996b; Wimpenny and Waters 1987) might prove particularly fruitful in replicating higher complexity gradient-rich environments.

Concluding Remarks

The results of the present study thus expand our knowledge of the vast microbial diversity supported by the gradient-rich environments of salt marshes. It enlightens

the ecological and biotechnological significance of the salt marshes, and the opportunities to detect novel microbes in such habitats across the UK. The findings of this study also suggest further implications of studying microbial communities in gradient-rich environments, especially using the methodological expansion of gel-stabilised gradient plates for microbial cultivation and isolation. Furthermore, the prospective research work proposed here suggests gradient-rich natural environments will be a fruitful avenue of exploring their microbial treasure in the future.

Appendix 1 (Lab Material List)

List of chemicals, instruments and microbial cultures used in the present study

Abbreviation: n/a = Not available

Material used	Brand /Supplier	Product Code
2x PCRBIO Taq Mix red	PCRBiosystems	PB10.13-02
Absolute ethanol	Fisher bioreagents	BP2818-500
Agar technical	Oxoid	LP0012
Agarose (Electran DNA grade)	BDH Prolabo	438792U
Amoxycillin/Clavulanic acid (30 µg) discs	Oxoid	CT0223B
Ampicillin (10 µg) discs	Oxoid	CT0003B
Anaerobic indicator strips	BioMérieux SA	96 118
API® 20E kit	BioMérieux SA	20 100
API® 50 CH bacterial identification kit	BioMérieux SA	50 300
API® 50 CHB/E medium	BioMérieux SA	50430
API® ZYM kit	BioMérieux SA	25 200
Bacillus thuringiensis	DSMZ Culture Collection, Germany	DSM2046^{T}
bead beating sample tubes	Fisher Scientific	11681350
Biolog turbidimeter	Biolog Inc.	3587
BiologGEN III MicroPlatesTM	Biolog Inc.	1030
Boric acid	Scientific Laboratory Supplies	CHEB12
Calcium chloride	BDH Chemicals	27587
CAPS buffer solution (pH 9.5–11)	Sigma	C2632
Caps with O-ring for bead beating sample tubes	Fisher Scientific	11621270
CAPSO buffer solution (pH 9–10)	Sigma	C2278
Carbenicillin disodium	Melford	C0109
Cavity glass slides, single cavity 15 mm	Academy	n/a
Cefotaxime (30 µg) discs	Oxoid	CT0166B
Cephalexin monohydrate (assay 95%)	Duchefa Biochemie	C0110.0005
Cetyl trimethylammonium bromide	Fisher Scientific	10203330
Chloramphenicol (30 µg) discs	Oxoid	CT0013B
Ciprofloxacin (5 µg) discs	Oxoid	CT0425B
Crystal violet (0.5% alcoholic solution)	Pro-lab diagnostics	PL.7000/25
Dipotassium phosphate (K2HPO4)	Fisher Scientific	EC 231-834-5
Eppendorf tubes 1.8 ml capacity	Fisherbrand	05-708-129
Erythromycin	Duchefa Biochemie	E0122.0025
Escherichia coli	DSMZ Culture Collection, Germany	DSM 30083^{T}
Ethidium bromide	Sigma-Aldrich	E1510
Forward primer 27f, (5' AGAGTTTGATCCTGGCTCAG)	Sigma	n/a
Gas chromatography	Agilent GC	6890
GasPak glass jar	BioMérieux SA	96 124
GENbox anaerobic generator sachet	BioMérieux SA	96 124

Appendix 1 continued…

Material used	Brand /Supplier	Product Code
GenEluteTM PCR clean-up kit	Sigma-Aldrich	NA1020
GeneRuler DNA Ladder mix	Thermo Scientific	SM0333
Glass slides	Clarity	C362
Glucose	Timstar	GL2858
Glycerol	Benchmark Supplies	GL2878
Haloarcula marismortui	DSMZ culture collection, Germany	DSM 3752^T
Halomonas taeanensis	DSMZ culture collection, Germany	BH539^T
Hand refractometer	Atago, Japan	S-28E
HEPES buffer solution (pH 6–8)	Sigma	83264
Hydrochloric acid	VWR	20255.29
Hydrogen N5.0, (CP Grade H2), compressed gas cylinder	BOConline	290628-L
Hydrogen peroxide, 6% w/v, L.R., purity 5.75%	Timstar Lab Supplies LTD.,	681234
Inoculation Fluid A (IF-A)	Biolog Inc.	72401
Kovac's reagent	Sigma Aldrich	67309
Levofloxacin (5 µg) discs	Oxoid	CT1587B
Lugol's Iodine / Grams Iodine	Pro-lab Diagnostics	PL.7000/25
Magnesium chloride	Sigma Life Science	M8266
Magnesium sulphate	Sigma Life Science	1001690671
Marine broth	BD DifcoTM	2216
MES buffer solution (pH range 4-6)	Sigma	M1317
Methanol HPLC grade	Fischer Chemicals	M/4000/PB15
Micrococcus luteus	National Collection of Type Cultures (NCTC)	NCTC 2665^T
Mineral oil	bioMérieux	70100
Multi-rotator	Grant-bio	PTR-35
Nalidixic acid (≥98%)	Sigma Aldrich	101878117
n-Hexane	Fischer Chemicals	11/0421/PB17
NIT 1 + NIT 2 reagent	BioMérieux	70442
Nitrogen N5.2 (CP Grade N_2), compressed gas cylinder	BOConline	110628-L
Nutrient agar	Oxoid	CM0003
PCR thermal cycler	Bio-Rad	T100
Peptone	Oxoid	LP0037
pH indicator strips (non-bleeding)	MColorpHastTM, Merck,	1.09584.0001
pH meter	Mettler Toledo	n/a
Phenol:Chloroform:Isoamyl alcohol (25:24:1)	Acros Organics	ACRO327111000
Polyethylene glycol 6000 (PEG 6000)	Fisher	p/3677/08
Portable temperature and pH meter	Hanna	HI-9811-5
Potassium bromide	BDH	101954F
Potassium chloride	Sigma Life Science	101418775
Potassium phosphate	Fisher Scientific	P/4800/53
Pseudoalteromonas peptidolytica	DSMZ Culture Collection, Germany	F12-50-A1(T)

Appendix 1 continued…

Material used	**Brand /Supplier**	**Product Code**
Pseudoalteromonas piscicida	JCM Culture Collection, Riken BRC	JCM 20779^T
Pseudoalteromonas. flavipulchra	DSMZ Culture Collection, Germany	NCIMB 2033^T
R2A agar	Oxoid™	CM0906
Reverse primer 1492r, (5' TACGGYTACCTTGTTACGACGAC)	Sigma	n/a
Safranine solution 1%	Timstar	SA5262
Salinisphaera shabanensis	DSMZ Culture Collection, Germany	DSM 14853T
Sea water analysis kits (phosphate, iron, ammonium, nitrite)	Merck	1.14661.0001, 1.14660.0001, 1.14657.0001, 1.14658.0001
Silicon grease	ACC Slicons	S6M494
Sodium bicarbonate	Timstar	S05610
Sodium bromide	BDH Lab Reagent	30116
Sodium chloride	Sigma Aldrich	1002551088
sodium hydroxide (NaOH, Certified ACS)	Fischer Chemicals	S/4920/60
Sodium phosphate dibasic	Sigma-Aldrich	S0876
Sodium phosphate, monobasic, anhydrous,	Sigma	S-0751
Sodium phosphate/ trisodium phosphate	Aldrich	342483
Sodium sulphate	Scichem	S0325
Spectrophotometer	VWR	UV-1600PC
Sterile cotton tipped inoculator swabs	Inoculatorz™	3321
Streptomycin sulphate	Alfa Aesar	J61299
Strontium chloride	Aldrich-ACS	25552-1
Sudan Black B	Sigma Aldrich	101799195
Tertiary butyl methyl ether (HPLC grade)	Fischer Chemicals	M/4496/17
TestOxidase™ reagent	Pro-Lab Diagnostics	PL 390
Tetracycline (30 µg) discs	Oxoid	CT0054B
Tris Borate EDTA buffer	Sigma-Aldrich	T4415
Trisodium citrate	Applichem	A4522
Vancomycin (30 µg) discs	Oxoid	CT0058B
VP 1 + VP 2 reagents	BioMérieux	70422
Water bath	VWR	VWB 12
Wettable surface squared petri dishes (10 cm x 10 cm x 2 cm)	Sarstedt	82.9923.422
Whatman Grade AA discs (6mm dia.)	GE Heakthcare Life Sciences	2017-006
Yeast extract	BD	288620
Zirconia beads (0.1 mm dia.)	Stratech	11079101Z-BSP
Zn powder	SciChem	Z1015
ZYM A reagent	BioMérieux SA	70494
ZYM B reagent	BioMérieux SA	70493

Appendix 2 (Result Table)

Phylogenetic identification of pure bacterial cultures isolated from Fingringhoe Wick and RSPB Marshside salt marshes on EzBioCloud BLAST tool, based on a comparative analysis of their QCed, partial 16S rRNA gene sequences of different sizes
BLAST analysis of an isolate with varying nucleotide length resulted in matching genus level identity with no or moderate variations in their similarity ratio

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud Blast	Similarity %	Phylum	Class	Order	Family
FINS_1-1-11	259	Bacillus mycoides DSM 2048(T)	100	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-1-11	667	Bacillus mycoides DSM 2048(T)	100	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-2-2	294	Pleionea mediterranea MOLA:115(T)	98.63	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Alcanivoracaceae*
FINS_1-2-2	808	Pleionea mediterranea MOLA:115(T)	98.27	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Alcanivoracaceae*
FINS_1-4-5	379	Halomonas niordiana ATF 5.4 (T)	99.74	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*
FINS_1-4-5	805	Halomonas taeanensis BH539(T)	98.38	Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Halomonadaceae*

Appendix 2 Continued…

Isolate ID	Nucleotide length (bp)	Closest match on EzBioCloud Blast	Similarity %	Phylum	Class	Order	Family
FINS_1-3-5	428	Alkalihalobacillus hwajinpoensis SW-72(T)	99.53	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
FINS_1-3-5	934	Alkalihalobacillus hwajinpoensis SW-72(T)	99.79	Firmicutes	Bacilli	Caryophanales	*Bacillaceae*
MARW_1-1-18	563	Pseudidiomarina sediminum c121(T)	99.64	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*
MARW_1-1-18	806	Pseudidiomarina sediminum c121(T)	99.75	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*
MARW_1-2-23	589	Planomicrobium flavidum ISL-41(T)	98.3	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
MARW_1-2-23	682	Planomicrobium flavidum ISL-41(T)	99.12	Firmicutes	Bacilli	Caryophanales	*Caryophanaceae*
MARW_1-8-13	932	Marinobacter flavimaris SW-145(T)	98.82	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*
MARW_1-8-13	1209	Marinobacter maroccanus N4(T)	99.83	Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*

Acknowledgements

At this time of submitting my PhD thesis, I would like to express my wholehearted gratitude to all those people who have supported me in my PhD journey and helped me to accomplish what I planned in the last five years. I will be carrying your guidance and suggestions with me in the future.

Andre and Paul, thank you so much for showing your confidence in me and granting me a PhD/GTA position, and for all the support and guidance you have given me. Thank you, Jayne, for joining the supervisory team and for your fruitful suggestions. The completion of PhD study with the supervision of all three of you has helped me to cross another milestone in my life and learn skills for my future endeavours.

My salt marsh sampling visits would not have been well organised and safe without the guidance of the managers and wardens at RSPB Marshside and Hesketh Out salt marshes. Many thanks to their teams. Countless thanks to Terry McGenity, Raied Abed, and researchers from DSMZ and HZI institute, Germany for their valuable guidance and help in my sample and data analysis. Thanks a lot Michelle, Thom, Kirsty, Rajiv, Andy Devaynes, Lina, Matt, Marta, Marie, Gill, and our lovely administrative team of Vicky, Louise and Claire. Studying and working in a foreign land wouldn't have been so exciting and fruitful without your kind support.

I am always over the moon when I describe how blessed I am to have a very supportive family. My Mom, my sisters Sandhya and Sonali, and my dearest niece Cherry. Thanks for being a source of my confidence, strength and encouragement. Dad you are not amongst us now, but I am sure you would have been very pleased on this occasion. Tons of thanks to my lovely husband Andrew, who has always been there for me with all the help I have needed while completing my studies - assisting me in checking my thesis drafts, cooking delicious meals for me, and all the help that I have asked for. Thanks to my very supportive in-law family too – Chris, Dave, Sarah, Karl, Ethan and Eva. I cannot stop thanking God who brought me here for PhD study and allowed me to meet these wonderful people who are part of my life now. Above all, I want to give glory to God for being the Way-Maker in my path.

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