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The use of traditional and unconventional culturing methods for the discovery of antimicrobial compounds derived from marine microorganisms

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Abbreviations:

Sa	*Staphylococcus aureus*
Ec	*Escherichia coli*
Bs	*Bacillus subtilis*
Pa	*Pseudomonas aeruginosa*
Am	*Alteromonas macleodii*
Pi	*Phaeobacter inhibens*
MH	Mueller-Hinton
FMAP	Fortynnet (diluted) marine agar plates
WC	Winogradsky column
MIC	minimum inhibitory concentration
"O"	organic (as in, $WC1_O$)
"A"	aqueous (as in $WC1_A$)
DMSO	dimethyl sulfoxide
HPLC	High performance liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
MIC	Minimum inhibitory concentration
BGC	biosynthetic gene clusters

Foreword

First and foremost, I need to thank Hans-Matti Blencke for his support, flexible attention and politely pretending that I didn't find something wrong to do every day, and Ida for her expertise and generosity with her time. I'd also like to thank Klara and Ataur, who, although we met only occasionally, always met me with openness and positivity.
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1 Introduction

1.1 Antibiotic resistance as a threat to public health in the present and future

The discovery of antibiotics and their widespread availability revolutionised healthcare in the mid-20th Century. However, bacteria have always evolved to resist new drugs invented to combat them (1). According to *The Review on Antimicrobial Resistance* commissioned by the UK government, AMR could kill 10 million people a year by 2050. In 2019, 4.95 million deaths were associated with AMR, with 1.27 million of those being directly attributed (1, 2). Antimicrobial resistance (AMR) occurs when genetic changes in a bacteria population cause the antibiotics used against them to become less effective. Antibiotics work as a selection pressure, as only the members of microbial communities able to survive and reproduce will do so, carrying their advantageous traits into the next generation (3). In areas of the world where public health surveillance is low and there is little data, it is difficult to estimate the prevalence of resistance and how severe the impact of AMR is. It is therefore also hard to estimate the highest priority pathogens in different locations (2). Comprehensive data collection and analysis on AMR, plus a robust understanding of the background both scientific and in terms of public health, are essential to tackle the AMR crisis efficiently.

The leading resistant pathogens associated with deaths are *Escherichia coli* (Ec), *Staphylococcus aureus* (Sa), *Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Pa). These six pathogens were responsible for ≈26.5% (4) of AMR associated deaths in 2019 (2). Consequently, it is important to tackle these pathogens before more, stronger AMR strains emerge. In this study, antibiotic-sensitive strains of these bacteria were used as preliminary targets to find compounds that work on some of these genera, using marine bioprospecting techniques. Since 1968, only three new antibiotics have entered the market with a novel mechanism of action: Linezolid, Daptomycin and Teixobactin. This presents a problem for tacking AMR, and new drugs that can control the growth of bacteria causing infections are in serious demand (5-7).

1.2 Possible ways to reduce the impact of antimicrobial resistance on public health

The extent of the AMR crisis will be determined by three factors: firstly, the increasing number of resistant phenotypes, which is an evolutionary response to the widespread use of antibiotic drugs and the selection pressure they enact. Secondly, the growing connectedness of populations, which makes containing and stopping the spread of disease very difficult. Thirdly, the extensive and often unnecessary use of antimicrobials, which makes this selection pressure much more widespread and therefore more likely to be overcome by pathogens. Of the three, the growing population and globalization are the hardest to limit. Conversely, better management of antimicrobial use is possible. This is a combined focus of both the fields of bioscience and public health, increasing the diversity of antimicrobial strategies and normalizing antibiotic use as a last resort, rather than a first line treatment (3).

1.2.1 Responsible use of antibiotics

Penicillin was initially introduced as a “silver bullet”, a simple solution to a complicated problem; due to its effectiveness, it became widely and successfully used to treat infections during the Second World War. However, by the 1950s penicillin-resistance was so prevalent that other solutions had to be found to prevent a reversal of progress (8). Other Beta-lactam antibiotics were developed in response to this threat (9), restoring the ability of doctors to cure most bacterial infections, but the first cases of methicillin-resistant *Staphylococcus aureus* (MRSA) were discovered in the 1960s. Resistance to nearly all antibiotics developed has been found since, including vancomycin which was initially developed to treat MRSA and initially thought to be resistant to resistance development (8). As long as the selection pressure exerted by antimicrobials is widespread, it will provoke an equally serious adaptive response from microbial communities (3).

Antimicrobials are often overprescribed because all patients require, to a greater or lesser extent, fast if not urgent care. If a patient is in a critical condition, a cocktail of antibiotics might be applied. To select more specific antibiotics, patient samples would have to undergo tests, and during this time the infection would likely worsen. Until more rapid tests are available, this will likely continue to be the case. The case against overprescription is more reasonably argued for control between general practitioners and non-urgent cases, where the appropriate treatment can be chosen

after the necessary diagnostic tests. Another way that prescription practices may be changed to limit AMR includes normalizing different dosing regimens. Until recently, doctors were encouraged to make prescriptions under the assumption that higher doses for longer periods would reduce the likelihood of infection relapse. However, this has recently been shown not to be the case, and that by limiting courses to the minimum dose and period required to achieve a positive outcome, the selection pressure on microbes will be limited (3).

Outside of the clinic, governments should play an active role in modulating the perceptions of the public and commercial regulation. Considerations must be made to regulate agricultural use of antibiotics. Widespread use in this industry is due to the increased yield and quality of produce that can be achieved. However, this selection pressure when applied to land practices has severe effects on the microbial ecology. There is also commercial gain to be had from advertising antibiotic use to the public, especially in countries where antimicrobials are not strictly regulated or require a prescription. Thirdly, where bacterial vaccinations are available, they can be preemptively used to protect the vaccinated individual from the risk of AMR strains developing in response to future antibiotic use, and unvaccinated individuals from being subjected to exposure (3). In these cases, governments have a responsibility to educate their populations and regulate industry practices (3, 10).

1.3 Development of new antimicrobial drugs

The pipeline for antibiotic development starts with target identification, deciding which physiological process to inhibit (**Figure 1**). This is usually done with the help of genomics and bioinformatics to recognise conserved sequences across the target organisms (11). This is followed by target validation, confirming the functional role of the potential target in the disease phenotype (12). Inhibition/binding assays are then run on libraries of compounds to identify ‘hits’, compounds that bind to and inhibit the activity of the molecular target, for instance, an enzyme or receptor. From here, ‘lead molecules’ are selected from ‘hits’ to find those that have characteristics that make them more promising leads. These are then altered using medicinal chemistry techniques, such as adding functional groups, to produce optimised antimicrobials for preclinical trials (11).

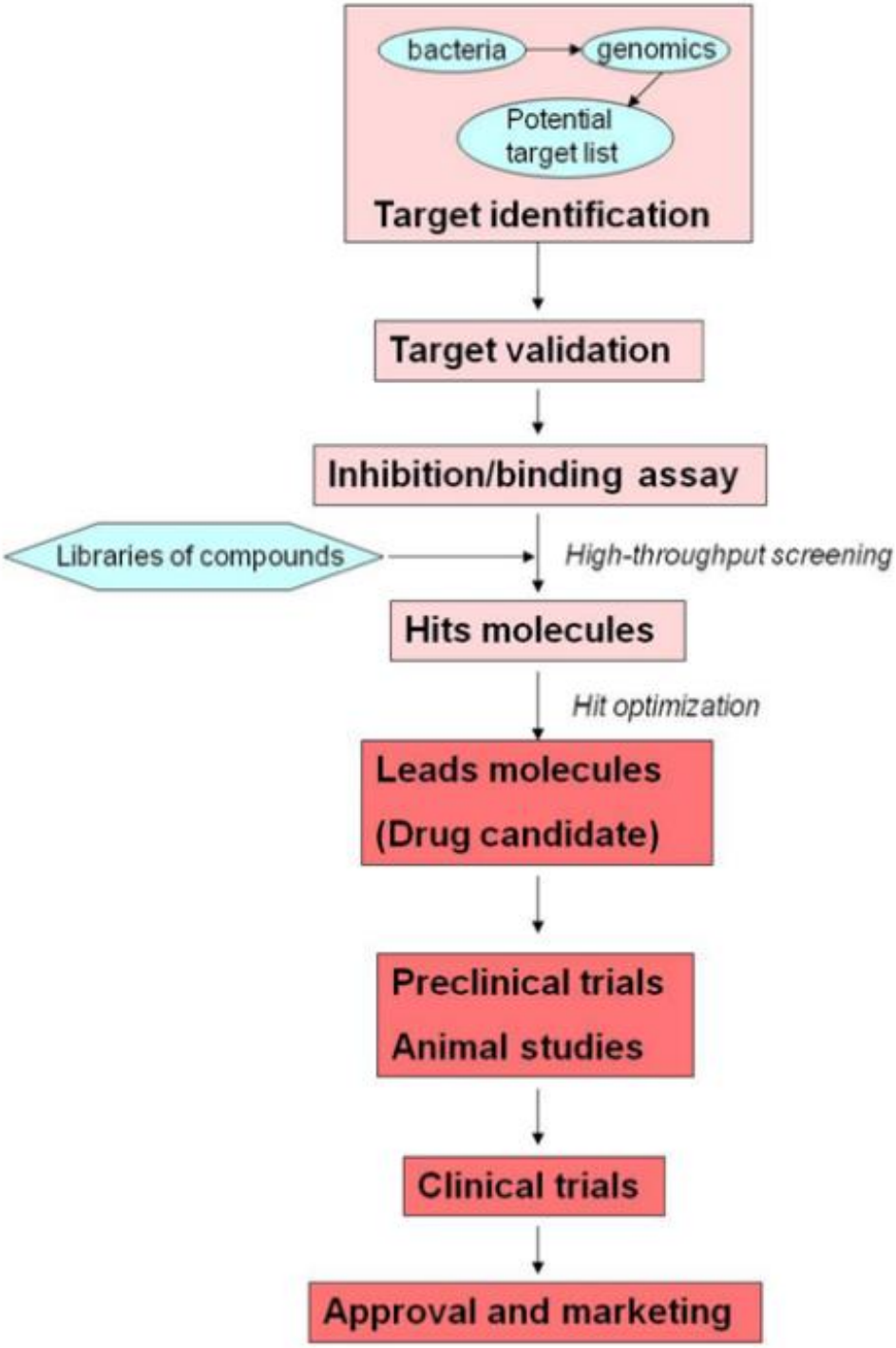


Figure 1.The process of discovery and development of antibiotic drugs. Adapted from Oancea et. al. (11).

The development of new antimicrobial drugs is a challenging pipeline which faces logistical, regulatory, and economic obstacles. The funding for finding new antimicrobial compounds is diminishing from pharmaceutical and biotechnology companies due to a lack of financial incentives. As the need for new antibiotics is a

global necessity, the logic of this is not necessarily intuitive. But as older antibiotics are still effective for treating most infections and the burden of AMR is made worse by frequency of use, new antibiotics are seen as a last resort (13).

Scientifically, challenges start at the beginning of the pipeline: identifying targets. Different antibiotic classes target different cellular processes in bacteria to interfere with physiological functions and eventually lead to cell death. The rate at which AMR develops is also related to the cellular process targeted. For instance, resistance to ansamycin antibiotics such as rifampin that target transcription develops rapidly, whereas resistance to β-lactam antibiotics which target cell wall peptidoglycan biosynthesis develops markedly slowly, particularly those that target transpeptidases (11).

Furthermore, different types of bacteria present their own challenges. Antibiotics geared towards antibiotic-resistant strains, such as MRSA and vancomycin-resistant *E. faecialis*, must look for alternative mechanisms. The Pseudomonas-gap is an observed phenomenon whereby members of this genus show a high resistance to biocides and antibiotics. This is likely to be the result of one or more intrinsic physiological factors and is thought to be attributable to the Gram-negative outer membrane. When a new antibiotic is tested for activity, the high cation content of their membranes means that there is a ‘‘gap’’ seen in efficacy between *Pseudomonas* and other Gram-negative bacteria (14).

Candidate compounds can be entirely synthetic (designed by medicinal chemists), natural products (extracted from existing organisms), or somewhere between the two (taken from nature and modulated for a more efficacious response). Of antimicrobial drugs approved between 1981-2019, just 22.2% were completely synthetic, found by random screening/modification of an existing agent. All other agents were either natural products derived from or inspired by a natural product (15), or semi-synthetic (16). One reason for this is that antimicrobials are found in nature, as many organisms have had to find ways of minimising infection or the effects of infection for survival (17), not least bacteria which compete for space and resources (18).

1.4 Marine bioprospecting as a source of novel antimicrobial drugs

Bioprospecting is the exploration and screening of biological matter including plants, microorganisms and animals that can be further developed, often to produce new products including medicines (19). Although there have been concerns that bioprospecting may pose challenges to conservation, these have largely been unconfirmed for marine bioprospecting for pharmaceuticals, as generally small samples are taken, and strategies involve synthesis and culture as opposed to large scale extraction. As a result, it seems to minimally impact the environment (20). Furthermore, many marine organisms produce secondary metabolites that exhibit unique structural features not exhibited by terrestrial organisms (21). There is great potential for marine bioprospecting in northern Norway, as any organisms that can survive and proliferate in the Arctic Sea have had to adapt to extreme conditions. In the case of microorganisms, many have done so with the help of specialised compounds. Despite the hostile environment, biodiversity is high, and much of it remains unexplored (22).

1.4.1 Organisms of interest

Some marine organisms are known to be particularly good sources of compounds for bioprospecting. The ability of marine bacteria to produce bioactive compounds has long been recognised, the first antimicrobial metabolite isolated in 1966. Bottom dwelling organisms are collected, taxonomized, and microbiological information is identified (22). Sponges are the most prolific marine organisms with respect to their antimicrobial properties, but these compounds are likely not produced by the sponge itself. Rather, they are produced by sponge-associated microbes, producing potent compounds effective against HIV-1, influenza, and *Candida albicans* (23).

In 1929, Alexander Fleming published his discovery of penicillin, stemming from the detection of airborne mould particles which had contaminated culture plates of *Staphylococcus sp*. Fleming noticed that once the mould had grown, the edges caused lysation of the *Staphylococcus* colonies. This was not the first antimicrobial compound discovered from a microbe, nor would it be the last. In 1929, Alexander Fleming published his discovery of penicillin, which was due to the detection of airborne mould particles, which had contaminated culture plates of *Staphylococcus*

sp. Fleming noticed that once the mould had grown, the edges caused lysation of the *Staphylococcus* colonies. This was not the first antimicrobial compound discovered from a microbe, and nor would it be the last (24). Antibiotics in clinical use are almost entirely products of microorganisms or derived from their products, such as their metabolites. They are especially good targets for bioprospecting because as the evolution of resistance mechanisms has progressed over billions of years, so has the evolution of these antimicrobial compounds. Bioprospecting for new compounds is labour-intensive because often compounds that are found are not novel or are found at low yields that are difficult to work with. On the other hand, the number of bacteria species' that are available to work with are immense. It has been argued that biodiscovery projects should focus on chemical novelty and bioactivity, using the most appropriate scientific methods, firstly by approaching under-explored environments and focusing on the most promising candidates (25). However, to investigate an isolate, it must first be cultured.

1.4.2 The great plate count anomaly

Many life-sustaining environments on earth such as marine sediment, seawater and soil are home to diverse populations of microbes that have never been cultured (26). These populations have never been characterised by their observed physiology, rather, their characteristics only inferred by their genomics and environmental characteristics (27). The drive to cultivate pure cultures fell out of favour for a time because the use of metagenomics allowed the diversity of cultures to be identified without the need for pure cultures. However, interest in culturing methods has increased recently to observe the role each species plays in its environment, such as the nutrients it consumes, and the bioactive metabolites it produces (28).
Unfortunately, there is a significant challenge to this, which is the "great plate count anomaly", a phrase coined by Staley and Konopka in 1985 to describe the difference between the cell count in an environmental sample under microscopy, and much lower number of viable colonies that are able to grow on agar (29). In fact, it is estimated that only 1% of marine bacteria is cultivatable in artificial media (30). According to Mu et. al. (2021), there are three main reasons with which we can categorise 'unculturable' bacteria, and each group requires different culturing techniques. These include a) dominant active bacteria, which require a better simulation of their environment, b) rare active bacteria, which require better or

different nutrient enrichment and may respond well to culturomics techniques, and c) dormant bacteria, which are alive but not proliferating. As bacteria can only be considered viable if they are growing, they require conditions that will provoke their resuscitation (31).

1.4.3 Traditional bioactivity-based screening of easy to grow isolates

The aforementioned discovery of penicillin ushered in a ‘golden era’ of antibiotic discovery, during which half of the antibiotics used today were discovered. This was partly due the “Waksman platform” which was an early screening platform developed to capitalize on the opportunity presented by Fleming (24) used to screen soil samples for antibacterial agents from *Streptomyces*. The method was similar of that of how penicillin was found, but the application of this serendipitous discovery to a systematic screening paved the way for the production of the major classes of antibiotics to be discovered and classified over the next 20 years (32).

The screening of bioactive compounds requires a sequence of assays to assess the potential of molecules, based on their chemical properties. Two approaches can be applied: a classical pharmacological approach, or target based-drug discovery. The classical approach screens chemical libraries or natural products against a disease model or bacterial cells, to identify compounds that have the desired therapeutic effect target based-drug discovery starts with a disease-associated protein target and chemical libraries are screened to find candidates that bind with high affinity. This method became widely used in the 1990s when genomics technologies were also popularized (33).

Traditionally in natural product research, compounds are extracted from a plant or organism, and the extract is then partitioned; both are achieved through the use of solvents (34). If the screening is for antibiotics, the extracts are then tested in an antibacterial assay such as an agar diffusion method (35). The extract can then be run through a chemical analysis, such as a MALDI-TOF or LC-MS, to give a sense of the compounds present. The extract can be fractionated by chromatographic separation, and the fractions tested again for inhibitory effect and chemical content to elucidate the compounds responsible for bioactivity, and the minimum dose needed for the required response (34, 35).

For marine bacterial isolates that can be cultivated by traditional methods, samples must be taken from habitats such as sea water, sediment, or soil. They then must be grown in marine media that mimics seawater and contains relevant nutrients. For this, many types of marine media are available. The bacteria then must be isolated by growing colonies on marine agar plates. The isolated bacteria can then be cultivated through the use of marine liquid media, before screening for antimicrobial activity (36).

1.4.4 Accessing the antimicrobial potential of difficult to grow microorganisms

With the apparent limited potential of synthetic compound libraries, it is estimated that, at the current rate that antibiotics are being discovered, 10^7 new bacterial isolates will have to be screened before the next class of antibiotics is described. To increase the rate that species are isolated, microbiologists will need to gain access to the "uncultured majority" (37). Although it seems clear that this is because natural environmental conditions are not being adequately reproduced, it is difficult to solve as it is unclear what aspects are missing. Nutrients, pH, osmotic pressure and temperature can all be considered but together present an infinite number of possible conditions, making optimal conditions hard to investigate. Some efforts for addressing this issue include optimised media, simulated environments, host associated environments and co-culture (37).

1.5 Growing the difficult to grow

The issue with cultivating isolates is that, by definition, the bacteria's normal conditions for growth cannot be replicated, as organisms rarely grow in complete isolation. Bacteria interact with other organisms and are expected to have a mutualistic, commensalistic, or parasitic relationship with other populations (38). As antimicrobial compounds are produced by bacteria to compete with other, dissimilar populations, it would stand to reason that they may only be prompted to produce certain compounds in the presence of these other bacteria to carve out their niche (31). One example is the species *Streptomyces tenjimariensis*, whose production of istamycin, an aminoglycoside, is provoked when stimulated by co-culture with various marine bacteria (39).

1.5.1 Growth in communities: Winogradsky columns as sources for bioactivity

The second experiment also addressed the necessity for more interactive, naturalistic cultivation conditions, aiming to test the antimicrobial activity of biofilms from Winogradsky columns. Winogradsky columns are vertical miniature ecosystems that consist of a clear cylinder of sediment with a carbon source such as tissue at the bottom and are incubated with light. It is possible to observe changes in the species composition and the abundance of microbes over time as ecological gradients within the column develop, creating diverse niches that allow enrichment of certain populations (40). These changes are known as community succession (41, 42) and involve an evolving natural selection due to the production of nutrients by one set of bacteria that are used by another population. Aerobic bacteria form near the source of air and autotrophic photosynthetic bacteria form at the edges in response to light, whereas heterotrophic bacteria form near the carbon source (41). The benefit of this method is the provision of enrichment, especially for anaerobic photosynthetic bacteria (43). As anaerobic bacteria are largely found in sediment, as opposed to oligotrophic bacteria which grows in seawater, which is well oxygenated (44). this is a good model for demonstrating marine bacterial growth (45). Although Winogradsky columns have been around since the 1880s (46), little if anything has been published about the antimicrobial activity produced by microbial communities enriched in the Winogradsky columns. It was unclear before this experiment if this was because there was nothing to report, or because these tests had not been undertaken, perhaps because the advent of bioprospecting came much later than the invention of Winogradsky columns.

1.5.2 Typical composition of biofilms in Winogradsky columns

There are benefits to obtaining study populations from a closed system, such as the formation of light-dependent surface biofilms (40). Biofilms are surface-associated multicellular communities of bacteria (47). Their formation is initiated by bacteria forming a monolayer attachment on surfaces in natural, aqueous environments. Surfaces include plants and animals, or submerged objects such as rocks, wood, metal, or plastic. Over time, the biofilms become more complex, both in the formation of layers of organisms (41) and extracellular matrices produced by the constituent cells, which contribute significant diversity and organisation to the community therein

(47). The matrix provides a dynamic environment for bacterial life, the building of which is a defined, stepwise process, that changes over time. It retains old constituents of dead bacteria, including DNA, and extracellular enzymes that process and sequester nutrients, contributing to species diversity. Extracellular polymeric substances (EPS) immobilize bacteria, keeping them in proximity to one another and allowing interactions to occur such as horizontal gene transfer (HGT), quorum sensing and the formation of a synergistic microbial community. EPS is made up of a wide variety of components, which as a result is a slow process to break down, and requires a variety of enzymes (48).

Biofilm formation is a concern in methodological research for two reasons. Firstly, they can cause problems both clinically and in industry. Biofilms form a protective layer around the bacteria associated with infections and biofouling, and the inhibition of establishment of biofilm formation is a target for research for the AntifoMar project here at UiT. Secondly, biofilms provide a possible target for bioprospecting, as they contain a rich source of bacteria.

Biofilms would be harder to obtain and preserve from samples taken directly from the sediment, but they have the benefit of being reliably enriched by a few types of bacteria (40). As well as being a microenvironment which allows interactions between taxa with interacting metabolisms, it also leads to the enrichment of taxa that were initially rare in the sediment (40).

Obligate anaerobic bacteria are a challenge to culture in the laboratory, as equipment is required to provide an oxygen-free environment. Since Hungate first developed the first system for culturing highly oxygen sensitive microorganisms, such as sulfate reducing bacteria and archaea, a range of systems such as anaerobic containers, chambers, and GasPak systems have become available that are less complicated and do not require the level of training as his system of replacing atmospheric oxygen. Anaerobic cultures also require complex media, containing supplements, carbon sources, metals, and growth factors. Selective culture conditions may require the use of antibiotics (29).

1.5.3 Enrichment and isolation of typical bacteria from biofilms in selective media: sulfate reducing bacteria

One group that require sensitive conditions are sulfate reducing bacteria (SRB). These bacteria were long considered to be obligate anaerobes but are now known to possess enzymes that protect against oxygen toxicity and sometimes prefer to live at the anoxic-oxygenic interface rather than under completely anaerobic conditions (49). SRB are a group unified by the chemical reaction (50):

$$4\ AH_2 + SO_4^{2-} + H^+ \rightarrow 4\ A + HS^- + 4\ H_2O$$

They are often found in anoxygenic sediments and the water of lakes and oceans, and use sulfates, sulfites, thiosulfates and elemental sulfur instead of oxygen in their respiratory chain, playing a recycling role in nature (51). They have unique enzyme systems and metabolic pathways from not only common aerobic organisms, but also from other anaerobic bacteria. Facultative anaerobes can survive in both oxygenic and anoxygenic conditions and are sometimes also capable of reducing sulfate for energy. However, they use a different pathway from SRB, the 'assimilatory pathway', which is for incorporation of sulfur into biological compounds (50). The 'dissimilatory pathway' is bioenergetic and used only by SRB; it is conserved only in species of sulfate reducing bacteria. This requires only three enzymes that are structurally much simpler than those used in the assimilatory pathway, but the various species of SRB use variations of the enzyme sulfite reductase. This is because facultative anaerobic bacteria must manufacture enzymes for varying levels of oxygen, so the third step of the assimilatory pathway is to use one of many types of sulfite reductase depending on the current environmental oxygen level. As such, they contain far more genetic material. Furthermore, the assimilatory pathway and dissimilatory pathway result in different end products, the dissimilatory pathway ending with hydrogen sufide (52). Accordingly, SRB theoretically should produce structurally different respiratory metabolites. However, their potential to produce drug leads, their ecological role, and their bioactivity is severely limited in part by the difficulties in isolating and cultivating them (53).

One factor to consider when choosing growth conditions for SRB is the degree to which they are aerotolerant. Rather than being obligate anaerobes, as previously thought, it has been revealed that many species of SRB contain members (strains)

that are capable of surviving in high oxygen environments but behave differently. Firstly, they form aggregates, resulting in a higher tolerance to oxygen exposure. Secondly, it was found that the species *Desulfovibrio vulgaris* upregulates a set of 19 proteins and downregulates another set of 35 proteins under aerobic conditions, resulting in a downregulation of cell division, nucleic acid and protein biosynthesis, and, interestingly, detoxification. The downregulation of toxification enzymes specifies the toxicity of oxygen for SRB cells. Peroxidases are upregulated, which help to defend the SRB bacteria against oxidative stress. It is also remarkable that SRB can move towards oxygen to perform reduction, despite the deleterious effects of oxygen on SRB. This appears to serve an ecological role in transitioning the habitat from an oxic to anoxic environment, protecting themselves and other anaerobic species and creating the right level of redox potential for their growth (54). To optimise the methods for SRB growth and feasibility, it was accordingly noted that SRB can survive some oxygen exposure (55).

SRB are often found to closely interact with other species in biofilms, including *Clostridium*, *Bacteroides*, *Escherichia* and *Pseudomonas*, and phototropic green sulfur bacteria (56). This is of interest in the context of optimising isolation experiments because SRB are difficult to separate from their satellites. A dilution culture is one way to separate SRB from other bacteria; it was a technique first used by environmental microbiologists to improve the number of species that could be cultured from environmental samples and to detect minority populations. It involves the successive dilutions of a sample in media until no bacterial growth is seen (29). Another approach is the use of selective media. Kováč et. al. published a modified version of the classic Postgate medium for growing intestinal SRB, based on their biochemical and physiological properties, and the conditions of the large intestine where they are found. Modifications to the Postgate media include increased sulfate, decreased lactic acid, and the addition of compounds such as Na_2SO_3 to remove satellite organisms (52).

1.6 Purpose, Aims and Hypothesis

The main aim is to evaluate the content of marine biofilms and different marine bacteria for antimicrobial potential.

Hypothesis: antimicrobial compounds can be produced in the presence of other bacterial species; in the context of Winogradsky columns that simulate a naturalistic environment, and in the proximity to other species in the case of marine bacterial isolates.

For this main aim and hypothesis, the following subgoals are defined:

1. Document any antimicrobial production by marine bacteria from different marine sources, using a co-culture approach.
2. Document the bacterial content of biofilm in Winogradsky columns (WC) from the seashore, using different techniques.
3. Evaluate the antibacterial production in the bacterial content from Winogradsky biofilms by performing diffusing assays, MIC assays in combinations with chromatographic methods.

2 Materials and methods

2.1 Biological material

The test material that was used in the study is from two sources. The content for the Winogradsky columns were collected in spring 2022 and are listed in Table 1**.**

Table 1. Source of bacteria from the shore near Tromsø airport.

Purpose	Origin of sediment	Date collected	WC experiment /duration (months)
Winogradsky column bacteria	The shore from the intertidal zone near Tromsø airport	21.01.2023 (9:30 am)	WC-1 /10
		31.10.2022 (11:50 am)	WC-2 /12 WC-3 /3 WC-111,112 /5
Sulfate reducing bacteria	The shore from the intertidal zone near Tromsø airport	01.11.2022 (12:15 pm) 16.01.2023 (1:30 pm)	

Winogradsky column bacteria – cultivated in natural and artificial sunlight for 10, 12, 3 months. 10 ng high molecular weight genomic DNA was extracted from these bacteria for meta barcode 16S sequencing.

The cruise bacteria collected from different marine material on the marine bioprospecting cruise on RV Kronprins Haakon, in August 2020, are listed in **Table 2**. These marine bacteria had been subjected to 16S analysis to suggest the genus and possible genes coding for BGCs. All bacteria were received in the form of frozen glycerol stocks and stored at -80˚C.

LC-MS Spectra of biological material from the **DeepSeaQuence project** was used as a reference material (project number 315427), cited in **Figure 17.**

Table 2. List of bacterial test strains from 2020 research cruise. The genera were confirmed by metagenome sequencing.

Sl. No.	Strain ID	Origin of strains	Genus (Taxonomy 16S)
1	MBP3358	*Porifera ind.*	*Shewanella*
2	MBP3359	*Porifera ind.*	*Shewanella*
3	MBP3373	*Synoicum turgens*	*Shewanella*
4	MBP3376	*Synoicum turgens*	*Aliivibrio*
5	MBP3377	*Synoicum turgens*	*Shewanella*
6	MBP3379	*Synoicum turgens*	*Psychromonas*
7	MBP3380	*Synoicum pulmonaria*	*Shewanella*
8	MBP3381	*Synoicum pulmonaria*	*Shewanella*
9	MBP3383	*Synoicum pulmonaria*	*Shewanella*
10	MBP3384	*Synoicum pulmonaria*	*Sinobacterium*
11	MBP3384 #4	*Synoicum pulmonaria*	*Pseudoalteromonas*
12	MBP3402	*Porifera ind.*	*Pseudoalteromonas*
13	MBP3403	*Porifera ind.*	*Shewanella*
14	MBP3404	*Porifera ind.*	*Thalassotalea*
15	MBP3405	*Porifera ind.*	*Shewanella*
16	MBP3406	*Porifera ind.*	*Shewanella*
17	MBP3626	Sediment from 5450m	*Alkalihalobacillus*
18	MBP3631	Sediment from 5450m	*Paenisporosarcina*
19	MBP3635a	Sediment from 5450m	*Moritella*
20	MBP3636	Sediment from 5450m	*Jeotgalibacillus*
21	MBP3637	Sediment from 5450m	*Jeotgalibacillus*
22	MBP3638	Sediment from 5450m	*Sporosarcina*
23	MBP3639	Sediment from 5450m	*Jeotgalibacillus*
24	MBP3640	Sediment from 5450m	*Flavobacterium*
25	MBP3641	Sediment from 5450m	*Jeotgalibacillus*
26	MBP3642	Sediment from 5450m	*Peribacillus*
27	MBP3643	Sediment from 5450m	*Alkalihalobacillus*
28	MBP3644	Sediment from 5450m	*Alkalihalobacillus*
29	MBP3645	Sediment from 5450m	*Jeotgalibacillus*
30	MBP3646	Sediment from 5450m	*Bacillus*
31	MBP3647	Sediment from 5450m	*Paenibacillus*
32	MBP3648	Sediment from 5450m	*Paenisporosarcina*

2.2 Bacterial reference strains

The bacterial refence strains (targets) that were used for testing antibacterial activity are listed in **Table 3**. The bacteria were stored as glycerol stocks at -80˚C until use.

Table 3. List of bacterial reference strains.

Bacterial Species	Strain ID	Abbreviation
Stapholococcus aureus	ATCC 9144	Sa
Pseudomonas aeruginosa	ATCC 27853	Pa
Escherichia coli	ATCC 25922	Ec
Bacillus subtilis	1S34	Bs
Alteromonas macleodii	DSM 100460/LMG 2843	Am
Phaeobacter inhibens	DSM 24588 (2.10)	Pi

2.3 Chemicals and kits

The chemicals/kits that were used in this project are listed in Table 4.

Table 4. Overview of kits used in the project.

Chemical/Kit	Producer	Product number
Soil DNA extraction kit	Qiagen DNeasy® Powersoil® Pro Kit (50)	47014 lot 169012669
16S Barcoding Kit 1-24	Oxford Nanopore Technologies	SQK-16S024
Flow Cell Priming Kit	Oxford Nanopore Technologies	EXP-FLP002
Flongle Flow Cell (MinION adapter)	Oxford Nanopore Technologies	FLO-FLG001 Flow cell–ID - ALD070

The platform bomb.bio was used for DNA extraction and PCR purification. The following reagents were used, with specified amendments. For DNA extraction, Bomb.bio protocol *#7.1 BOMB gDNA extraction using GITC lysis* was used, reagents found in ***Table 5*.** For PCR clean-up, protocol #4.2 BOMB clean-up and size exclusion using carboxyl beads was used. Reagents found in ***Table 6*** *(57).*

Table 5. The reagents needed for DNA extraction, in accordance with Bomb.bio protocol #7.1.

Bomb.bio DNA extraction protocol reagents	
Sera-Mag™ Carboxylate-Modified Magnetic Beads & SpeedBeads (cytiva)	20 µl per sample, 2 ml for a 96-well plate (doubled concentration of beads)
TE buffer, pH 8.0: 10 mM Tris 1 mM EDTA 3% RNase A	140 µl per sample, 14 ml per 96-well plate
Lysis buffer pH 7.5 Reagent concentrations: GITC 4 M Tris HCl pH 7.5 mM Sarkosyl 2% EDTA 20 mM No antifoam was used	240 µl per sample, 24 ml per 96-well plate for 50 ml: 23.64 g 2.5 ml 1 M stock 1 g 2 ml of 0.5 M stock
Isopropanol	720 µl per sample, 72 ml for a 96-well plate
80% ethanol	600 µl per sample, 60 ml for a 96-well plate
Elution buffer pH 8.5: 5 mM Tris-HCl	70 µl per sample, 7 ml per 96-well plate

Table 6. The reagents for PCR purification in accordance with bomb.bio protocol #4.2.

Bomb.bio DNA PCR purification protocol reagents	
Binding buffer Reagent concentrations: NaCl 2.5 M Tris HCl pH 8.0 mM PEG 6000 EDTA 1 mM Tween 20 0.05% Carboxyl-coated paramagnetic beads 2%, washed	100 µl per 50 µl sample for 50 ml: 25 ml of 5 M stock 0.5 ml of 1 M stock 10 g 0.1 ml of 0.5 M stock 0.25 ml of 10% stock 2 ml of stock
80% ethanol	2 × 200 µl per sample, 40 ml for a 96-well plate
Elution buffer pH 8.5: 5 mM Tris-HCl	40 µl per sample, 4 ml per 96-well plate

2.4 Media

Composition for liquid and solid media, and the procedure to make them are listed in **Table 7**.

Table 7. Overview of media used in the project and their compositions.

Type of Media	Reagents, masses	Procedure
FMAP liquid media	reagents for 1L: Trypton 5g marine broth 15g MillQ water 700ml sea water 300ml	Reagents are mixed with 500ml milliQ water, and heated and mixed until completely dissolved. Both solutions are then autoclaved and added together.
FMAP solid media (0.7% agar)	reagents for 1L: Trypton 5g marine broth 15g MillQ water 700ml sea water 300ml Agar 7g	Reagents are mixed with 500ml milliQ water, and heated and mixed until completely dissolved. Agar media is made separately with the other 500ml water. Both solutions are then autoclaved and added together. Liquified agar-containing media can be plated. 30 mL media is used for each co-culture plate.
Modified postgate liquid media	reagents for 1L: Na_2SO_4 0.5g KH_2PO_4 0.3g K_2HPO_4 0.5g $(NH_4)_2SO_4$ 0.2g NH_4Cl 1g $CaCl_2 \cdot 2H_2O$ 0.04g $MgSO_4$ $7H_2O$ 0.1g lactate syrup 60% 3.333g yeast extract 1g $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ 0.007g $C_6H_5O_7Na_3 \cdot 2H_2O$ 0.3g ascorbic acid (20% solution) 500 ul MillQ water 1L	Reagents aside from ascorbic acid are mixed with 500ml milliQ water, and heated and mixed until completely dissolved. Both solutions are then autoclaved and added together. Ascorbic acid is added through a syringe filter once cooled.

Modified postgate solid media (1.5% agar)	reagents for 1L: Na_2SO_4 0.5g KH_2PO_4 0.3g K_2HPO_4 0.5g $(NH_4)_2SO_4$ 0.2g NH_4Cl 1g $CaCl_2 \cdot 2H_2O$ 0.04g $MgSO_4$ $7H_2O$ 0.1g lactate syrup 60% 3.333g yeast extract 1g $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ 0.007g $C_6H_5O_7Na_3 \cdot 2H_2O$ 0.3g ascorbic acid (20% solution) 500 ul MillQ water 1L Agar 7.5g $NaSO_3$ 7.5g	Reagents aside from ascorbic acid are mixed with 500ml milliQ water, and heated and mixed until completely dissolved. Agar media is made separately with the other 500ml water. Both solutions are then autoclaved and added together. Ascorbic acid is added through a syringe filter once cooled. Liquid agar media can be plated and left for 2 days to remove condensation.
2.5% Mueller-Hinton liquid media (broth)	Reagents for 200 ml: 5g MH broth 200 ml milliQ water	Reagents are mixed with a magnetic stirrer and autoclaved.
2.5% Mueller-Hinton solid media (1.5% agar)	Reagents for 200 ml: MH broth 5g MilliQ water 200 ml agar 3g	Reagents are mixed with a magnetic stirrer and autoclaved before plating.

2.5 Project workflow

The project consisted of three preliminary investigations (**Figure 2**). The first was a co-culture experiment testing marine bacteria collected from a research cruise in 2020 that were cultivatable by traditional methods. The second and third were cultivation experiments using sediment samples. For the second experiment, Winogradsky columns were made to cultivate anaerobic biofilms, the extracts of which were then examined for antimicrobial activity. The third experiment attempted to isolate and cultivate sulfate-reducing bacteria by dilution and through the use of selective, modified media.

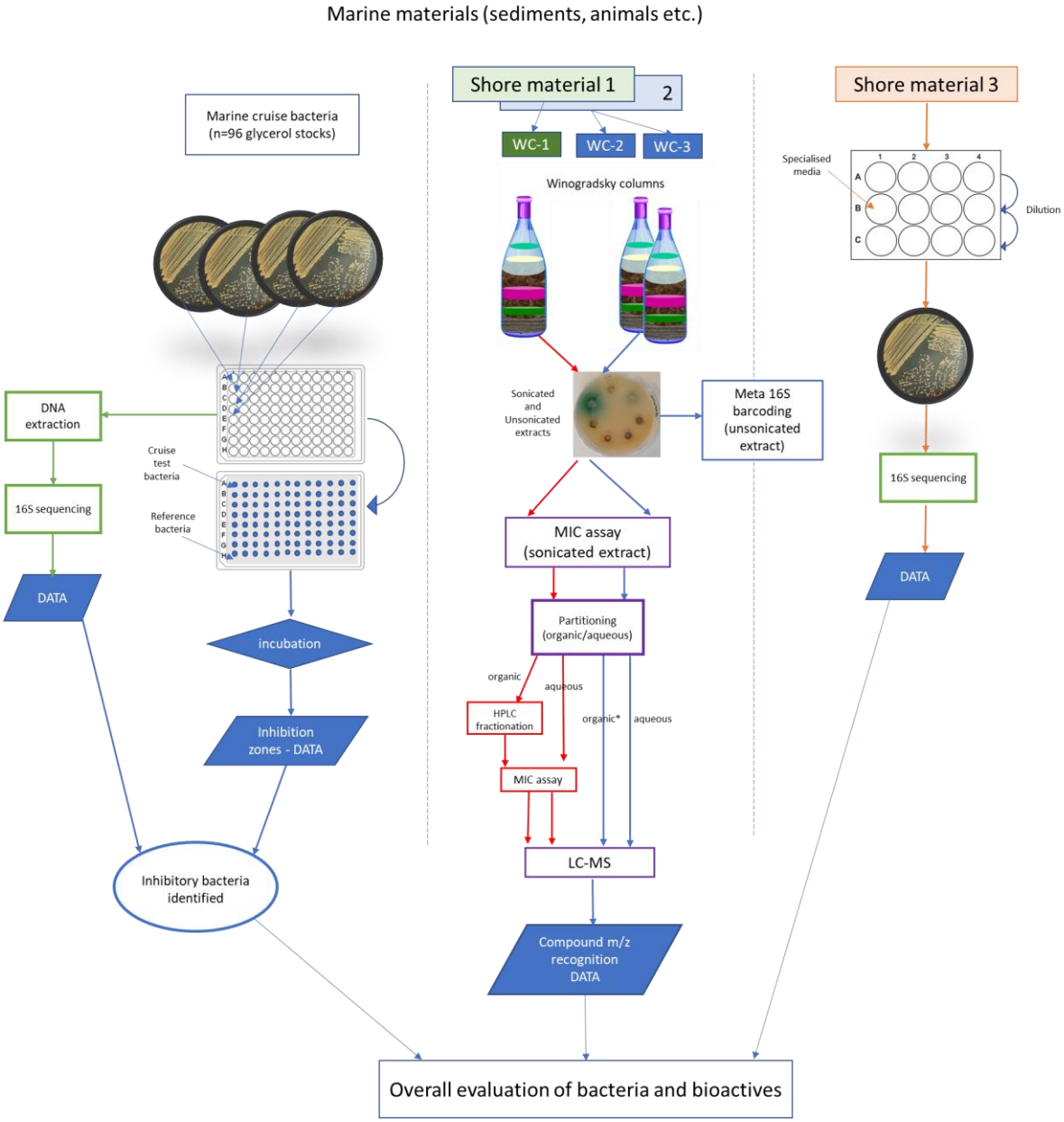


Figure 2. The order in which methods were performed for the three experiments, depicted in a flow chart.

2.6 Co-cultures for antimicrobial activity testing

Colonies of the marine bacteria (see **section 2.1**) were transferred from the petri-dishes and used to make liquid cultures in 600 µl FMAP media in a deep, 96-well plate with a breathable film, shaken at 600 rpm at 12˚C. Growth was observed every day. Co-culture experiments were done in accordance with the protocol for Microscale co-culture of marine bacteria (appendix 3), amendments are detailed below. Co-culture experiments were conducted using flatbottomed trays. MH solid media was used to make clinical reference bacteria co-culture plates and FMAP solid media was used for the plates of marine reference strains (*section 2.2*). Media that contained agar was kept at 55˚C until used, then cooled for 5 minutes while stirring before the clinical reference bacteria were added. For the FMAP plates, marine reference bacteria were spread on the surface with a glass rod evenly once cooled. Once solidified, marine bacteria samples were added to the co-culture plates in the same format as grown in the 96-well plates. An amount of 5 µl of each bacteria test culture were added to the clinical bacteria co-culture plates and 3.5 µl was added to the marine bacteria test plates. Plates were kept at room temperature for up to an hour to dry down the samples (this was more successful on FMAP plates, samples on MH plates were less dry) and incubated at 12˚C. Every 2-3 days the plates were removed, observed for inhibition zones, and re-incubated at a higher temperature until 32˚C. The shape of the inhibition zone was noted. The genera of the bacteria were identified by corroboration with the Sanger 16S sequencing results, as was the genus of any surrounding bacteria that appeared to favour inhibition zone formation. The marine bacteria that showed a notable inhibition zone were tested in petri dishes, in isolation and with any neighbouring bacteria suspected to induce AM production.

2.7 Winogradsky column (WC) experiments

Winogradsky columns were made from biological material listed in **Table 1**. Sediment was included in a clear plastic bottle (Råjuice 25 cm, diameter 7.5 cm) with paper at the bottom as a source of carbon for heterotrophic bacteria. The bottles were left in artificial and natural light for a variable number of months as specified in Table 1 before deconstruction. A variation of the protocol as described by Hockett et. al. (2017) was used (58). It was modified such that the biofilms were extracted from the sides of the column with 0.9% NaCl and mechanical force. If the sediment was stuck

to the plastic surface, this was preserved. The biofilm surface and attached surface was washed with 0.9 M NaCl solution; for WC1, ≈7ml, for WC2 and WC3, 1:1 volume of NaCl to sediment, and the suspension was pipetted into 15 ml falcon tubes. 1 ml of the biofilm samples were frozen and stored for posterity and further tests. The rest was sonicated for activity testing (2.12.1).

2.8 Isolation and cultivation of sulfate reducing bacteria

Sediment for the production of sulfate reducing bacteria is listed in Table 1 and was diluted with seawater in 15 ml culture tubes.

The cultivation of SRB bacteria is outlined as follows, derived from the protocol described by Kushkevych (59) with the following modifications: 1-4 ml of shore matter was topped up to the brim of a 15 ml falcon tube of anoxic Postgate media. Tubes were brim-filled and closed to provide anaerobic conditions, a variation of the protocol was such that liquid paraffin was not added to cover and Mohr's salt solution was not used as an indicator. The composition of modified Postgate medium can be found in Table 4. Temperature for environmental samples was kept at 25˚C. Anaerobicity was maintained using GasPak bags and indicator sachets. Biochemical tests to test whether SRB bacteria were present were skipped in favour of directly sequencing DNA (2.10).

2.9 genomic DNA isolation by magnetic Beads

DNA was extracted using the BOMB protocol for genomic DNA extraction (gDNA) (appendix 1). TE including RNAse was used to suspend the cells and to degrade RNA that might interfere with the PCR reaction. Lysis buffer was used to disrupt the cells and release the DNA into the buffer. Isopropanol was used to precipitate the DNA and coated magnetic beads were used for extraction. Extracted DNA was washed with ethanol and dried. Elution buffer was added to remove the DNA from the beads. Amendments include a) doubling the concentration of the magnetic beads in the binding buffer, b) in step 3: the inclusion of 3% RNAse in the relevant TE buffer as the protocol gave two different concentrations to use, and pipetting to mix until step 5, after which this was not done in case of DNA shearing, c) samples were also centrifuged to pellet after step 11 (second ethanol wash). DNA was stored on ice during lab work and kept overnight at -20˚C. The gDNA was used as a template to amplify 16S rRNA sequences by PCR.

2.10 Identification of isolates by amplification of 16S rRNA genes and sequencing

gDNA from the isolates was used as template for PCR reactions to amplify the 16S rRNA genes using a PCR mastermix containing 12.5 µl DreamTaq Green 2x PCR master mix (Thermo Scientific), 1 µl forward (27) and reverse (1492) primer (60) from 10 µM stocks and 10.5 µl RNAse free water. The PCR was run at standard conditions with 55 °C annealing temperature. A gel electrophoresis was run at 120 V for 20 mins to check for successful amplification of the 16S rRNA genes and approximation of their concentration. When confirmed, the resulting amplicons were cleaned up (bomb.bio protocol 4.2) and used as a template for Sanger 16S sequencing. Cycle sequencing contained 4 µl template, 1 µl Big dye Master Mix (Applied Biosystems), 1 µl forward (27) or reverse (1492) primer (60), 2 µl big dye buffer and 12 µl RNAse free water. The cycle sequencing programme was run 96˚C for 5 mins, and the following for 40 cycles: 96˚C for 10 seconds 55˚C for 10 seconds and 60˚C for 4 minutes. The tubes were then cooled to 4˚C until removed. 16S sequencing was performed using either the forward or reverse primer – therefore only a partial 16S sequence was obtained. For the co-culture bacteria, 27f was used, for the SRB bacteria, 1492r was used. The partial 16S sequences were used to identify the isolates at genus level using BLAST by comparison to the 16S sequences in NCBI databases.

2.11 Characterizing biofilms from Winogradsky columns by meta 16S rDNA meta-sequencing

Unsonicated bacteria/biofilm suspension was prepared for meta 16S barcoding using the soil DNA extraction kit to extract the DNA (appendix 8) and the 16S Barcoding Kit 1-24 (Oxford nanopore technologies) (SQK-16S024), along with the accompanying protocol (appendix 5) to quantify and barcode the DNA, given that biofilm bacteria species are not able to be isolated. The DNA libraries were quantified with Qubit so that libraries could be pooled in the optimal ratios, and to optimise cluster density during sequencing. Meta 16S barcoding was performed using flongle flow cells with an adapter in the MinION™ (Oxford Nanopore technologies). The meta 16S barcoding results – the quantities and taxonomies of the bacteria identified – were analysed in Epi2me. The sequencing of Winogradsky column DNA generated 589 classified reads from the first run and 258 from the second run.

2.12 Extraction and activity testing

2.12.1 sonication of biofilms

Biofilm extracts were sonicated in 45 second pulses. This disrupted the cell membranes, reducing the number of living cells and releasing antimicrobial compounds from the cytosol.

2.12.2 Diffusion assay

The sonicated lysates were then pipetted into wells in Mueller-Hinton soft agar plates infused with the bacterial species listed in **Table 3.** 0.9 M NaCl was used as a negative control, and 64 or 32 µg/ml ampicillin was used as a positive control. For WC1, 90 µl was included, for WC2 and WC3, 120 µl per well was included.

The soft agar plates were prepared as follows: Individual colonies from fresh dilution streaks on LB plates used to inoculate overnight cultures in 5 ml liquid MH media, shaking at 600 rpm at room temperature. The MH agar media as described in Table 7 was stirred and cooled at room temperature for 5 minutes. 5 ml of overnight cultures were then stirred into 500ml MH agar media for a further 30 seconds before plating into petri dishes.

2.12.3 MIC assay

Reference strains for the minimum inhibitory concentration (MIC) assay were chosen based on the results from the biofilm plates. Bacteria were grown overnight in 5 ml liquid MH media, shaking at 600 rpm., then 20 µl was added to 5 ml fresh MH broth so they would be in the exponential growth phase after two hours, at which point they were tested using a *Thermo Scientific Life Science Analyzer: BioMate 3S Spectrophotometer*. Bacteria from this second bacteria containing MH broth was added to a third 5 ml fresh MH broth. The concentration included was chosen based on the optical density (OD_{600}) (appendix 7). Bacteria in MH media (25-45 µl) and the same volume of aqueous extract of a known concentration was added to the same well of a 384-well plate. The absorption was measured over a 24-hour period as read by the plate reader (Envision, PerkinElmer, Massachusetts), every 30 minutes measuring and shaking for 30 seconds at 600 rpm. Two controls were included: a positive control of 32 µg/ml ampicillin and milliQ water as a negative control. For extracts where DMSO was used, 2.5% DMSO was also included as a control.

2.13 Chromatographic analysis of active compounds

2.13.1 Solid phase extraction of biofilms

The sonicated samples were freeze dried. Samples were separated into an organic and aqueous phase by the addition of 500 µl 60% acetonitrile (ACN) and 1% TFA, shaken in the cold room (4˚C) overnight, then separated. The aqueous samples were then desalted by solid phase extraction, using the following steps: 2 ml 100% ACN to condition the column, 2 ml MilliQ H_2O for equilibration, addition of sample, 3 ml H_2O to wash, 1 ml 80% ACN to elute from the column. The water was dried from both phases by vacuum centrifuge. Eppendorfs were weighed before and after drying to determine the mass of the sample. For WC1 samples were then diluted to 1 mg/ml, for WC2 and WC3 they were diluted to varying concentrations.

2.13.2 HPLC and LC-MS analysis

Select samples were separated into fractions by high-performance liquid chromatography (HPLC) using a Waters preparative HPLC system equipped with a photodiode array (PDA) detector and an XBridge C18, 5 µm, 10 × 250 mm column (Waters Associates, Milford, MA, USA). The separation was performed using linear gradients of acetonitrile (95% in water) and water, both eluents containing 0.1% TFA (Sigma-Aldrich) with a flow rate of 0.3 mL/min.

Molecular weight and purity of the compounds was confirmed using a high-resolution 6540B quadrupole time-of-flight (Q-ToF) mass spectrometer with a dual electrospray ionization (ESI) source, coupled to a 1290 Infinity UHPLC system, controlled by MassHunter software (Agilent, Santa Clara, CA, USA). The compounds were separated using a Zorbax Eclipse Plus C18, 1.8 µm, 2.1 × 50 mm column (Agilent). A gradient running from 3–20% acetonitrile containing 0.1% formic acid over 15 min with a flow rate of 0.4 mL/min was applied for the determination of the retention times of the extracts.

2.13.3 Software programmes for data analysis

The following software was used: Masshunter in Agilent (LC-MS), Benchling for 16S DNA sequence analysis, Microsoft Excel for data analysis.

2.14 Statistical tests

The Shannon index is a measure of species evenness (61), taking into account the number of species and the relative abundance of species in a community (62), although can be calculated for any taxonomic level (63).

Equation 1 $$H = -\sum[(p_i) \times ln(p_i)]$$

H – the Shannon diversity index

p_i – proportion of individuals in a community (reads in a taxon ÷ sum of all reads)

Σ - sum

ln – natural logarithm

3 Results

The marine bacteria co-culture, Winogradsky column biofilm analysis, and the cultivation of sulfate reducing bacteria were treated as three separate experiments.

3.1 Antibacterial activity detected from marine bacterial co-cultures

The first experiment aimed to characterise existing stocks of marine bacteria, which were isolated but not identified by taxon or protein fingerprint. The goal was to determine if these bacteria, although known to be “culturable” by traditional methods, could produce antimicrobial (AM) compounds that are not produced in pure culture with the addition of another cultivation method, co-culturing. A co-culture is a technique for cell cultivation wherein at least two different bacterial populations are grown together with some degree of contact between them. These systems are necessary for the study of any cell-to-cell interaction, including observing natural interactions between populations, provoking unnatural reactions from a population, or improving successful culturing of a population (64).

Marine bacteria from the research cruise in 2020 shown in Table 3 were tested using two approaches. For the first, bacteria glycerol stocks from the cruise were selected randomly and grown on agar plates. From these, those bacteria which had grown well were selected, as the co-cultures would also be performed on agar.

The second approach was more selective; bacteria were chosen for the co-culture assay based on their genome sequences and for co-culture based on whether they appeared to have promising biosynthetic gene clusters.

The marine bacteria were co-cultured with one of six reference strains, including *Bacillus subtilis* (Bs), three clinical reference strains, *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sa), *Escherichia coli*, and two marine test strains, *Phaeobacter inhibens* (Pi), *Alteromonas macleodii* (Am). The clinical reference bacteria, although not AMR strains, were chosen due to their AMR relevance.

The marine sample bacteria were then grown and some produced inhibition zones, which denoted antimicrobial activity. These were clear areas around the sample of marine bacteria where the reference strains were unable to grow. 96-samples of

marine bacteria were included in a known formation (the same as grown in the 96-deep-well plate) and grown on co-culture plates containing one reference strain.

The co-culture plate for *Alteromonas macleodii* (Am) provided no results.

3.1.1 Marine bacterial isolates with activity against *S. aureus*

The test plate for *S. aureus* can be seen in Figure 3. Marine bacteria samples produced antimicrobial compounds which were effective in inhibiting *S. aureus* by the formation of irregular but clear inhibition zones.

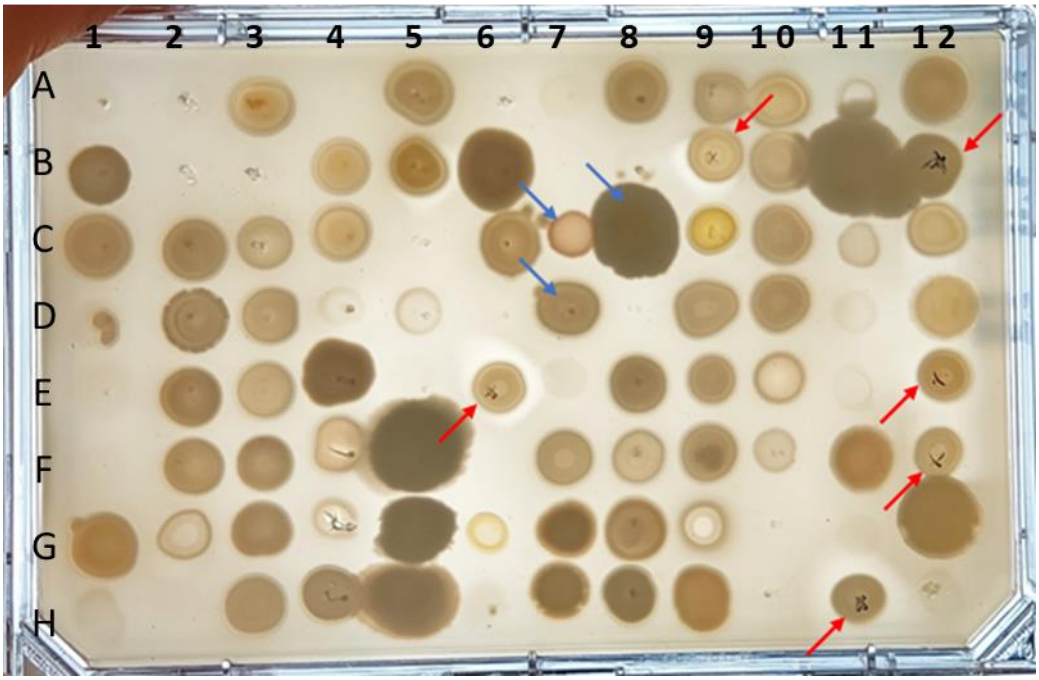


Figure 3. The S. aureus *inhibition by compounds produced by marine co-culture bacteria with marine bacteria from 2020 cruise in a known formation. Successful anti-microbial effects are denoted by clear surrounding inhibition zones. Red arrows indicate producers of antimicrobial compounds; blue arrows indicate suggested inducers.*

Suspected inducers were identified based on the bias of the shape of the inhibition zone. A larger formation in the direction of another marine bacteria species indicated it was a possible inducer. Suspected inducers are identified in **Table 8,** indicated in blue.

Table 8. Marine isolates inhibiting S. aureus*.*

ID	*Genus*
5B	*Pseudoalteromonas*
	possible inducers:
6B	*Psychrobacter*
6C	*overlap of two sequences, one Psychromonas*
5C	*Marinobacter*
6D	*overlap of two sequences, one Marinobacter*
	possible inducers:
5D	*overlap of two sequences, one Paenisporosarcina*
7C	*Sporosarcina*
7D	*Sporosarcina*
9B	*Pseudoalteromonas*
	possible inducers:
8C	*Shewanella*
11H	*Aliivibrio*
	possible inducers:
12B	*overlap of two sequences, one Pseudoalteromonas*
12E	*Pseudoalteromonas*
12F	*Pseudoalteromonas*

A possible inducer of the bacteria in 11H, *Aliivibrio sp.* was not determined, as the inhibition zone was not perfectly round but surrounding marine bacteria did not appear to grow well.

3.1.2 Marine bacterial isolates with activity against Bacillus subtilis

The test plate for *B. subtilis* showed marine bacteria produced antimicrobial compounds which were effective in inhibiting *B. subtilis* by the formation of small inhibition zones, as seen in Figure 6, and indicated in **Table 9**.

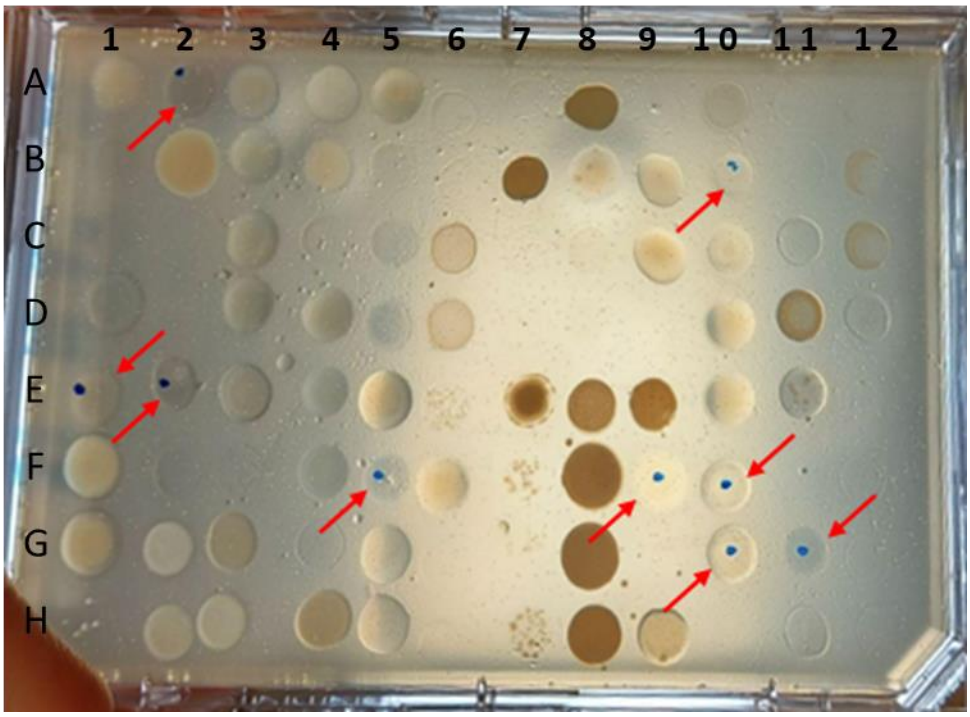


Figure 4. the B. subtilis *inhibition by compounds produced by marine co-culture bacteria. Marine bacteria are plated in a known formation. Successful anti-microbial effects are denoted by clear surrounding inhibition zones. Red arrows indicate producers of antimicrobial compounds.*

The irregularity seen in the *S. aureus* test plate was not seen, and growth inhibition appeared to be less successful.

Table 9. the bacteria that produced compounds that inhibited B. subtilis growth, as identified by their 16S sequences in NCBI.

ID	***Genus***
2A	*Sinobacterium*
2C	*Shewanella*
6D	*Marinobacter*
7A	*Arenibacter*
7B	*unidentified*
10F	*Psychromonas*
11D	*Aliivibrio*
11E	*Shewanella*
11F	*Shewanella*

The second co-culture plate conducted for *B. subtilis* (**Figure 5**), containing bacteria selected for their BGC clusters also showed inhibitory activity.

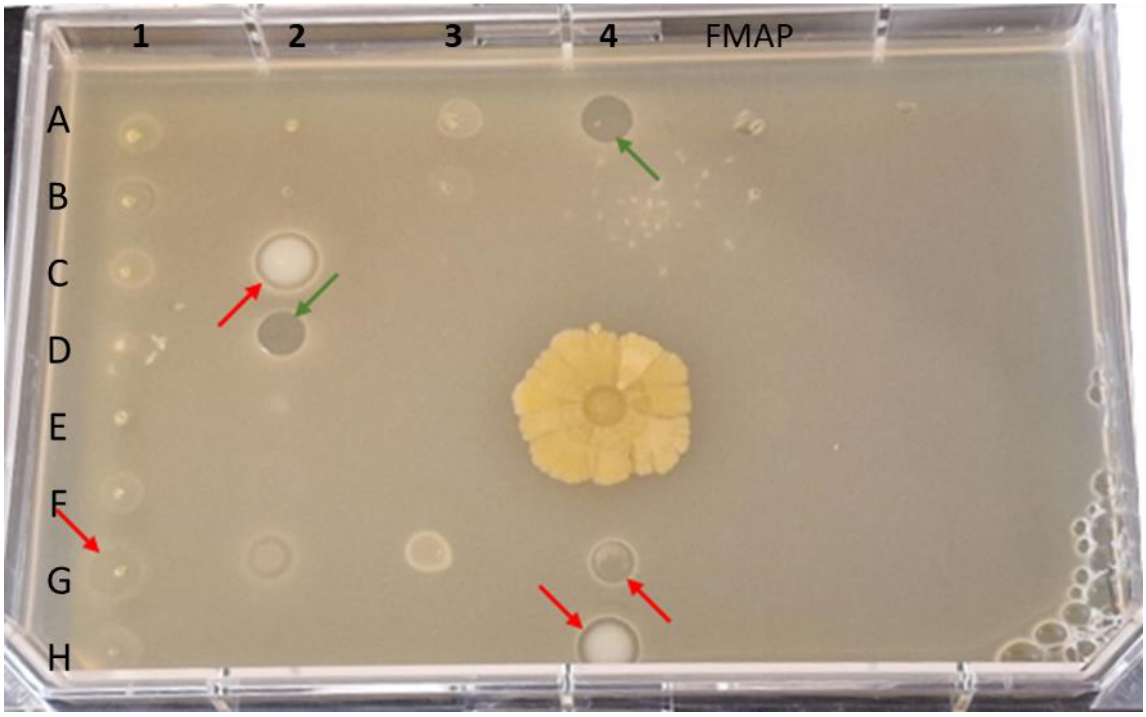


Figure 5. The activity of compounds produced by marine test bacteria against B. subtilis*, performed in co-culture, with test bacteria from 2020 cruise in a known formation. Successful anti-microbial effects are denoted by clear inhibition zones. Red arrows indicate producers of antimicrobial compounds, green arrows indicate where antimicrobial activity was found, but bacteria did not grow.*

The inhibition zones in 2D and 4A were produced after marine samples were pipetted, but the bacteria themselves did not grow. This may be an indication that the compounds were produced when the bacteria were cultured but were not produced on the co-culture plate in response to the reference strain.

The identities of the marine test bacteria that caused these inhibition zones were identified by their genome sequences (**Table 2**).

3.1.3 Marine bacterial isolates with activity against *P. aeruginosa*

The test plate for *P. aeruginosa* showed marine bacteria produced antimicrobial compounds which were effective in inhibiting *P. aeruginosa* by the formation of small inhibition zones (**Figure 6**).

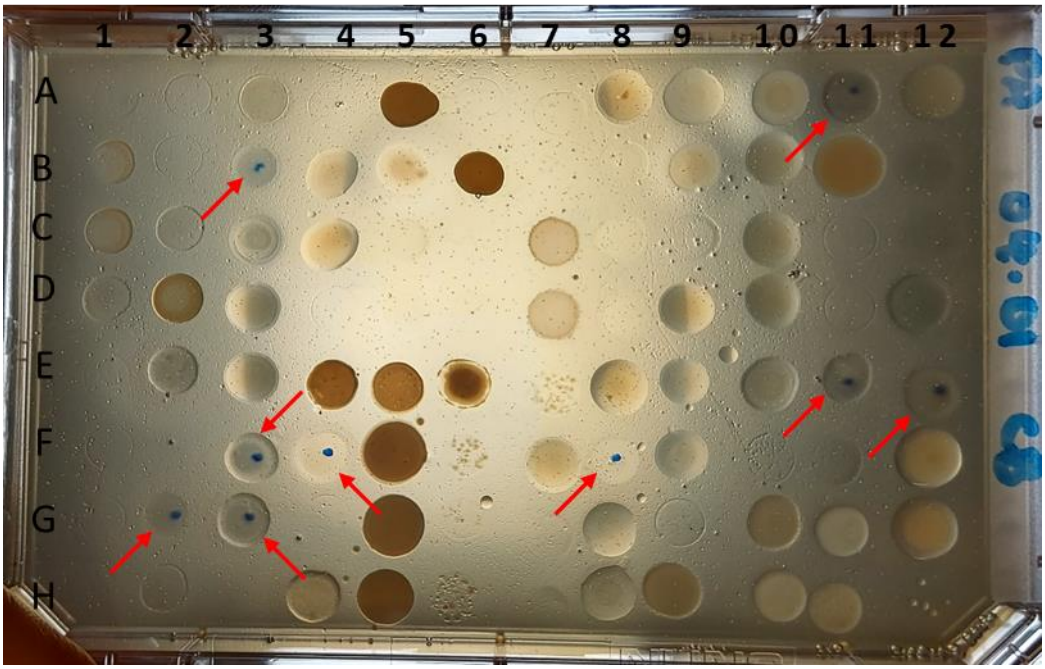


Figure 6. the P. aeruginosa *inhibition by compounds produced by marine co-culture bacteria. Marine bacteria are plated in a known formation. Successful anti-microbial effects are denoted by clear surrounding inhibition zones. Red arrows indicate producers of antimicrobial activity.*

Like *B. subtilis*, the irregularity seen in the *S. aureus* test plate was not seen, and inhibition appeared to be less successful. The bacteria that inhibited growth of *P. aeruginosa* were verified in NCBI. Their genera are recorded in **Figure 10**.

Table 10. the bacteria that produced compounds that inhibited P. aeruginosa growth, as identified by their 16S sequences in NCBI.

ID	***Genus***
2G	*Pseudoalteromonas*
3B	*Moritella*
3F	*unidentified*
3G	*unidentified*
4F	*Shewanella*
8F	*Pseudoalteromonas*
9E	*Pseudoalteromonas*
9F	*Pseudoalteromonas*
10E	*Shewanella*
11A	*Shewanella*
11E	*Shewanella*
12E	*Pseudoalteromonas*

The second co-culture plate conducted for *P. aeruginosa*, containing bacteria selected for their BGC clusters, also showed inhibitory activity (**Figure 7**).

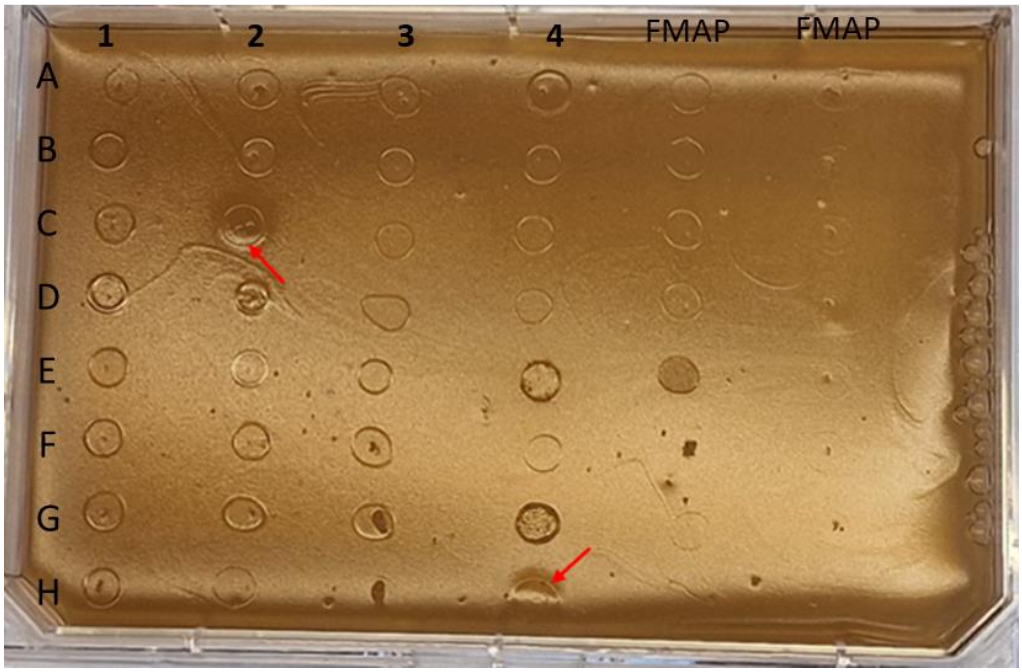


Figure 7. The activity of compounds produced by marine test bacteria against P. aeruginosa, *performed in co-culture, with test bacteria from 2020 cruise in a known formation. Successful anti-microbial effects are denoted by clear inhibition zones. The marine bacteria were tested in an 8×4 format. The last two rows were controls (FMAP media). Red arrows indicate producers of antimicrobial activity.*

The genera were recognised as *Sinobacterium* and *Shewanella* from the metagenome data given in **Table 2.**

3.1.4 Marine bacterial isolates with activity against *P. inhibens*

The test plate for *P. inhibens* showed marine bacteria produced antimicrobial compounds, which were effective in inhibiting *P. inhibens* by the formation of surrounding inhibition zones, as seen in **Figure 8**.

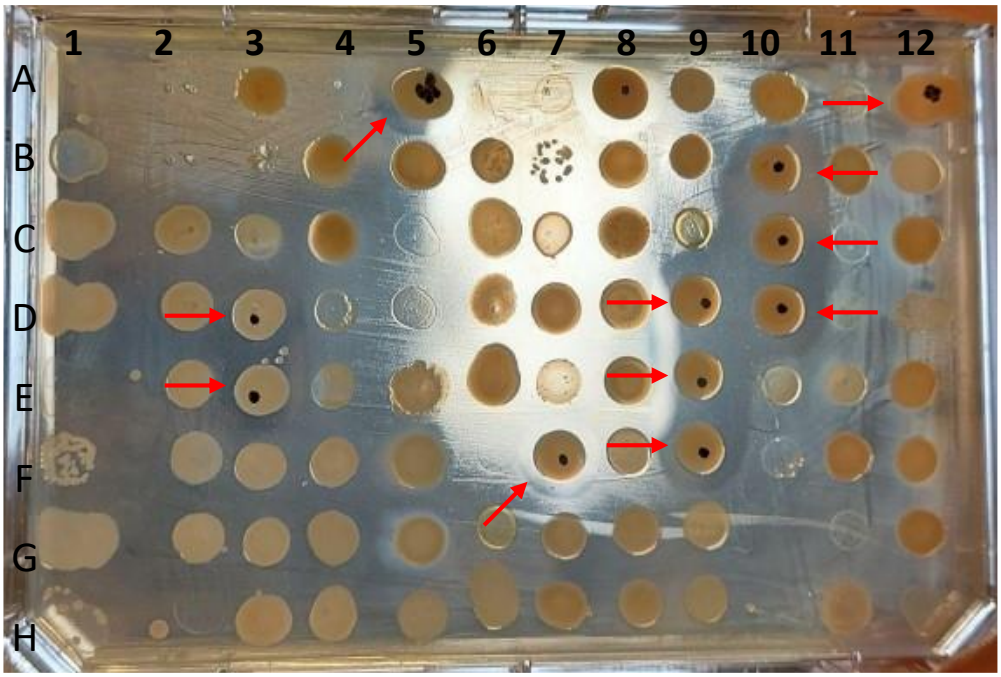


Figure 8. P. inhibens co-culture plate, with marine bacteria from 2020 cruise in known formation. Successful anti-microbial effects are denoted by clear inhibition zones, and blue dots in the case of low visibility. Red arrows indicate producers of antimicrobial activity.

The inhibition zones were larger than those on the other test plates. They were also notably regular in size and shape. Positive results are summarized in **Table 11.**

Table 11. The bacteria that produced compounds that inhibited P. inhibens growth, and their possible inducers, as identified by their 16S sequences in NCBI.

ID	***Genus***
3D	*Pseudoalteromonas*
3E	*Sinobacterium*
5A	*Pseudoalteromonas*
7F	*Pseudoalteromonas*
8A	*Pseudoalteromonas*
9D	*Pseudoalteromonas*
9E	*Pseudoalteromonas*
9F	*Pseudoalteromonas*
10B	*Pseudoalteromonas*
10C	*Pseudoalteromonas*
10D	*Pseudoalteromonas*

Most of the inhibitory bacteria came from one genus, *Pseudoalteromonas.* The regular inhibition zone formation indicated that this was produced in direct response to the reference strain, rather than to the surrounding bacteria.

3.2 Bioactivity and bacterial composition of biofilms in Winogradsky columns

Three Winogradsky columns were made from marine sediment and incubated to cultivate biofilms, mostly from anaerobic bacteria, that could be used for antimicrobial activity testing **(Figure 9).** WC1 was cut into 11 sample areas, WC2 into 5 and WC3 into 4. for WC2 and WC3, greater surface areas were taken in order to retain more material and preserve a higher concentration of compounds per extract.

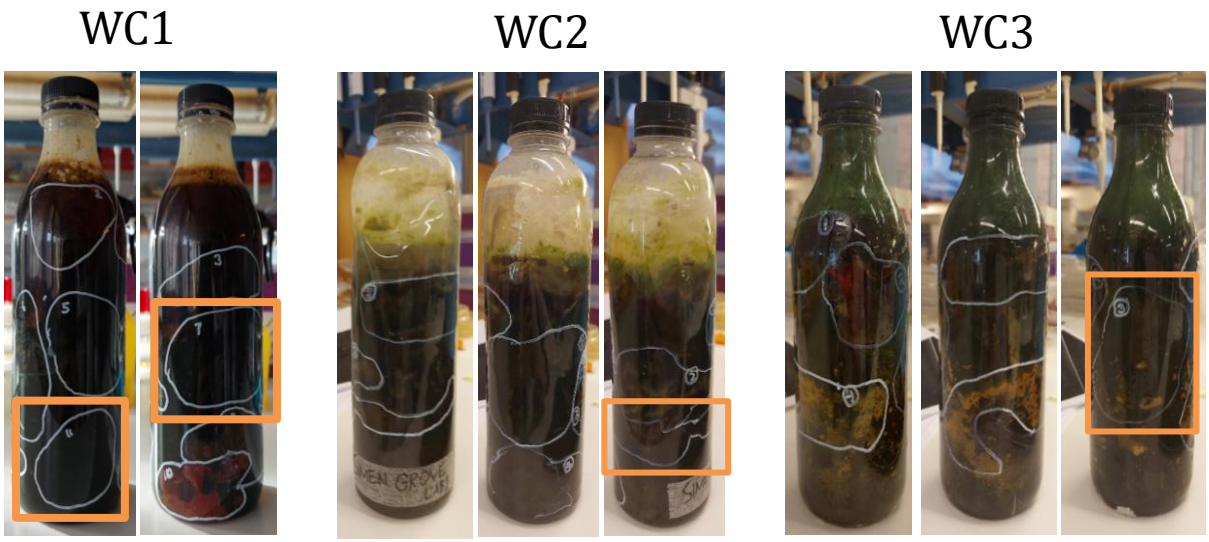


Figure 9. Winogradsky columns WC1, WC2 and WC3. Patches of the column denoting areas of biofilm to be tested were outlined in white and labelled. Areas of like-colour were grouped together and considered one homogenous patch.

The most efficacious biofilms from each column were $WC1_{O}$-7, $WC1_{O}$-11, $WC2_{O}$-3 and $WC3_{O/A}$-3. They were each from the middle or lower half of the column, dark in appearance, with little colour. Some mustard yellow biofilm patches could be seen.

3.2.1 Biofilms from Winogradsky columns inhibit growth of Gram-positive bacteria

The biofilm extracts were tested in agar plates containing the reference strains (**Table 3, Table 4**) for initial activity. Inhibition zones indicated the extracts were causing the growth of the reference bacteria to be inhibited. Larger inhibition zones corresponded to greater inhibitory activity **(Figure 10).**

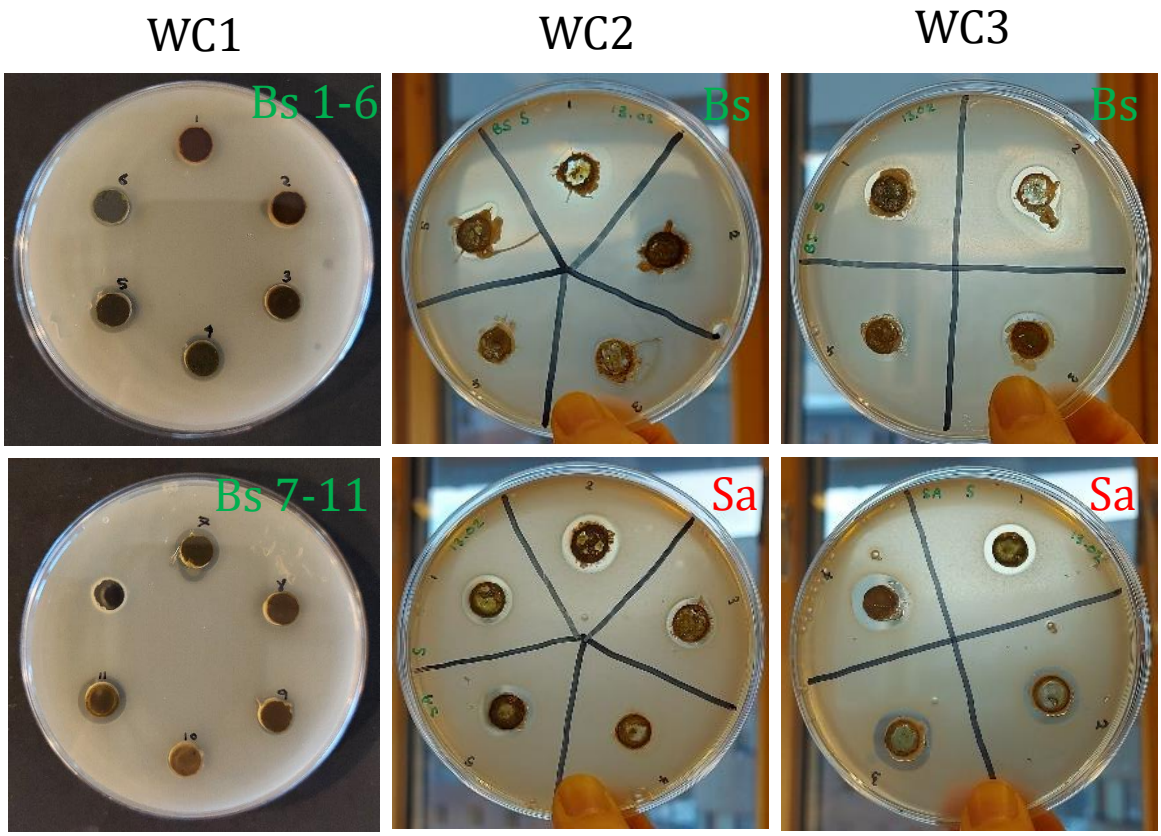


Figure 10. Preliminary antimicrobial activity shown in plates of sonicated biofilm extract from WC1 against Bs (left, top extracts 1-6, left bottom extracts 7-11), WC2 (centre top - Bs screening, centre bottom - Sa screening) and WC3 (right top - Bs screening, right bottom - Sa screening) right bottom. Samples with greater inhibitory efficacy against each test strain showed larger inhibition zones.

For WC1, the only reference strain that showed results was *B. subtilis*, so this was chosen as the reference strain for the MIC assay. Sonicated extracts for WC1-7 and WC1-11 inhibited *B. subtilis*. For WC2, sonicated extracts WC2-2 and WC2-5 showed inhibition of *B. subtilis* growth, and all sonicated extracts showed a clear inhibition of *S. aureus* growth except for extract WC2-4. All sonicated WC3 extracts showed some inhibition of *B. subtilis* growth; extract WC3-4 produced the smallest inhibition zone. Samples affected *S. aureus* growth with clear inhibition zones.

The extracts were split into two phases, an organic phase and aqueous phase with the addition of ACN. This was done under the assumption that the polar compounds would stay in the aqueous phase, and the non-polar compounds would move into the organic phase. The aqueous phase was also tested, but none showed efficacy in inhibiting *B. subtilis* at maximum concentrations. Further experiments were conducted on the organic phases.

Extracts were purified and tested for the minimum inhibitory concentration needed to inhibit growth of the reference bacteria against which they showed activity in the plates. This was calculated using a dilution series, starting with a known concentration. Extracts $WC1_O$ 3-11 were tested in a serial dilution against *B. subtilis*. Ampicillin, H_2O, 2.5% DMSO were used as controls. It is evident from **Table 12** that all extracts tested inhibited *B. subtilis* growth at high concentrations, even those that did not show efficacy in the preliminary plates.

Table 12. The MIC of organic fractions of biofilm extracts from Winogradsky column 1 required to inhibit growth of B. subtilis in ug/mL.

Extract	**Minimum inhibitory concentration**
	B. subtilis (ug/mL)
$WC1_O$-1	Not tested
$WC1_O$-2	Not tested
$WC1_O$-3	500
$WC1_O$-4	250
$WC1_O$-5	500
$WC1_O$-6	250
$WC1_O$-7	125
$WC1_O$-8	500
$WC1_O$-9	500
$WC1_O$-10	500
$WC1_O$-11	125

The most potent inhibitors of *B. subtilis* growth were extracts $WC1_O$-7 and $WC1_O$-11, which was in keeping with the result from the screening plates in **Figure 10.**

*Table 13. the minimum inhibitory concentration of each biofilm extract from WC 2 and WC 3 dissolved in an organic solvent, ACN, needed to inhibit growth of the reference strains. *Important note: the MIC for Bs and Sa cannot be compared as the assays were performed separately and prepared differently.*

Extract	**B. subtilis (ug/mL)**	**S. aureus (ug/mL)**
$WC2_O$-1	>1000	>500
$WC2_O$-2	500	>2000
$WC2_O$-3	250	2000*
$WC2_O$-4	>1000	>1000
$WC2_O$-5	1000	>1000
$WC3_O$-2	5000	>3000
$WC3_{O/A}$-3	>2250	3000*
$WC3_O$-4	2000	>1000

The high concentration required to produce a result for *B. subtilis* is likely due to the mass of matter that could not be dissolved, which was included in the calculation to work out the concentration of the extracts but was not included in the MIC assay.

It is important to note that the MIC for *B. subtilis* and *S. aureus* cannot be compared. Between the two assays, some insoluble matter was removed before the compounds were dried down and weighed, affecting the recorded mass. This caused the concentration required to inhibit *S. aureus* to come across as more concentrated. On the other hand, the MIC assay was run for *S. aureus* later, after some samples had been fractionated and undergone further processing.

WC1 extracts were then separated into fractions by HPLC, and the fractions were tested for activity in MIC assays separately to discern which compounds were responsible for the inhibitory effect, but few fractions returned a significant result. The only fractions of note were fraction 5 in extracts $WC1_O$-7, 8 and 10.

Despite the apparent potency of $WC1_O$-11 in the test plates, no individual fraction inhibited the growth of *B. subtilis*. A further exploratory experiment was conducted whereby the fractions in $WC1_O$-11 were combined in tens (fractions 1-10, fractions 11-20 etc.) to see if there was a cumulative effect between fractions. However, this did not return a positive result.

Because of this result and time constraints, just one extract, from WC2, $WC2_O$-2, was fractionated by HPLC for comparison to see if the fractions were capable of producing individual inhibitory responses. As this was not successful, the rest of the extracts went straight into LC-MS testing and analysis.

3.2.2 Chemical content of Winogradsky column biofilm extracts from HPLC/LC-MS

Fraction 5 of extracts $WC1_O$-7, $WC1_O$-8, $WC1_O$-10 were active. The spectra and chromatogram for fraction 5 were compared in positive ion mode. Extract 9 fraction 5 was not active but included for comparison. Extracts 10 and 8 showed similar spectra and 7 and 9 were similar, but there was no definite, substantial peak shared between 7, 8 and 10 that was not shared by 9, so the spectra were compared in negative ion mode in Figure 11.

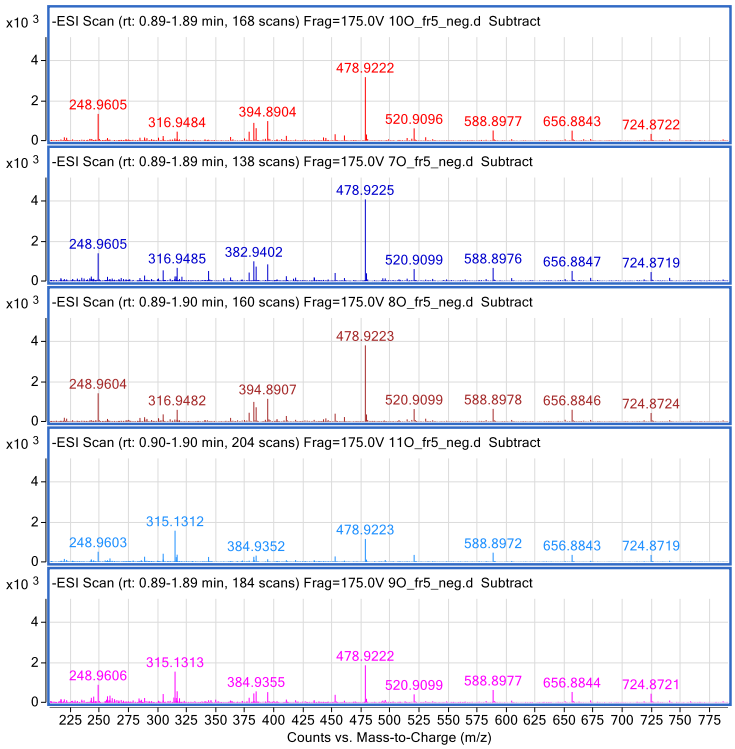


Figure 11. The compounds in fraction 5 for WC1o-7, WC1o-8, WC1o-9, WC1o-10, WC1o-11 found by LC-MS in negative ion mode.

As well as Fraction 5 of WC1$_O$-9, WC1$_O$-11 was also included for comparison because fraction 5 was inactive, but the extract showed high efficacy. It was observed that the concentration of compound with m/z 478.9223 was approximately twice as abundant in the active fractions as the non-active fractions as seen in *Figure 11*. The atomic composition could not be found in *MassHunter.*

Two other compounds were noted to be more abundant in the more active extracts, WC2$_O$-3 and WC3$_{O/A}$-3. It is important to note that WC3$_{O/A}$-3 was treated differently to the other extracts; as it could not be separated into an organic and aqueous phase, only one “phase” was retained. Upon examination of the spectra for other extracts, it became evident the compounds existed in all WC2 and WC3 organic extracts to some degree, depicted in Figure 12.

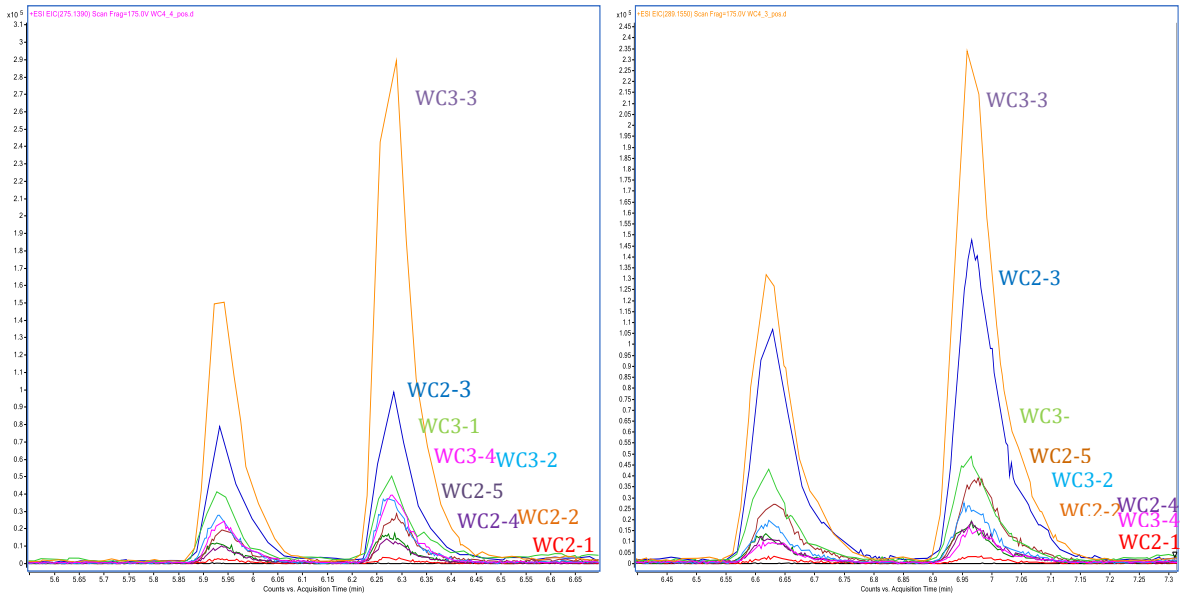


Figure 12. Two compounds of interest found in all WC2 and WC3 organic extracts. Graphs show the counts vs. acquisition time for the compounds found in positive ion mode for peaks with mass to charge ratio (m/z) 289.1553 at 6.63 minutes and 275.1395 at 5.94 minutes for extracts from WC2 and WC3.

It is important to note that peaks in *Figure* 12 cannot be directly compared visually because the concentration injected differed. To account for this, the abundances were normalised for visual comparison in Figure 13.

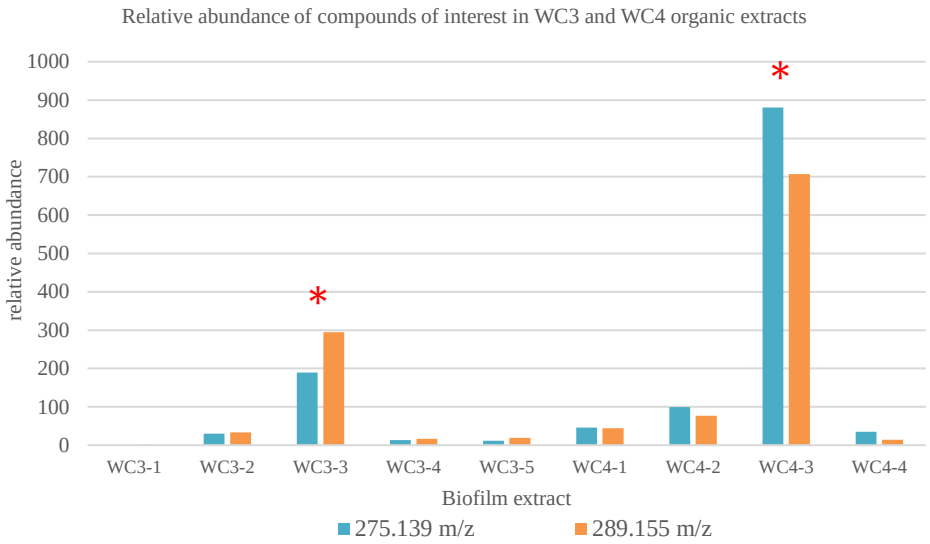


Figure 13. The relative abundances of compounds with mass-to-charge ratio m/z 275.139 and 289.155 in each of the WC2 and WC3 organic extracts. "Relative abundance" was calculated by (area under curve × concentration (mg/ml) × volume injected into the LC-MS) ÷ 1000 (for readability). Red asterisks indicate the most efficacious extracts.

Because the most efficacious extract of WC3 was not split into two phases, and retained more of these compounds, the aqueous phases of some of the other extracts were tested by LC-MS, where these compounds were also found.

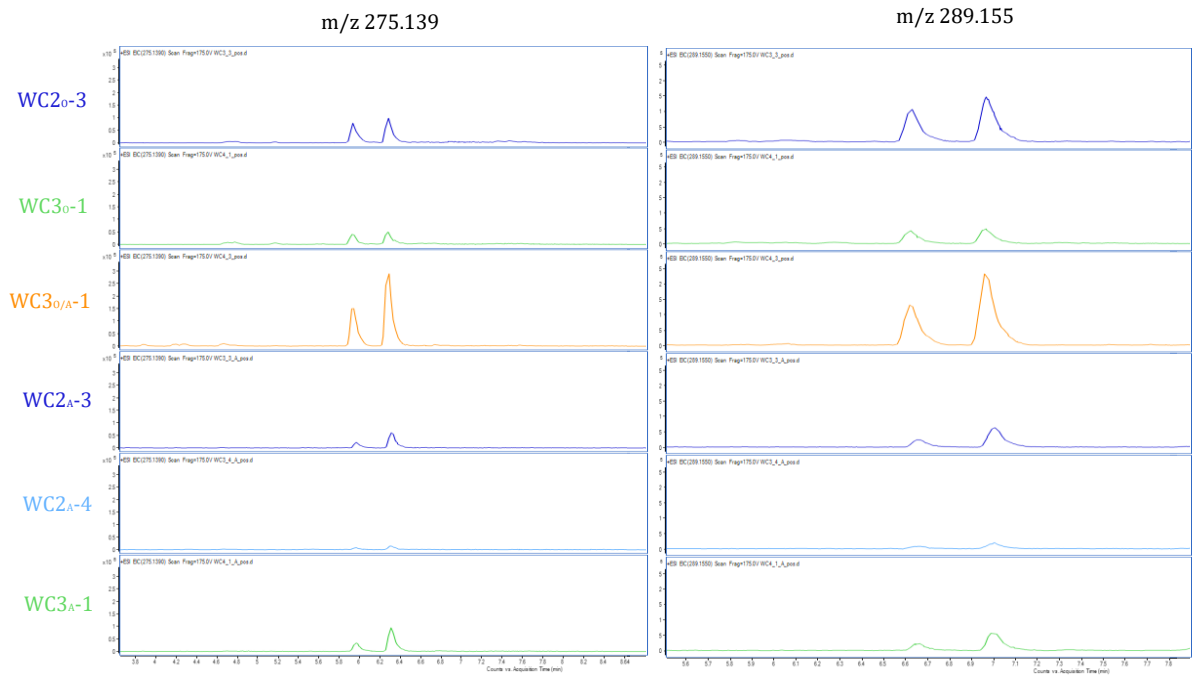


Figure 14. The coupounds of interest in the aqueous phases of WC2 and WC3 biofilm extracts. LC-MS spectra of compounds with mass-to-charge ratio m/z 275.139 (left) and 289.155 (right) in three organic (top) and two aqueous (bottom) extracts. $WC3_{O/A}$-3 is in orange. Spectra of the same colour indicate they are from the same extract (green $WC3_O$-1 and $WC3_A$-1, dark blue $WC2_O$-3 and $WC2_A$-3).

Isomers of both compounds were detected in extract WC3-3. These had the same mass to charge ratio but were detected at different acquisition times.

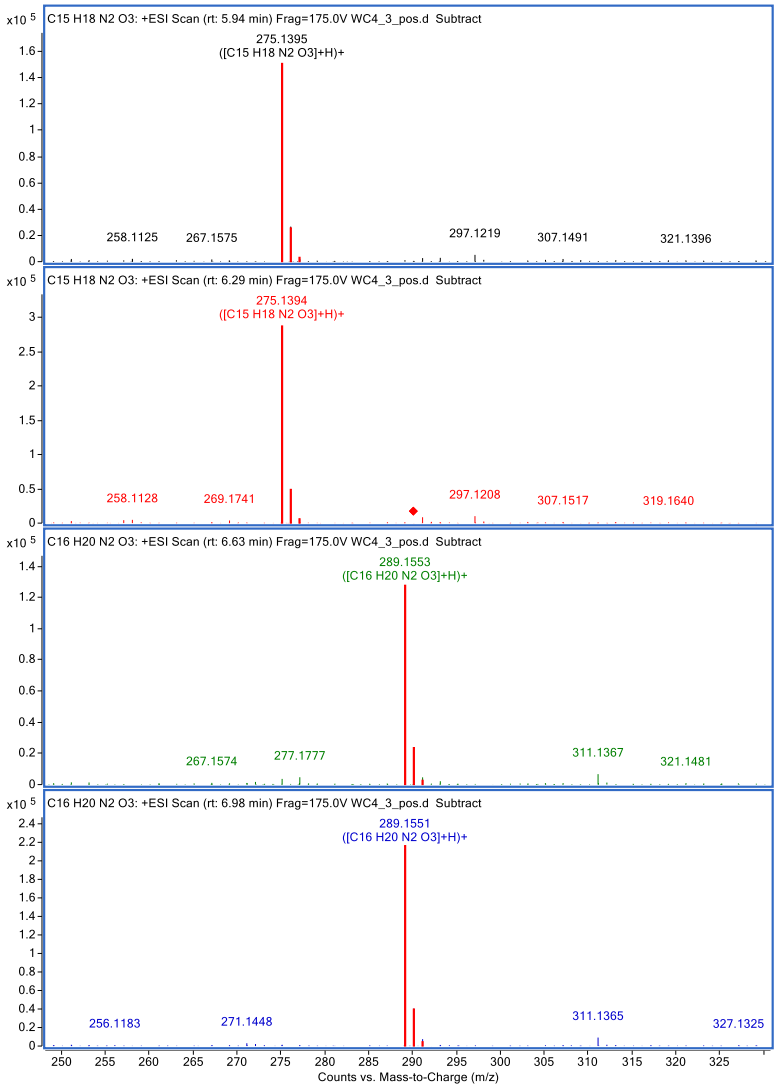


Figure 15. Isomers of the compounds detected with mass-to-charge ratio m/z 275.139 (top two spectra) and 289.155 (bottom two spectra) in extract WC3-3. Spectra show the count vs. mass-to-charge ratio (m/z) for the isomers detected of compounds.

The chemical formula was predicted for the two compounds. An unusually high score was given for the first suggestion for each compound, and the second suggestion for each compound was given a much lower relative score.

Table 14. Possible chemical formulae for the compounds found with mass to charge ratio (m/z) 289.1553 at 6.63 minutes and 275.1395 at 5.94 minutes, in positive ion mode. Found in Agilent MassHunter. Score indicates the statistical certainty of the software for this formula.

RT (min)	Formula	m/z	score
6.63	C16 H20 N2 O3	289.1553	97.7
	C14 H18 N5 O2	289.1553	85.86
5.94	C15 H18 N2 O3	275.1395	98.97
	C13 H16 N5 O2	275.1395	86.82

The same compounds could be seen in many organic extracts of WC1. However, because this was not part of the initial experiment plan, not all extracts were tested and the masses were unknown. Consequently, the relative abundance could not be compared in any quantifiable way but could be correlated. There appeared to be a relationship between the amount of the compounds detected and the apparent efficacy of the extract.

LC-MS could not be run on $WC1_O$-11 as an extract due to a lack of material after fractionation but select fractions of $WC1_O$-11 had been run, and in one fraction these peaks can be seen in abundance.

Table 15. An indication of the relative abundances of the compounds found with m/z ratios 289.155 and 275.139 for the WC1 biofilm extracts. A more quantitative measure, the area under curve, is in appendix 6.

	peak EIC 275.139	peak EIC 289.155
Extract	relative abundance	relative abundance
WC1-1	very low (factor of 1)	very low (factor of 1)
WC1-2	Not tested	Not tested
WC1-3	low (factor of 10)	high (factor of 100)
WC1-4	high (factor of 100)	high (factor of 100)
WC1-5	Not tested	Not tested
WC1-6	Not tested	Not tested
WC1-7	high (factor of 100)	high (factor of 100)
WC1-8	low (factor of 10)	low (factor of 10)
WC1-9	Not tested	Not tested
WC1-10	Not tested	Not tested
WC1-11	high, but only from one fraction	high, but only from one fraction

A polymeric pattern was recognised in all biofilm extracts, appearing in fraction 4 in fractionated extracts. It was identified by regular intervals in the LC-MS spectra, in this case, of 67.9 m/z. An example from one extract is represented in Figure 16. Of the repeating units, the compound furthest to the right on the x-axis represents the longest polymer chain, and each successive interval towards the origin represents the polymer with one less repeating unit.

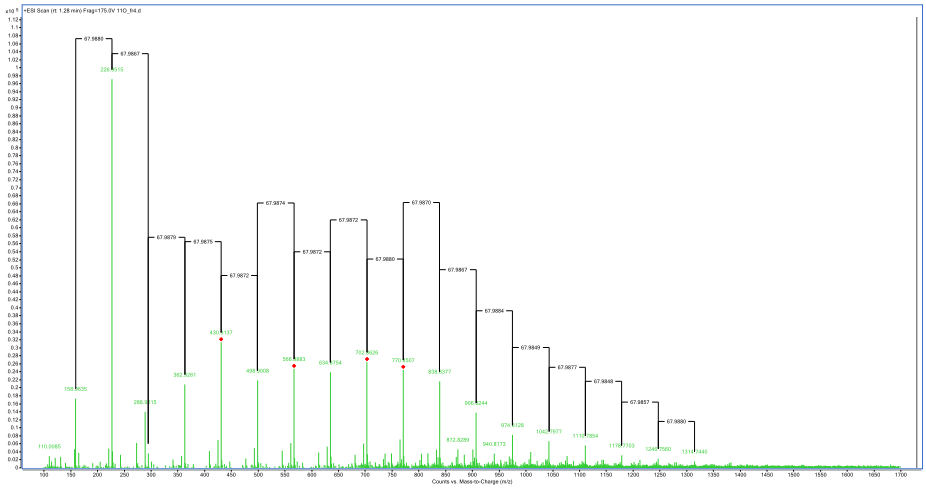


Figure 16. The polymeric pattern found in all biofilm extracts shown in an LC-MS spectrum of fraction 4 of WC1$_O$-11.

This LC-MS pattern was also found in biofilm material in unpublished data from the DeepSeaQuence project (project number 315427) from deep sea hydrothermal vents, but not in unpublished LC-MS data from marine organisms, or marine bacteria.

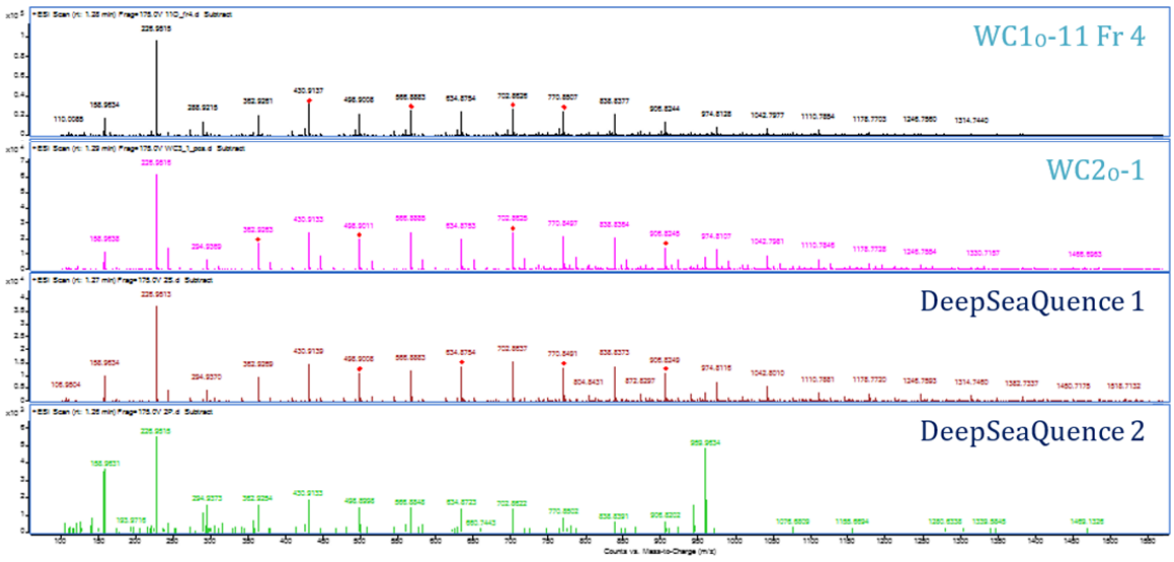


Figure 17. The polymeric pattern found in marine biofilms, LC-MS spectra of found in positive ion mode with a retention time of ≈1.26-1.29 minutes. The top 3 spectra are from the Winogradsky column biofilms, and the bottom two are from deep sea vents (DeepSeaquence project number 315427)

3.2.3 Taxonomic information from meta 16S barcoding analysis

A meta 16S barcoding analysis was performed for WC2 and WC3 extracts to identify the bacteria in the different biofilms, with the aim of correlating bacteria populations and biofilm activity. The results were found from Epi2me. However, this did not produce the expected results; ≈ 20 000-80 000 total reads were expected as opposed to 1 000-2 000. A replicate was run with the same DNA but there was no consistency between the two datasets. For this reason, the results could only be qualitatively analysed.

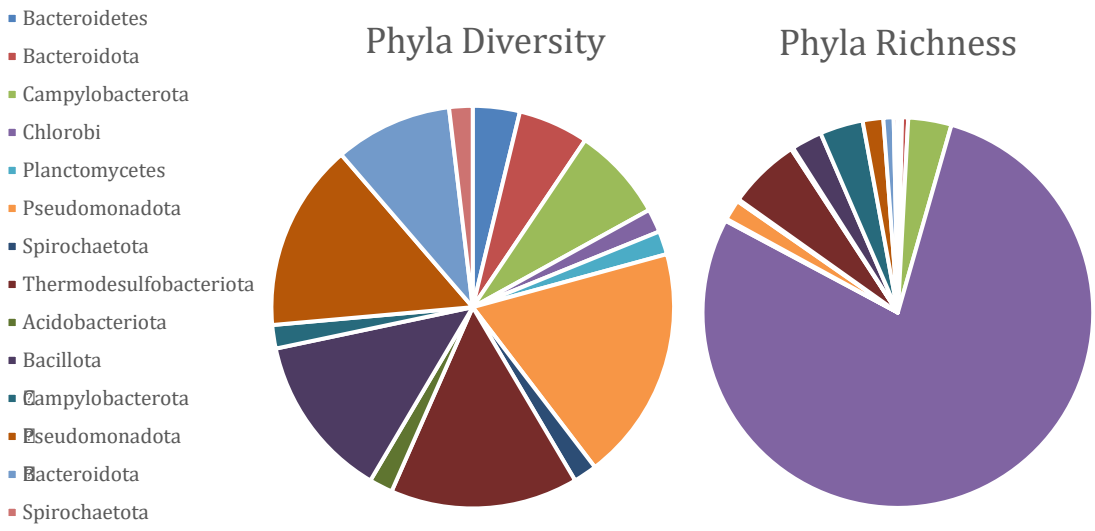


Figure 18. the Phyla diversity and richness of bacteria found across WC2 and WC3.

Only the following could be garnered: the genus *Prosthecochloris* was by far the most abundant genus, accounting for 460 out of 581 bacteria detected and recognised. The next most common genus accounted for only 11 reads.

Preliminary data for the project was obtained the previous semester in 2022. The meta 16S barcoding was successful, but no antibacterial activity testing was obtained for these biofilms. The data was run in 4 barcoding sets. The 2022 dataset was much larger, consisting of 96757 identity reads, providing a much more reliable sample. much less information was retrieved than was needed to identify and analyse the populations present in columns WC2 and WC3. It was also clear that although the phylum *Chlorobia* makes up a large fraction of the 2022 data, there is a proportional overrepresentation of *Chlorobia* in the WC2 and WC3 data compared to the data from 2022.

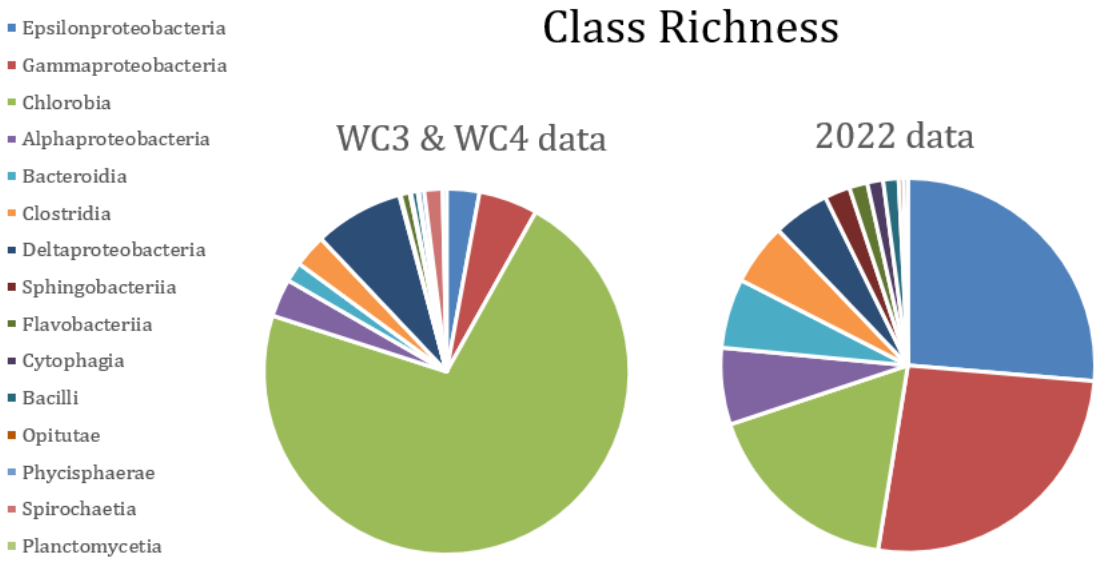


Figure 19. the class richness of bacteria from Winogradsky columns. Left: WC2 and WC3, right: 2022 data. WC2 and WC3 data = 581 reads, 2022 data = 96757 reads.

The data from the 2022 data also gave a better indication of the diversity in Winogradsky columns. Examples of the most prevalent genera in barcoding set 2, which contained the largest number of samples, can be seen in *Figure 20*.

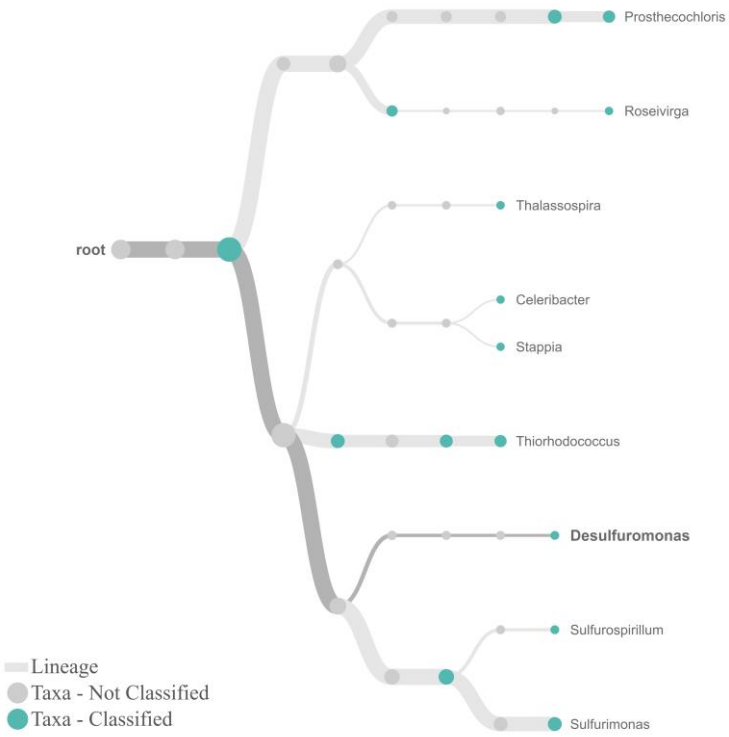


Figure 20. Some of the most abundant genera in winogradsky column biofilms and how they are related, presented in a phylogenetic tree as presented in epi2me. Data from 2022 dataset, barcode set 2.

The abundance of the genera in Figure 20 are depicted in Figure 21, where the genera richness can be compared.

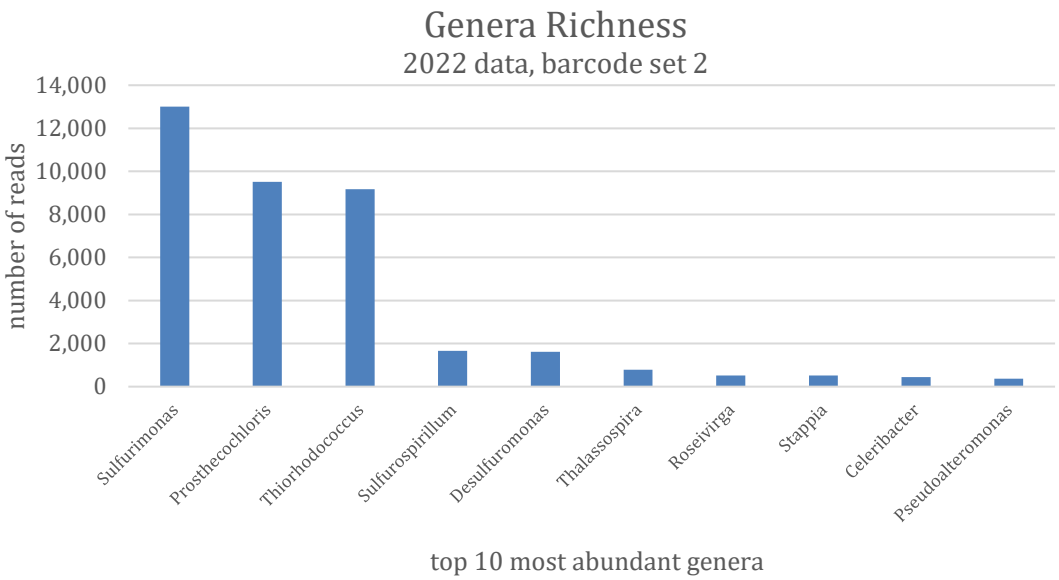


Figure 21. Relative abundances of the top 10 most abundant bacterial genera from Winogradsky column biofilms from meta 16S barcoding, 2022 dataset, barcoding set 2.

The *S*hannon index was calculated *from Equation 1* for the bacterial **genera** detected in WC2, WC3, and two WCs from 2022 (**Table 1**). WCs from 2022 that had obtained data for 4 biofilm extracts were selected so that all WCs would be analysed for a similar number of extracts.

Table 16. the genera evenness of WC-2, WC-3, WC-111 and WC-112 biofilm extracts, denoted by the Shannon index to 4 decimal places. The total number of reads classified and the genera richness (n=genera) are also represented.

	Genera level reads classified	Genera richness	Shannon index
WC-2	218	10	0.0664
WC-3	152	12	0.8491
WC-111	3821	137	2.0143
WC-112	4582	73	1.5955

The recorded appearance of biofilms from the 2022 data was correlated with their appearance to obtain qualitative data, shown in **Table 17**. Barcodes were grouped for their descriptions of colour and position in the WC. “Water” or “water/sediment” denoted the biofilm was cut from the top of the column, “sediment” denoted it was taken from further down. Data was grouped for green biofilms, but only if they were

found near the top of the WC. Data was also collated for purple biofilms but separated into water-level biofilms and sediment-level biofilms. The data for dark coloured biofilms of the sort that showed highest efficacy was too little and poorly described, so only two biofilms were looked at that adequately fitted the description. Both were described as “dark” and were found at a sediment level.

Table 17. Qualitative data about the bacterial populations in biofilms, correlated with their colour and position in the column.

Biofilm colour	Biofilm position	Notable dominant bacteria
Green	Water or water/sediment	*Prosthecochloris*
Purple	Water	*Thiorhodococcus* > *Sulfurimonas*, Roseivirga present
Purple	Water/sediment	*Thiorhodococcus* = *Sulfurimonas*, *Roseivirga* present
Purple	Sediment	*Thiorhodococcus* < *Sulfurimonas*, no *Roseivirga*
Dark green	Sediment	*Desulfuromonas* most dominant
Dark brown/ black/grey	Sediment	*Pseudoalteromonas* dominant, no *Sulfurimonas, Prosthecochloris, Thiorhodococcus*

Biofilms generally contained a high amount of *Sulfurimonas* and *Thiorhodococcus,* but purple biofilms showed a higher proportion of *Thiorhodococcus* over others. *Prosthecochloris,* found in most samples and predominant in green biofilms, was also the type of bacteria that was overwhelmingly found in the barcode results for WC2 and WC3.

In the dark brown/black/grey biofilm, the only dominant bacteria type was Pseudoalteromonas, and the biofilm did not contain any significant proportions of the genera that appear to otherwise predominate WC biofilms (*Sulfurimonas, Prosthecochloris, Thiorhodococcus*). The dark green sediment biofilm contained *Desulfuromonas*, a sulfate reducing bacteria. Although 16S is not usually accurate to a species level, it is notable that Epi2me classifies 383 out of 415 reads as the species *Desulfuromonas svalbardensis*.

3.3 Sulfate reducing bacteria could not be successfully cultivated from sediment using modified Postgate media

The bacteria were grown in serial dilution in modified Postgate media. The culture was then plated on modified Postgate agar, as described in Table 7, to grow single colonies. Once cultured, the DNA was extracted and sequenced to find the genera of the bacteria cultivated.

Table 18. The genera of the bacteria grown in modified Postgate media as identified from 16S Sanger sequencing and identified with 16S sequence data from NCBI.

ID	Genus	Respiration	Gram-stain
1a	*Yersinia sp.*	Facultative anaerobic	-
1c	*Massilia sp.*	Facultative anaerobic	-
1e	*Serratia sp.*	Facultative anaerobic	-
1f	*Pseudomonas sp.*	Facultative anaerobic	+
2b	*Clostridium sp.*	Obligate anaerobe	+
2c	*Yersinia sp.*	Facultative anaerobic	-
2d	*Hafnia sp.*	Facultative anaerobic	-
2e	*Lelliottia sp.*	Facultative anaerobic	-
3	*Klebsiella sp.*	Facultative anaerobic	-

Most of the genera identified from the data were aerobic bacteria, but facultatively anaerobic. Most were also Gram-negative.

4 Discussion

In this project we looked for antibacterial activity produced by bacteria isolated from different marine materials collected in a marine bioprospecting cruise in 2020 and from biofilm material from different Winogradsky columns. It was possible to identify strains and biofilm material that contained antibacterial activity against different bacteria and thus is a good starting point for further analysis. In the next paragraphs, different aspects of the results will be discussed in more detail.

4.1 Are marine isolate interactions affecting the production of compounds active against *S. aureus*?

The co-culture experiment contained multiple samples of *Pseudoalteromonas.* When *S. aureus* was used as the reference strain, some *Pseudoalteromonas* samples produced a response and not others. It is possible that the production of antimicrobial compounds was influenced by the surrounding bacteria species on the plate. This is further indicated by the variable shape of the inhibition zone, which, as opposed to that seen in the *P. inhibens* co-culture plate, is irregular but very directed (**Figure 3**). It is also possible that the species identification is not reliable, as they were identified by their 16S sequences only. This would depend on how similar this region of the genome is between species of the same genus. It is also possible that this is a difference between strains. However, in order to compare the genetic content for biosynthetic pathways, the whole genomes would have to be obtained.

Conversely, the inhibition zones of the *P. inhibens* co-culture were uniform in size and shape, and were produced by all samples of *Pseudoalteromonas*, so there was no reason to assume that the inhibition zones were influenced by surrounding bacteria. Production of virulence factors is energetically expensive (65), and selective production may be a metabolically favourable way to produce antimicrobial compounds. This would indicate that one marine species can provoke another neighbouring marine species to produce antimicrobials that are effective against *S. aureus*, but not *P. inhibens*. This might also indicate that the antimicrobial compounds production is targeted, as the compounds produced are effective against a particular genus or species and are only produced in response to bacteria they will inhibit.

Both *S. aureus* and *Sporosarcina sp.* are Gram-positive. The antimicrobial producers *Marinobacter* and *Pseudoalteromonas*, which are both Gram-negative, may have produced antimicrobial compounds in response to the Gram-positive cell wall, which consists of an anioinic, thick peptidoglycan layer with embedded teichoic acids. Many bacterial species produce cationic antimicrobial peptides (AMPs) that have a number of ways to interact with the cell wall, such as by electrostatic forces (66). The bacteria presumed to be influenced by *Sporosarcina sp.* could not be reliably sequenced and appeared to be a combination of two different species, one of which was identified as a *Marinobacter* species. Little is written about the compounds produced by *Marinobacter* that are effective against *S. aureus,* but, interestingly, Silva et. al. (2018) found *Marinobacter psychrophilus* to be active against another clinical strain, *M. luteus*, but not at all against *S. aureus* (67), suggesting co-culture could be an important factor in the positive result of this experiment.

The *Pseudoalteromonas* species that produced compounds effective against *S. aureus* also had an extremely directed inhibition zone. Given their relative distance and the direction of the inhibition zone, it is possible that *Sporosarcina sp.* could have also induced production of these compounds, as they are just behind the bacteria marked as a possible inducer. This should be validated with confirmatory plates. Many *Pseudoalteromonas* species have shown antimicrobial activity against *S. aureus*, including several halogenated and non-halogenated alkaloids, Bacteriocins and Bacteriocin-Like Inhibitory Substances (68), and even compounds that produce anti-methicillin resistant *S. aureus* substances (69, 70). However, the compounds produced in this experiment would require further investigations to be identified. The first step would be to sequence the genome of the isolate to confirm its species and identify any potential biosynthetic gene clusters.

It may be that the biased shape of the inhibition zone is produced because compound production is being inhibited in the negative direction, rather than induced by a colony in the positive direction. If compounds are not induced by suspected inducers in the initial confirmatory plates, suspected inhibitors should be tested in further confirmatory studies. Thinner layers of agar might have been used, as this may have affected the success of these assays here. The explanation for this is that a thinner layer of agar would contain fewer target bacterial cells. This would make the assay more sensitive for detections of antibacterial activity.

4.2 Genera *Sinobacterium and Shewanella* may be a source of antimicrobial compounds against *P. aeruginosa* and *B. subtilis.*

In both co-cultures for *P. aeruginosa* and *B. subtilis*, the clearest inhibition zones were made by species of *Sinobacterium* and *Shewanella*. Interestingly, all samples of *Shewanella* that caused inhibition were isolated from marine sponges. The symbiotic relationship between marine bacteria and host sponge is a known resource in antimicrobial bioprospecting, and the bacterial compositions differ greatly between sponges in sea water and sediment samples (71, 72). Furthermore, the marine sponge *Callyspongisa diffusa* is known to be associated with *Pseudomonas aeruginosa*, and also contain the bacteria *Shewanella algae (72).* It therefore stands to reason that the strains of *Shewanella* that grow in marine sponges may benefit from the production of secondary metabolites that will promote their survival over *Pseudomonas*-like species. (72) *Shewanella* is from the taxonomic class *gammaproteobacteria*, from which a great deal of information is currently being generated. The Gram-negative species of this class possess the potential for bioactive metabolite assembly due to being richer in *nrps* and *pks* BGC clusters. Polyketides from marine products are valuable leads (73). These compounds form the basis for many antibiotics used today, such as rifamycin (74). The species *Shewanella algae* is known to produce polyketide macrolides, some of which have been characterised, and even sucessfully tested against antibiotic-resistant pathogens including *MRSA* and vancomycin resistant *E. faecalis* (75). Other species have also been found to produce antimicrobials, such as *Shewanella halifaxensis,* which is known to produce 2-benzyl-4-chlorophenol (76), a broad-spectrum antimicrobial agent often used in disinfectants (77).

Conversely, it was not possible to find anything in the literature on the ability of the genus *Sinobacterium* to produce antimicrobials. The sample was isolated in this instance from the colonial ascidian *Synoicum pulmonaria (78)*. The species was identified as *norvegicum* by metagenome, as part of ongoing work. *Sinobacterium norvegicum* was isolated and characterised in 2015 off the coast of Bergen from the great scallop, *Pecten maximus (79)*. This gap in the literature may be an avenue for further research, especially if it is a species specific to the Norwegian sea or the surrounding areas. The lack of research into the potential of *Sinobacterium sp.* could

also indicate co-culture is a necessary factor for antimicrobial production. This could be further explored by recombinant DNA technology and heterologous gene expression. By inserting the gene cluster into a plasmid and inducing expression via the promoter, the bacteria will not have to be co-cultured again to obtain the compound of interest.

4.3 Sodium ion clusters interfere with the results from LC-MS analyses of biological matrices

When questioning the chemical content of biofilms, it is important to consider the matrix, as it undoubtedly has chemical properties that vary with the nature of its microbes (47). The biofilm matrix has been called the “dark matter of biofilms” because of the wide variety of matrix biopolymers and the difficulty in analysing them. They differ depending on the microorganisms present, temperature, nutrients available, and the forces acting upon them. The ability to produce EPS may offer an advantage to certain bacteria over others, as the production of polymers pushes the daughter cells of producing bacteria to more oxygen rich areas (48).

However, it was later found in the literature that there is a known complication that gives this polymeric appearance. Biofluids and other complex samples containing matrices often contain high levels of salt. This is likely for the biofilm extracts from Winogradsky columns, as they simulate a marine environment. The ions in salt affect the ESI (Electrospray Ionisation) process. It has been found that the addition of Na^+ ions reacting with a strong acid, such as formic acid, which was included with the HPLC solvents, can lead to the formation of cluster ions. These sodium formate units form clusters, and sequentially lose units of 67.98 Da as they dissociate, the exact mass seen in the biofilm samples (80).

LC-MS is the principal method for studying biological matrices, a group that includes biofilms. Ionisation signal alterations due to proteins, lipids, sugars, or salts, are known as ‘‘matrix effects’’. Although not well studied, they occur when these interfering substances co-elute with analytes (81) (82). It would hence be advantageous for future studies to ensure contaminants are removed, for instance by performing a solid phase extraction on the organic phases or the whole extract before performing an LC-MS, to improve the quality of any data obtained.

Regardless, matrix polymers may still be an interesting avenue for future research. Biofilms do contain polymers such as EPS that undoubtedly serve a biological role, and exhibit enormous specificity (48). Polymers such as polyisoprene have also been known to have antimicrobial effects, can be used to make antimicrobial surfaces, and can be fragmented to expose their chemically reactive ends and insert biocidal groups. As many polymers have also been known to have antimicrobial effects, such as polyisoprene, and can be used to make antimicrobial surfaces, and can be fragmented to expose their chemically reactive ends and insert biocidal groups. As many polymer-based surfaces are made from fossil fuels, there is a drive to find natural polymer sources (83). Polymer based surfaces have basic physical properties that reduce surface adhesion of bacteria, preventing biofilm formation by having a low interfacial energy with water. Because it is not energetically favourable to bind with that surface, the force needed to detach a biofilm is low, and binding is reversible (84).

4.4 Isomers may be partly responsible for biofilm extract efficacy

Of the preliminary plates for WC1 biofilm extracts, WC1-11 was one of two samples which shows the strongest inhibition of *B. subtilis* growth (**Table 12**). However, once fractionated, none of the fractions showed any activity. One possibility is that too many of the inhibitory compounds were lost through the fractionation and purification process. The loss of activity could also be due to an initial synergism of many compounds, perhaps with a similar mechanism or potentiation effect, with no individual fraction containing enough to produce inhibition.

$WC2_{O}$-3 and $WC3_{O/A}$-3 were the extracts that showed the highest potency. It was noted that two compounds existed in these extracts at a higher concentration than any of the other fractions. These peaks could also be seen in the $WC1_{O}$ extracts. WC1-7, WC1-11 and WC1-4 in particular, which were the most efficacious extracts, showed a relatively high concentration of these compounds, although their exact concentrations were not known.

Only a select few fractions of $WC1_{O}$-11 were run on the LC-MS, but one fraction showed a high proportion of these compounds. There may have been other fractions that contained isomers of these compounds, and there is good reason to believe this

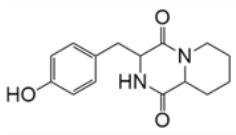
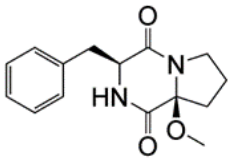
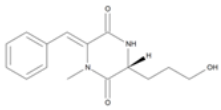
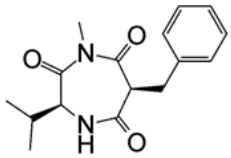
is the case, as when all of the HPLC fractions of $WC2_O$-2 were processed through the LC-MS, it was evident that the compounds exist in many fractions as isomers. The loss of the cumulative effect of these isomers might explain the high efficacy in the extract, compared to the lack of inhibition in any one fraction.

This might be further explained by additional dilution of these isomers when the extract was separated and tested in two phases. $WC3_{O/A}$-3 could not be separated into organic and aqueous phases, possibly due to a low salt concentration, and was therefore tested as one. Of all the extracts, it showed the highest potency against Sa. This led to the decision to test what remained of the aqueous phases and obtain their spectra by LC-MS. It was found that the aqueous phases of WC2 and WC3 extracts did in fact contain these compounds, some more than others. The organic extracts that showed the lowest activity in the MIC assay, retained a significant amount of these compounds in the aqueous phase. This result suggests that future experiments should not separate the two phases for activity testing, and the whole extract should undergo solid phase extraction. It also suggests the extracts should not be fractionated, rather, the isomers should be collected for testing against the reference strains.

This loss of activity also suggests that the logic behind separating the extract into two phases may be flawed. It was assumed that anything that dissolves in the aqueous phase would not be able to also dissolve in the organic phase due to its polarity, but the peaks have the same m/z ratio and retention time, which confirms these peaks most likely represent the same compounds. This may be because the compound ionises, but not well. This may result in the compound ending up in both phases, and neither phase contains enough of the compound to produce a result. In drug design, the extent of ionisation is an important consideration as polarity impacts solubility, affecting a compounds ability to cross the blood brain barrier (85).

The suggested formulae for the compounds were estimated with an unusually high certainty, indicating that they were most likely $C_{16}H_{20}N_2O_3$, and $C_{15}H_{18}N_2O_3$. There is a good chance that these compounds are related, as they differ in mass by only CH_2. In the MarinLit database, three compounds have been recorded with the chemical formula $C_{15}H_{18}N_2O_3$ and one with the formula $C_{16}H_{20}N_2O_3$ **(**Error! Reference source not found.**).**

Table.19. Possible identities from the database MarinLit that match the chemical profile of the compounds found in the biofilm extracts.

Compound name	Formula	Structure	Marine source	Ref
3-(4-hydroxybenzyl)-hexahydro-pyrido[1,2-a]pyrazine-1,4)-dione	$C_{15}H_{18}N_2O_3$		*Streptomyces* sp. LY1209	(1)
Gallaecimonamide C	$C_{15}H_{18}N_2O_3$		*Gallaecimonas mangrovi* HK-28	(2)
Pinodiketopiperazine A	$C_{15}H_{18}N_2O_3$		*Penicillium pinophilum SD-272*	(3)
Terretrione D	$C_{16}H_{20}N_2O_3$			(4)

Interestingly, one suggestion for the formula $C_{15}H_{18}N_2O_3$, pinodeketopiperazine A, and the suggested molecule for $C_{16}H_{20}N_2O_3$, territrione D, are both made by *Penicillium* spp., so come from the same genus of marine fungus. This may be an indication that these compounds are in fact related. However, to publish these findings, more proof is needed. The next step experimentally would be to isolate the two compounds in high enough concentrations to discern if they are active and to find their structure. This can be done preliminarily with MS-MS to see if they are related,

as the compounds will fragment in a similar way if the same pressure of gas is applied. The fragments can then be used to find the actual structures using NMR. However, these compounds are very different structures, so an alternative possibility is that the second compound is the same as the first with an additional –OH group, as this would also give the same mass.

4.5 *B. subtilis* inhibition by fraction 5 of select biofilm extracts may be due to a negatively ionisable compound

The compound that showed the most promise was in fraction 5 of extracts WC1-7, WC1-8, and WC1-10. This fraction contained a compound detectable in negative ion mode with m/z 478.9223, which was only half as abundant in other extracts of which fraction 5 was not active. The structure of the compound could not be found with any statistical confidence, but it is possible to know the compound is negatively charged when ionised and is dissolvable in the organic phase, suggesting that it is not highly polar. Even if the structure is not known, the compound can still be identified by its mass to charge ratio in future studies. This is useful, because if it is found again in other biofilm extracts, it can undergo further testing at other concentrations. Furthermore, if it is responsible for activity at this low concentration – the exact concentration of the compound is not known, but little biofilm was used for WC1 – this would indicate high efficacy. The compound has not been tested on any other bacteria subtype, which would be beneficial. However, because it was eluted in the fraction following the sodium cluster ion interference, it may be that the salt is actually to blame for growth inhibition.

4.6 Compound leads from Winogradsky columns may be discerned from correlative, not confirmatory findings

The methods for the Winogradsky biofilm experiments were amended after the protocols had been run for WC1. However, rather than optimising the procedure, it was found that both approaches had their benefits and costs. Taking fewer, smaller samples meant that the activity screenings were less sensitive but provided less data to sift through. There were fewer peaks in the spectra and chromatograms so when an effect was seen, it was easier to work out by elimination which compounds were having an effect. For example, for WC3, each biofilm was chosen because they had very distinct appearances. But because all extracts were efficacious in the preliminary plates, it was difficult to determine if efficacy could be correlated with the

appearance of a biofilm. Whereas for WC1, the highest efficacy biofilms, WC1-7, and WC1-11, were similar – they were dark (indistinguishable from the soil from the outside), as opposed to the mustard yellow, pink-purple or green biofilms that were more easily seen. This may mean that for a full analysis, both experiments (fewer, larger areas, and more, smaller areas) are required to both investigate potency and compare biofilms to make correlations with appearance and identify specific compounds of interest.

Not only did every extract inhibit *B. subtilis* growth to some extent, but once fractionated, almost no fractions inhibited B. subtilis growth. This posed a problem when finding the compounds responsible for the initial activity, as any peak could be antimicrobial and could just be a question of concentration, requiring a synergistic effect with compounds in other fractions to reach a threshold for efficacy. However, if high enough biofilm concentrations are obtained, single fractions will become efficacious, which would again reduce the number of peaks that could be attributed to the efficacy. A way to investigate this would be to keep the organic and aqueous extracts as one sample. The extracts that were not separated showed a higher efficacy, possibly due to compounds that were weakly ionisable being lost in both phases at a lower concentration. It would be even more beneficial to test the organic phase, aqueous phase, and combined phases at the same concentrations. The unseparated extract would give sensitive reading of efficacy, and the LC-MS of the phases would give a clearer indication of what was most potent. However, because product is inevitably lost through processing, this may not be an achievable aim.

This results-led approach also led to the exclusion of DMSO for WC2 and WC3 to avoid the high peak it produces in the HPLC. Fraction 5, which showed activity for some extracts, included a high chromatographic peak and there was some concern that DMSO was either a) producing this effect, b) obscuring the true compound peak, or c) making the other peaks appear comparatively lower and therefore harder to compare. The first two concerns turned out not to be the case, DMSO was not producing the peak as it evaporates upon drying but may have had the effect of reducing compound solubility. Future experiments should use DMSO if using the two phases-approach, as this may have caused some of the isomers to come out in the aqueous phase (3.2.2).

The colours of the biofilms were recorded with the plan to correlate them with their constituent species, and to discern if it possible to recognise the most promising biofilms as leads by their appearance. However, the metabarcoding results for these columns could not be obtained due to a problem with the kit. The most likely explanation for this was that the freezer in which were stored stopped working. By comparing the results with the 2022 dataset, it may be possible to get a sense the types of bacteria a biofilm is more likely to contain, in order to choose which to harvest. Firstly, the vast majority of bacteria were anaerobic and Gram-negative, and the biofilm extracts were only efficacious against *S. aureus and B. subtilis*, which are Gram-positive. It may be that there are fewer Gram-positive bacteria in the columns because the Gram-negative bacteria are recognising their antigens and producing antimicrobials that target them, as described in 4.1.

Although the most dominant genera were similar across all groups, there were some important qualitative differences. The green biofilms in or near the water contained a lot of *Prosthecochloris*, a type of green sulfur bacteria (86). This is also the type of bacteria that was overwhelmingly found in the barcode results for WC2 and WC3. All purple biofilms contained high proportions of a purple, non-sulfur bacteria, *Thiorhodococcus* (87). However, the biofilms in the sediment contained more *Sulfurimonas*, the ones in the water/sediment region contained an equal share of both genera, and the biofilms in the water contained more *Thiorhodococcus.* It is also notable that the higher biofilms contained *Roseivirga*, a pink coloured, strict aerobe (88). The two dark sediment biofilms produced interesting results. Firstly, the "brown/black/grey" biofilm contained none of the genera that seem to predominate other biofilm samples (*Sulfurimonas, Prosthecochloris, Thiorhodococcus).* The only dominant bacteria type was *Pseudoalteromonas,* a genus of bacteria known to produce a range of antimicrobial compounds and grow on traditional media, such as the FMAP plates used in the co-culture experiment. The dominant genus in the dark green biofilm was *Desulfuromonas*, a sulfate reducing bacteria, which, although metagenome sequencing would be required to confirm, was overwhelmingly classified as the species *Desulfuromonas svalbardensis.*

It was possible to discern that the most efficacious biofilms seem to be the ones that are dark in colour and are found in the middle and lower parts of the column, perhaps an indication that these biofilms are of bacteria that can survive in anaerobic

conditions. As both *Desulfuromonas* and *Pseudoalteromonas* are highly of interest, an experiment optimising their cultivation would be a promising avenue for research.

Future experiments should also take samples of the sediment within the samples as a control, as the biofilms may not be any more promising than the sediment. A final suggestion would be to run the protein spectra on the MALDI-TOF in conjunction with the results from the LC-MS to compare the results and indications of compound content in the samples.

4.7 The standardisation of Winogradsky column data presents a challenge for further research

Winogradsky columns may provide a rich resource for bioprospecting, as they provide many of the benefits of fresh sediment samples, such as the retention of habitat-specific compounds and interaction partners. Evidence of this ecosystem was present in our data (**Figure 21**); it was observable that the most prevalent genera in barcoding set 2 had little functional overlap, containing green and purple sulphur bacteria, sulphate reducers, nitrogen fixers, and heterotrophs. This may not only help with cultivation but also cause the bacteria to produce compounds that they would not in isolation (39).

There may be additional advantages; the column provides a closed system and environment for enrichment for biofilms to form, and their formation on the surface of the column is accessible for extraction. The instrument's content is dynamic in nature, which also makes the experiment hard to standardise. This is a problem because if a very potent compound was discovered through WC cultivation, the cultivation method might be too variable to predictably obtain the same result again, and the compound may never be obtained in a high enough concentration to resolve the structure by NMR. Firstly, it may be impossible to remove some of the content without disrupting the whole biosphere, changing the course of the experiment by interference. Consequently, it is difficult to directly test the bacterial composition of an individual column over time. However, the second-year Microbiology course here at UiT provides an opportunity, as it offers a large number of bottles taken from the same location, at the same time, on the same day. Although no individual column can be tested twice, by gathering enough data from enough columns, it might be possible to predict their most common colonies and their relative abundances at

different times. This might be a way to “normalise” the content of columns. To do this, all biofilm from several columns should be tested, and their similarity recorded.

The metagenomic analysis for WC2 and WC3 was not successful, but the finding that the problematic method skewed the proportions of bacteria detected is also an interesting result. The possibility that *Chlorobi* were unusually abundant in these columns cannot be discounted but given that these are green sulphur bacteria and none of the biofilms extracted were green, this seems unlikely. This may suggest that *Prosthecochloris* are more resilient to the changes that occurred or are a more amenable candidate to 16S sequencing. For instance, the cells might lyse more easily than other species, or may be more suited in some way to the barcode primers. Whether or not this leads to an overrepresentation of *Prosthecochloris* in normal samples remains to be determined. A standardisation should be performed whereby a sample with known species of bacteria and cell count are tested. This should be done before any new Winogradsky experiments are performed, where the species and abundance are not known.

In order to standardise the content of the columns, another experiment could be performed whereby a fresh column is inoculated with biofilms from a previous column, where the bacteria were known. This might be a way to enrich certain types of bacteria and would be of particular interest if SRB could be cultivated this way, with a more naturalistic environment and interspecies relationships. It was found that a sizeable number of bacteria in the 2022 data were sulfate reducing species, predominantly of the genus *Desulfuromonas*. The column could include enrichment that might promote the growth of SRB; Esteban et. al. found that the inclusion of calcium sulfate in the bottom of the column promotes H_2S production by sulfate reducers. Little information is given on how this was determined, but their sulfate-enriched columns showed more phylogenetic diversity sooner (40).

Unfortunately, the attempt to cultivate SRB in modified Postgate media in this experiment was unsuccessful so this would need to be addressed before sequencing, possibly with the use of a mini metagenome, which would not require complete separation of SRB from its satellites.

5 Optimisation and future prospects

Confirmation plates should be repeated so that inducers can be properly recognised. Thin layers of agar should be used to ensure the inhibition zones can be seen. For the *S. aureus* plates, samples of producers and suspected inducers should be placed at different distances to elucidate the necessary proximity. If the suspected inducers do not induce a response in the confirmation plates, suspected inhibitors should be tested. These would be identified by looking at the asymmetrical inhibition zones at the edges where less compound is being produced.

The bioactive potential for *Sinobacterium norvegicum* should be explored, as it shows promising bioactivity against Gram-negative bacteria which are difficult to target. For both this species and the known species of *Psudoalteromonas*, their predicted BGCs could be explored using recombinant DNA technology.

Where two marine test bacteria were of the same genus, and one produced a response and one did not, both isolates should undergo whole genome sequencing to establish their species and any differences in BGC clusters.

Several WC cultivation and harvesting practices have also been suggested. Firstly, biofilm extracts from a WC should be used to inoculate a sterile WC with the aim of producing a model for more replicable experiments. Secondly, darker, sediment-level biofilms should be harvested, as there is reason to believe they contain populations of interest, such as sulfate reducers or known producers of compounds with high bioactivity. The age of the WC at the time of harvesting should be recorded in case this has any effect on antimicrobial activity or an optimal time for deconstruction can be discerned. Lastly, the depth of biofilm in the column should be recorded, and different biofilms of the same colour should be noted, as their populations differ.

Several suggestions for improvements to analysis methodologies have also been suggested. Firstly, biofilm extracts should not be excluded from the MIC assays even if they did not produce a result in the in the diffusion assays. In fact, it may be preferable to forgo the diffusion assay altogether in favour of conserving material. Rather, the focus could be on the effects of Gram-negative bacteria on Gram-positive strains, especially while the method is still being optimised.

The biofilm extracts should be tested without partitioning initially and only then either fractionated or partitioned into phases, but not both at an early stage with little material. This should help to preserve the cumulative effect of isomers. Enough compounds are common between WC biofilm extracts that slightly changing the approach for each column gives new information that contributes to the overall picture of their potential for bioactivity without having too little information for analysis. If biofilms extracts are partitioned, DMSO should be used before the SPE to ensure better ionisation of the aqueous compounds.

It is important that salts are removed before performing LC-MS. An SPE should be performed on entire extracts, and an alternative to the strong acids used should be sought.

Finally, if SRB are able to be isolated in WCs, the media would need further optimisation, perhaps with agar alternatives (89).

6 Conclusion

Pseudoalteromonas, Sinobacterium and Marinobacter were documented as possible leads for further research as producers of antimicrobial compounds when co-cultured. Proximity to other marine species appears to play a role in production of antimicrobial compounds effective against *S. aureus*, but the exact role and species are yet to be verified.

Antimicrobial activity against Gram-positive bacteria was documented from extracts of biofilms that form on the inner surfaces of Winogradsky columns WCs may provide an opportunity for enrichment taxa that are hard to cultivate. Sediment-coloured biofilms from the lower portions of the column may be the most promising lead for antimicrobial discovery.

A series of bioactivity assessments were performed on WC biofilm extracts, the result of which was the detection of two compounds with antimicrobial potential. These compounds should be further investigated by purification and by retaining the isomers in the same extract for activity testing assays.

In light of these findings, the hypothesis can be confirmed; antimicrobial compounds can be produced in the presence of other bacterial species; in the context of Winogradsky columns that simulate a naturalistic environment, and in proximity to other species in the case of marine bacterial isolates.

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