UiT The Arctic University of Norway

Faculty of biosciences fisheries and economics

Bioprospecting of marine phytoplankton from large scale cultivation

Effect of culture conditions on bioactivity and biochemistry of the diatom *Porosira glacialis*

Renate Døving Osvik

A dissertation for the degree of Philosophiae Doctor

April 2021

«Jeg kniper
øynene igjen
og later som ingenting
(sa ingen
som forandret verden
noensinne)»

- **Trygve Skaug**

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Tromsø – April 2021

Marbio

&

Marine Microalgae and Microbiomes Research Group

Norwegian College of Fishery Science

Faculty of Biosciences Fisheries and Economics

UiT – The Arctic University of Norway

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Summary

The diatoms make up the largest and most important group of marine microalgae. They are responsible for a quarter of all inorganic carbon fixation in the ocean and are thus of immense importance for global CO_2 sequestration. This makes them good candidates for biological carbon capture and utilization (CCU) by mass cultivation using CO_2 emissions from industrial point sources. Large scale cultivation of marine diatoms will produce valuable biomass containing marine lipids and proteins with possible commercial application. Commercial production of biomass requires extensive knowledge on the species to be cultivated in terms of optimal cultivation conditions, but also on the bioactivity, cytotoxicity and how the species responds to changing cultivation conditions.

Most studies on the bioactivity and biochemistry of marine diatoms have been performed on species cultivated in the laboratory. Cultivation conditions are known to affect the biochemistry of diatoms, and large-scale cultivation will inevitably lead to a change in the environmental conditions. The present study was therefore designed to investigate how *P. glacialis* cultivated in large scale responds to changes in abiotic and biotic cultivation conditions.

The first study was designed to investigate whether the direct addition of factory smoke as a source of CO_2 would trigger production of bioactive or cytotoxic compounds in *P. glacialis*. Bioactivity testing revealed activity in anticancer, antibacterial and antibiofilm assays, in addition to cytotoxicity against human lung fibroblasts and effect on the development of sea urchin larvae. Nonetheless, the addition of factory smoke did not increase the cytotoxicity of *P. glacialis*, nor did it change the beneficial antibacterial and antibiofilm activity. These results are favourable for further development of large-scale production of diatom biomass.

P. glacialis was co-cultivated with zooplankton to investigate whether the bioactivity and metabolic expression of the diatom would change when exposed to grazing pressure. The bioactivity testing revealed that the presence of zooplankton increased the cytotoxicity of *P. glacialis* towards human normal lung fibroblasts. Investigation of active compounds and metabolomic analysis showed that the grazing pressure possibly influence the carotenoid concentration in *P. glacialis*. It was also found that the bioactivity might be traced to primary metabolites such as chlorophyll derived compounds. The study also showed that the use of OSMAC (one strain many compounds) could be a useful method for further investigation of the bioactivity of diatoms.

Investigation of the antibiofilm activity of *P. glacialis* resulted in isolation of two compounds showing inhibition of biofilm formation by *Staphylococcus epidermidis*. The compounds isolated were methyl 3-hydroxyoctadecanoate and a pheophorbide-like compound. This is the first time compounds with antibiofilm activity have been isolated from *P. glacialis* as well as the first evidence of such activity from both the isolated compounds.

Sammendrag

Kiselalgene utgjør den største og viktigste gruppen av marine mikroalger. De er ansvarlige for en fjerdedel av all uorganisk karbonfiksering i havet og er dermed av enorm betydning for det globale CO_2 opptaket. Dette gjør dem til gode kandidater for biologisk karbonfangst og -utnyttelse (CCU) i massedyrking ved bruk av CO_2 fra industrielle punktutslipp. Storskala dyrking av marine kiselalger vil produsere verdifull biomasse som inneholder marine lipider og proteiner med mulig kommersiell anvendelse. Kommersiell produksjon av biomasse krever stor kunnskap om arten som skal dyrkes med tanke på best mulige dyrkingsforhold, men også om bioaktivitet, toksisitet og hvordan arten reagerer på endrede dyrkingsforhold.

De fleste studier på bioaktivitet og biokjemi av marine kiselalger er utført på arter dyrket i laboratoriet. Det er kjent at dyrkingsforhold påvirker biokjemien til kiselalger, og dyrking i stor skala vil uunngåelig føre til en endring i miljøforholdene. Denne studien ble derfor designet for å undersøke hvordan *Porosira glacialis* dyrket i stor skala reagerer på endringer i abiotiske og biotiske dyrkingsforhold.

Den første studien undersøkte om direkte tilsetning av fabrikkrøyk som kilde til CO_2 ville endre produksjon av bioaktive eller toksiske forbindelser i *P. glacialis*. Bioaktivitetstesting avdekket aktivitet i alle tester: anticancer, antibakteriell og anti-biofilm. I tillegg viste resultatene toksisitet som hemmet utvikling av kråkebolle-larver og humane lungefibroblaster. Likevel økte ikke tilsetting av fabrikkrøyk toksisiteten til *P. glacialis*, og den hemmet heller ikke den gunstige antibakterielle og anti-biofilm-aktiviteten til algene. Disse resultatene er svært positive for videre utvikling av storskala produksjon av kiselalger.

P. glacialis ble dyrket sammen med dyreplankton for å undersøke om bioaktiviteten og utrykket av metabolitter ville endre seg når de ble utsatt for stress i form av beitetrykk. Testing av bioaktivitet avslørte at tilstedeværelsen av zooplankton økte toksisiteten til *P. glacialis* mot humane normale lungefibroblaster. Undersøkelse av aktive forbindelser og metabolittprofiler viste at beitetrykket muligens påvirker konsentrasjonen av karotenoider i *P. glacialis*, og at bioaktiviteten muligens kan spores til primære metabolitter som klorofyllderivater. Studien viste også at bruk av OSMAC (one strain many compounds) kunne være en nyttig metode for videre undersøkelse av bioaktiviteten til kiselalger.

Undersøkelse av anti-biofilm-aktiviteten til *P. glacialis* resulterte i isolasjon av to forbindelser som viser inhibering av biofilmdannelse av bakterien *Staphylococcus epidermidis*. De isolerte forbindelsene var metyl-3-hydroksyoktadekanoat og en pheophorbid-lignende struktur. Dette er første gang en forbindelse med anti-biofilm-aktivitet er blitt isolert fra *P. glacialis*, og det første beviset på slik aktivitet fra begge de isolerte forbindelsene.

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Renate Døving Osvik, Tromsø 25.04.2021

List of Papers

This thesis is based on the following papers:

Paper I

Osvik, R.O., Andersen, J.H., Eilertsen, H.C., Geneviere, A.M., Hansen, E.H. (2021). Bioactivity of a marine diatom (*Porosira glacialis* (Grunow) Jørgensen 1905) cultivated with and without factory smoke CO_2. *Industrial Biotechnology,* 2021. **17**(1):p. 38-48

Paper II

Osvik, R.O., Ingebrigtsen, R.A., Norrbin, F., Andersen, J.H., Eilertsen, H.C., Hansen, E.H. (2021). Adding zooplankton to the OSMAC toolkit: Effect of grazing stress on the metabolic profile and bioactivity of a diatom. *Marine Drugs,* 2021. **19**(2): p. 87

Paper III

Osvik, R.O., Ingebrigtsen, R.A., Hansen, K.Ø., Isaksson, J., Andersen, J.H., Eilertsen, H.C., Hansen, E.H. Compounds from *Porosira glacialis* with activity against biofilm formed by *Staphylococcus epidermidis*. (Manuscript)

Abbreviations

LC-PUFAs	Long-chained polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
ALA	Alpha-linoleic acid
LA	Linoleic acid
DOC	Dissolved organic carbon
PUA	Polyunsaturated aldehyde
CCS	Carbon capture and storage
CCU	Carbon capture and utilization
ROV	Remotely operated vehicle
NP	Natural products
MAG	Monoacylglycerides
Chrl	Chrysolaminarin
Fx	Fucoxanthin
Chl *a*	Chlorophyll *a*
DA	Domoic acid
Dxd	Diadinoxanthin
PBR	Photobioreactor
OSMAC	One strain many compounds
EtOH	Ethanol
MeOH	Methanol
DCM	Dicholorometane
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
UPLC	Ultra-high performance liquid chromatography
MS	Mass spectrometry
HR-MS	High resolution mass spectrometry
TOF	Time-of-flight

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1 Introduction

1.1 The microalgae group: diatoms

The diatoms impact our daily life to a higher degree than what most people recognize. Even if they only make up about 0.5% of Earth's photoautotrophic biomass, they are responsible for approximately 40% of the annual global primary production of organic carbon, which demonstrates their extreme growth rates compared to their terrestrial photosynthetic counterparts. Also, this translates to a higher CO_2 capture than all terrestrial rainforests combined [1-4]. Each year diatoms fixate a quarter of all inorganic carbon in the ocean and are therefore of immense importance for global CO_2 sequestration [5]. These microscopic photosynthetic organisms are in many ways the Oceans invisible forest and play an important role in regulating our climate [6].

The diatoms are a highly diverse group and can be found in any thinkable habitat on Earth [7]. They belong to the heterokontophytes, commonly referred to as brown algae due to their golden-brown coloured chloroplasts. About 12 000 species are described today, and a higher number is estimated to exist [8]. This number is debated, and ranges from 20,000 to 200,000

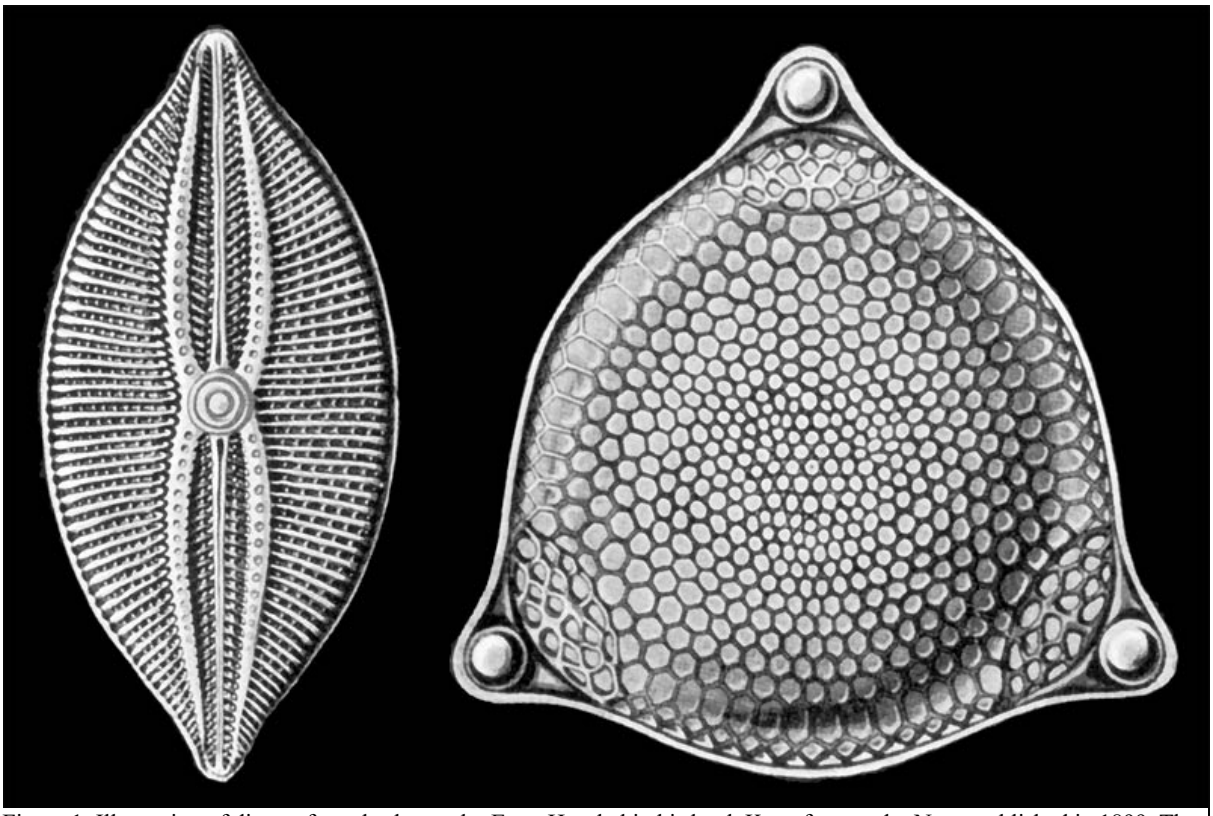

Figure 1: Illustration of diatom frustule shapes, by Ernst Haeckel in his book Kunstformen der Natur published in 1899. The cell to the left is a pennate diatom with bilateral symmetry. The cell to the right is a centric diatom with radial symmetry.

species [8, 9]. Nevertheless, the diatoms are regarded as the most successful group of microalgae with respect to diversity as well as biomass and primary production. The immense species diversity can be reflected in the extravagant morphological variety among the different genera of diatoms. The diatoms are divided into two groups based on the shape of their cell wall, called the frustule: centric diatoms or pennate diatoms, seen in Figure 1 [10]. The structure of the diatom frustule can be compared to a Petri dish, where the top half (epitheca) overlaps the bottom half (hypotheca). The frustule is composed by amorphous silica, giving them a glass cell wall [11]. Everyone who has observed diatoms in a microscope have seen the intricate structures of the frustule which is the true hallmark of the diatoms. The species we know today are mainly identified by the structure of their frustule, formation of chains or arrangement of setae (thin brush like structures protruding the frustule). Illustrations made by Ernst Haeckel at the end of the 19th century (Figure 2) show that diatoms have been studied for centuries, and displays the great morphological variation [10]. The *Thalassiosira pseudonana* genome (32.4 megabases) was the first diatom to be fully sequenced [12], and a genome sequencing of *Phaeodactylum tricornutum* (27.4 megabases) followed not too long after [13]. A comparison between the two showed that only 57% of the genes are shared, which can be compared to the same portion of genes shared between humans and fish. Revealing a great genetic diversity among these microalgae.

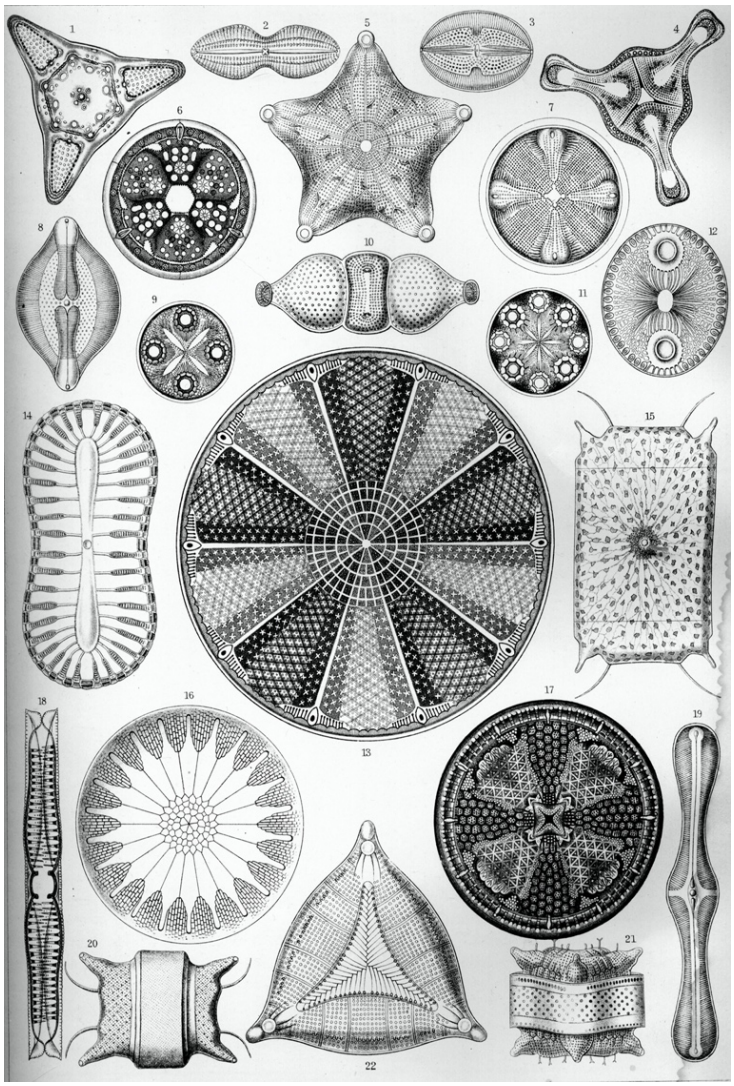

Figure 2: Illustration of a range of diatoms species by Ernst Haeckel in the book Kunstformen der Natur published in 1899.

Diatoms divide through binary fission. The two valves of the frustule divide, and both become the epitheca of the new cells, while a new hypotheca is formed to fit inside the epitheca. Thus, with each

mitotic division, the daughter-cells decrease in size, known as the MacDonald-Pfitzer hypothesis [14]. This decrease in cell size cannot continue eternally and a sexual reproduction with restoration of cell size therefore takes place periodically. This process can also happen during nutrient depletion, or as a response to unfavourable light conditions [14-16].

The diatoms are not only beautiful to observe in the microscope, but they also have a lot to offer on the inside. The diatoms are the dominant primary producers in the temperate and Arctic oceans, and they dominate all areas where fish production is high e.g., the Benguela, Humboldt and Canary upwellings as well as the Barents sea [17]. Being at the base of the marine food chain, they lay the foundation for all marine life (Figure 3). Diatoms and other phytoplankton are regarded as the main food source for zooplankton, early life stages of crustaceans, bivalves and fish larvae [18]. The valuable nutrients from the marine diatoms are transferred upwards in the food chain via zooplankton to fish and marine mammals, often ending up at our dinner table. Diatoms supply us with important fatty acids required in our diet. The marine long-chained polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) which are supplied through consumption of high-fat fish are originally synthesized by phytoplankton [19-21]. EPA and DHA are linked to reduction of cardiovascular disease, proper brain development and function and prevention of certain types of cancer [22]. EPA and DHA are synthesized from the two precursors alpha-linolenic

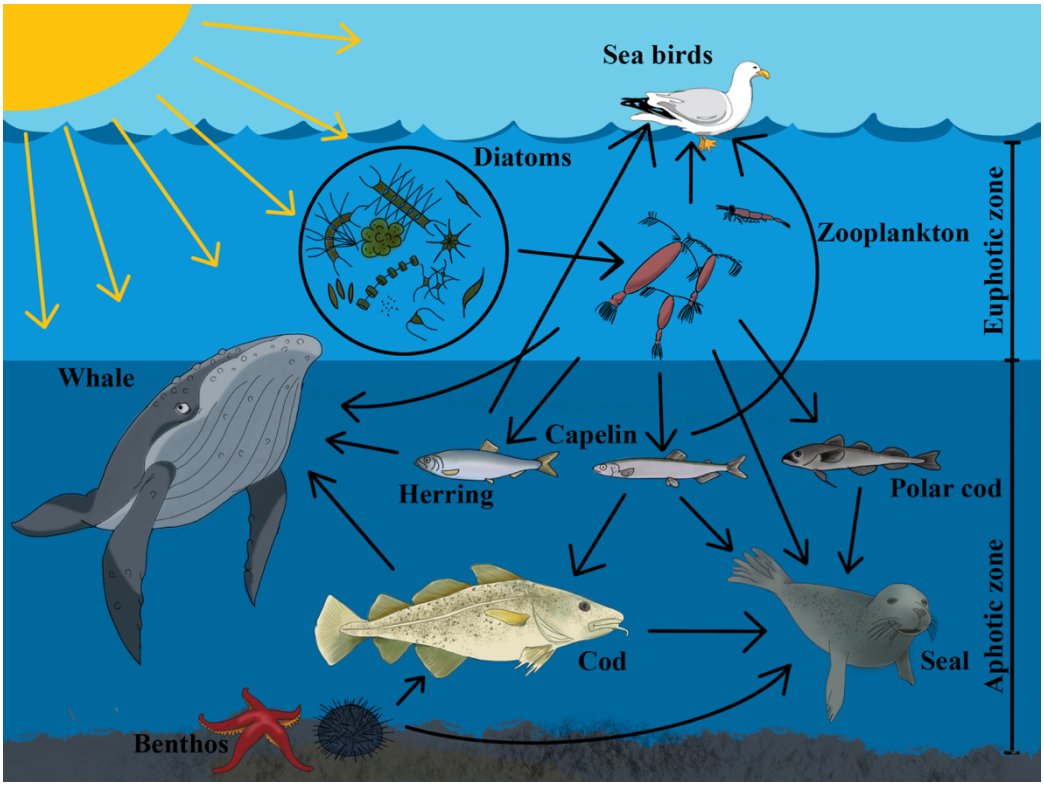

Figure 3: Marine food web of the Northern Norwegian coast. Figure: R. Osvik

acid (ALA, C18:3, n-3) and linoleic fatty acid (LA, C18:2, n6) which are the essential fatty acids. Humans and other mammals can convert ALA and LA to EPA and DHA, but the conversion rate is low [23]. Thus, the diatoms are essential for the survival of fish larvae, marine zooplankton [24, 25] and eventually, us [22, 26].

1.2 The spring bloom

The abundance and species composition of phytoplankton varies seasonally, and few species are present year-round [17]. Light is naturally the main limiting factor which hinders high phytoplankton production during the winter. Photoautotrophic organisms harvest visible light (400-700 nm) using photosynthetic pigments to produce organic bound carbon from CO_2 [27, 28]. The sun resides below the horizon during winter above the Arctic Circle, leaving little light for primary production. The March vernal equinox marks the beginning of spring in the

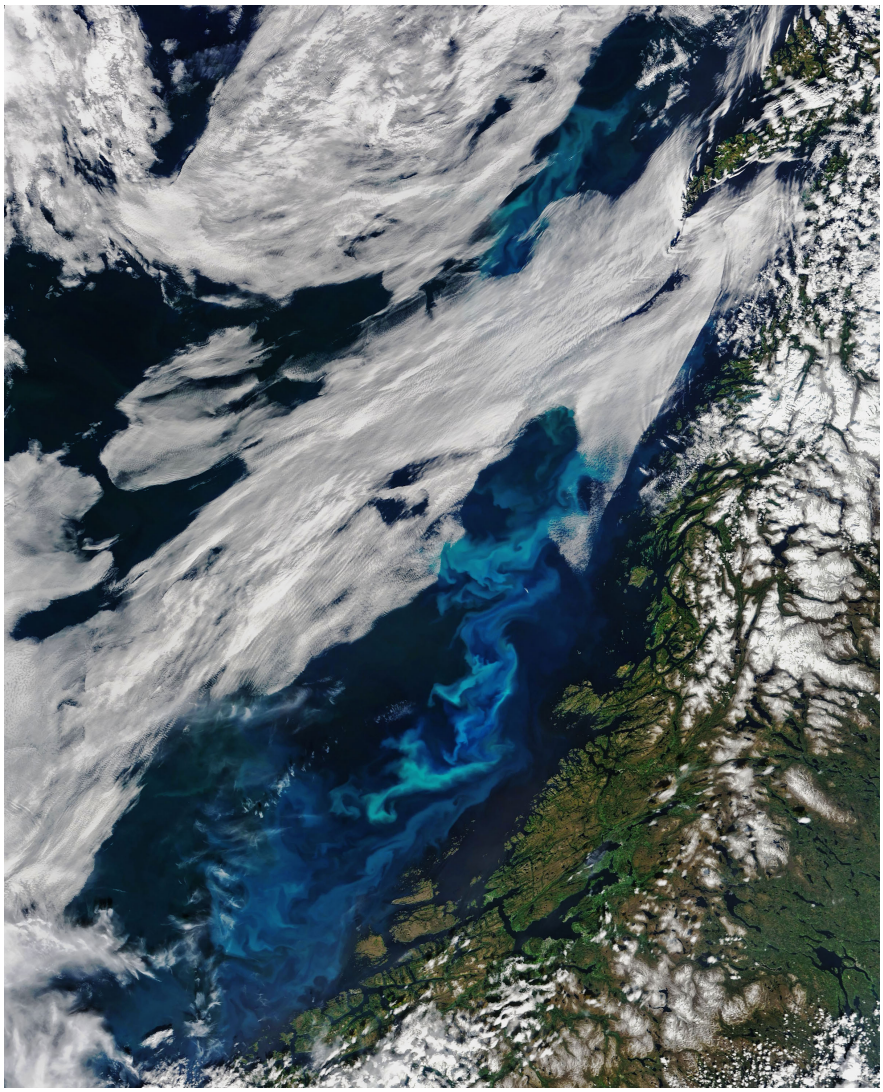

Figure 4: Satellite picture illustrating the immense prevalence of a microalgae bloom along the coast of Nordland and Trøndelag in Norway, June 2019. Photo: The Earth Observatory, NASA.

Northern Hemisphere. As the sun rises higher on the horizon and the daily photoperiod increases, the annual spring bloom commence. Upwelling from the ocean floor carries nutrients and phytoplankton spores up to the euphotic zone. The spores germinate into vegetative cells that divide further while species appear in successions [28, 29]. As mentioned, diatoms dominate the spring bloom in both the temperate and polar seas. During this bloom the diatom biomass grow exponentially, reaching cell numbers of 6-10 x 10^6 cells L^{-1} at its peak [17]. The spring bloom is such a massive event that its prevalence can be seen from space (Figure 4).

Equation 1:

$$6CO_2 + 6H_2O + \hbar v = C_6H_{12}O_6 + O_2 + heat$$

The photosynthetic equation (Equation 1) illustrates that carbohydrates are produced from inorganic carbon and water, using light as the energy source. By taking up nitrogen, phosphorus and other inorganic nutrient elements from the surrounding water, these primary producers are able to synthesize other nutritious compounds like proteins and lipids. Carbon and hydrogen are nearly always present in surplus in the ocean, while inorganic bound nitrogen, phosphorus and iron are often limiting in oligotrophic areas and at the end of bloom events [30]. Silicate is also essential for the growth of diatoms, and the access will gradually decline during the bloom.

Some diatoms secret polysaccharides functioning as a gel to keep cells of the same species together in close contact. This gel and the diatoms photorespiration release dissolved organic carbon (DOC), which is an important substrate for marine bacteria, thus diatoms do not only produce nutrients for higher trophic levels, but also for those below [30-32]. The nutrient limitation combined with grazing pressure leads to the end of the spring bloom. Parts of the remaining diatom cells will dissolve and become substrate for marine bacteria. What is left over will eventually sink to the bottom sediments where they form resting spores and await the right conditions for the next bloom to commence [28, 33, 34].

1.3 Planktonic interactions in the ocean – a network of chemical signals

The open ocean incorporates webs of interactions not visible to the naked eye. Microalgae, bacteria and zooplankton are termed “plankton” since they are the drifters of the ocean. They

lack strong means of movement or swimming and are at the mercy of the oceanic currents and waves. In this moving habitat, they are in constant contact with each other, for better or worse. Their relationship can be of a symbiotic, commensal or parasitic nature, or a predator-prey relationship [35].

1.3.1 Interactions between diatoms and bacteria

The first radial centric diatoms appeared in the Jurassic [14], thus their morphology and genome have evolved and diversified over approximately 200 million years. And at the same time, their relationship with bacteria has been shaped over an evolutionary timescale. The co-evolution can be reflected in the diatom genomes. Genome sequencing of *P. tricornutum* and *T. pseudonana* revealed a remarkably high number of genes transferred from bacteria to the diatoms [12, 13]. This shows that horizontal gene transfer from bacteria has been one of the main driving forces during the evolution of diatoms. Studying these planktonic relationships *in situ* is quite challenging as the oceans are in constant movement. Their terrestrial counterpart, the relationship between plants and microorganisms has been thoroughly studied. One example is the symbiotic relationship between plants and bacteria, where N_2 fixating bacteria live in the plants root nodule system, providing the essential N_2 for the plants [36]. These symbioses are easier to observe as they are confined to a limited area, and to a large extent, non-motile. Studies of diatom-bacteria interactions have mostly taken place in the laboratory, in the littoral zone, or on benthic diatoms [31]. Nonetheless, surveys of the plankton communities *in vivo*, like the Tara Ocean, have found that, similar to terrestrial plants, there are symbioses between diatom and N_2-fixating cyanobacteria in the marine environment as well, especially in low-nutrient areas [37, 38].

Interactions between diatoms and bacteria take place for the most part in the phycosphere of the diatoms. The term “phycosphere” was first published in 1972, and is defined as the region extending outward from the algal cell and is the aquatic analogue to the rhizosphere found in terrestrial plant roots [31, 39]. Several studies on the phycosphere have been conducted, with the aim of exploring the bacterial diversity that the diatoms are surrounded with. In a study by Sapp et al. [40] the bacterial community of the phycosphere of four different diatom species, *Thalassiosira rotula*, *Skeletonema costatum, Pseudonitzschia pungens* and *Guinarida delicatula*, were sequenced. The study revealed that Alphaproteobacteria and Gammaproteobacteria were the predominant bacterial groups associated with these diatoms and accounted for 43% and 37% respectively.

When cultivating diatoms, either in the laboratory or in an industrial scale, the cultures are rarely axenic. Bacteria will always be present, but usually they don't cause any problems for the diatoms. Cultivation of photoautotrophic diatoms does not require addition of organic nutrients, and the traditional diatom cultivation medium, Guillard F/2 [41], does not contain an organic carbon source. Yet, bacteria thrive in many diatom cultures. Most diatom species excrete an extracellular matrix mostly made up of carbohydrates. This matrix can often be seen in a diatom monoculture cultivated in the lab, as it tends to form a viscous gel surrounding the cells. The biomolecules form a perfect base of DOC for bacteria in the diatoms phycosphere [40, 42]. Just like multicellular eukaryotes house microbial communities (e.g. gut microbes), several studies have shown that diatoms secrete specific metabolites into their phycosphere to promote attachment of beneficial bacteria (e.g. rosmarinic acid) or supress the attachment of opportunistic ones (e.g. azelaic acid) [43]. Beneficial bacteria can be strains which diatoms rely on for synthesis of vitamin B12 (Cobalamin) [44, 45]. Thus, they can coexist and benefit from each other.

There are examples of far less beneficial relationships. Some algicidal bacteria can enter the phycosphere and release compounds capable of killing the diatom [31]. *Kordia algicida* is known to produce a protease with algicidal activity against species of *Skeletonema, Thalassiosira* and *Phaeodactylum* [46]. Diatoms also have defences against harmful bacteria. Antibacterial activity in diatoms has been linked to polyunsaturated fatty acids and has been found in several diatom species: *S. costatum, P. tricornutum* and *Navicula delognei* [47-49].

1.3.2 Interactions between diatoms and zooplankton

Marine herbivores, such as copepods, have been known to time their reproduction so that larvae development match with the peak of the spring bloom. This concept is part of what is termed the Cushing´s match-mismatch hypothesis [50]. By such timing the zooplankton stock can take advantage of the abundance of nutrients provided by the blooming primary producers. A mismatch on the other hand is bad timing that leads to transfer of less nutritious material up the food chain, affecting the next generation negatively [51]. Diatoms have traditionally been regarded as a significant food source for herbivores, and the nutritional value of the diatoms is highly important for the transfer of energy upwards in the food chain, supporting important fisheries [52-54]. Although, this predator-prey relationship was questioned in the beginning of the 1990s when several studies showed that certain diatom diets could have a negative effect on grazing copepods.

Ianora et al. reported in 1993 that a diet of *Thalassiosira rotula* seriously affected the hatching success of the eggs of the copepod *Temora stylifera* negatively [55]. Following this discovery, a series of studies uncovered the potential negative effect of a diatom diet on several copepod species [25, 56-58]. Diatoms showed a teratogenic effect meaning that the diet does not affect the grazer itself but rather the next generation [59]. Examples of familiar plant compounds with teratogenic effect are nicotine and alcohol, which both affect the foetal development in humans. In the case of copepods the teratogenic effect appears as production of abnormal eggs, interference with hatching of eggs or as malformation of nauplii larvae post hatching [54, 60]. The effect was shown to be dependent on density, as successful hatching of eggs increased with decreasing concentration of diatoms in the diet [61-63]. Oxylipins, especially polyunsaturated aldehydes (PUA) have been pinned as the main culprits of these harmful effects. Three low-molecular PUAs were isolated from *T. rotula, Skeletonema costatum* and *Pseudonitzschia delicatissima:* 2-trans-4-cis-7-cis-decatrienal, 2-trans-4-trans-7-cis-decatrienal, 2-trans-4-trans-decadienal which all had an effect on the embryonic development of *Calanus helgolandicus* [64]. Following this, a number of different PUAs have been isolated and both laboratory and field studies have confirmed the cytotoxic effect of diatoms [65, 66]. It was also discovered that a diatom diet did not only have a negative effect on copepods, but also other zooplankton species such as sea urchin larvae [67-71].

PUAs are not produced and excreted by the diatoms to scare grazing copepods away from eating them. They are rather produced enzymatically within seconds after cell rupture due to feeding, by oxidation of polyunsaturated fatty acids (e.g. EPA and DHA) [72-74]. It has also been suggested that cell lysis towards the end of a diatom bloom causes release of PUA [75]. The release of PUAs to the environment could also affect the grazing pattern of planktonic herbivores [76]. The number of studies confirming PUAs cytotoxic effect on zooplankton could suggest that the diatoms are not as valuable a food source as one thought. Although not all diatoms are known to produce PUAs. In a study by Wichard et al. in 2005 [77], 50 species (71 isolates) of diatoms were examined for production of PUA, and 27 were identified to contain PUAs, showing that it varies between species, and strains. Other studies have found no effect of PUAs on selected grazers [25, 78-81]. It has also been found that diatoms ability to produce PUAs can vary with season, and there are evidence that that stress due to nutrient deficiency can trigger PUA production [82, 83]. Although some species of diatoms have a negative effect on the hatching of copepods they are still a highly important nutrient source for the planktonic marine ecosystems (Figure 5).

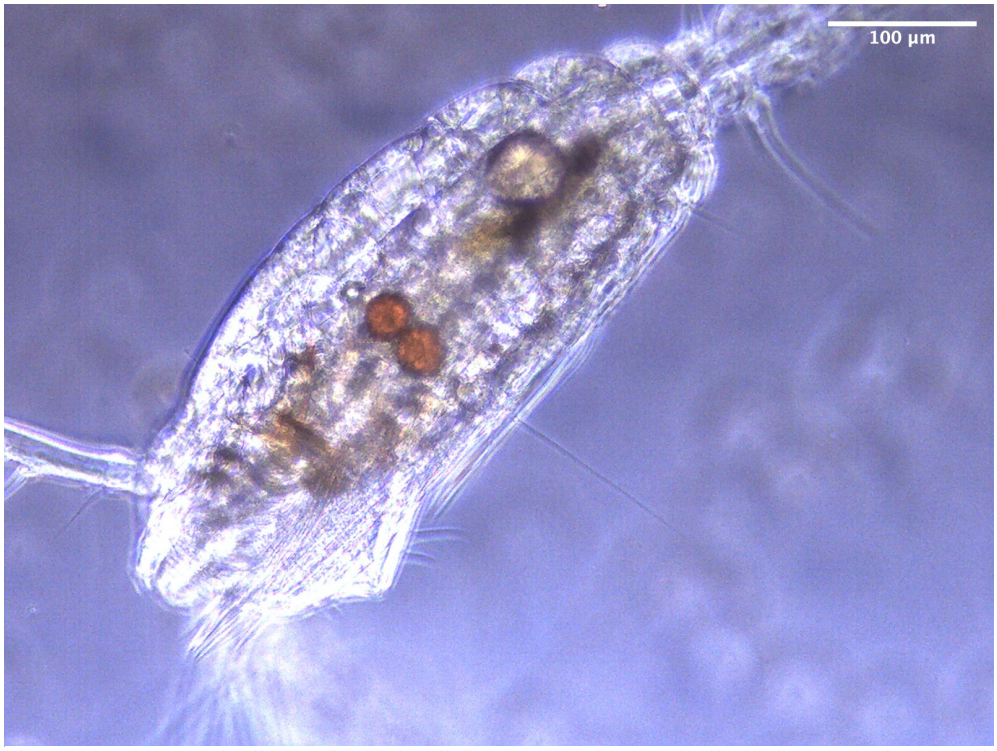

Figure 5: *Oithona* sp. feeding on the marine diatom *P. glacialis*. Intact cells can be seen in the stomach of *Oithona* sp. Photo: R. Osvik

Copepod species of *Calanus* sp. and *Pseudocalanus* sp. release chemical cues, taurine conjugated lipids, called copepodamides that affect the diatoms [84, 85]. These cues can be perceived by microalgae as a warning on the presence of grazers, and triggers both chemical and morphological changes in some diatom species. Chain-forming *Skeletonema costatum* reduce chain length in response to copepodamides [86, 87]. A reduction of chain length could sound counterintuitive as a defence mechanism. But the term "the bigger the better" does not apply here, as copepods have a lower grazing rate on singe cells [88]. Diatom *Pseudo-nitzschia seriata* responds by increasing production of domoic acid, the toxin known to cause amnesic shellfish poisoning, thus leading to harmful algae blooms [89]. These defence elicitors are the first to be identified in marine plankton and reveal how these small organisms can cause a cascade of chemical response in the planktonic food web.

1.4 From primary producers in the ocean to industrial producers on land - Mass cultivation and carbon capture and utilization

Similar to agricultural crops, marine algae can be cultivated and exploited for their valuable chemical contents. Microalgae have certain obvious advantages over traditional terrestrial crops: up to 20-fold higher productivity per area, does not compete with arable land, high growth rates (>100% increase in biomass per day), year-round production and high lipid content

[90]. The history of microalgae cultivation is a little over 100 years old. *Chlorella vulgaris* was the first green algae to be isolated and cultivated for the purpose of studying plant physiology [91]. Mass cultivation for production of biomass was first attempted in the late 1940s. Even though the industry has seen a growing interest in recent decades, less than 20,000 tons of microalgae biomass is produced globally each year (mainly phototrophic) [92]. This is a mere fraction of per million of the World's soy production. Common microalgae products are carotenoids (e.g. astaxanthin, fucoxanthin and ß-carotene), lipids and polysaccharides [93]. Renewable production of biofuels from microalgae received great attention some decades ago [94-99], but the focus has shifted as the production was not economically viable [100]. Despite their vast species diversity, nutritional importance and high lipid content, diatoms have not been among the most sought-after microalgae for mass cultivation. They are underrepresented on the list of commonly cultivated species. *Chlorella, Hematococcus, Rhodomonas* and *Nanochloropsis* are widely exploited species, while *Pheaocatylum tricornutum* seems to be the only diatom that is cultivated at a larger scale for production of fatty acids [101]. Borowitzka and Vonshak define large-scale production to be >10 tons dry mass per annum which is enough for commercial high value products, such as pharmaceutical or nutraceutical products [100]. Other diatom genera such as *Skeletonema, Thalassiosira* and *Chaetoceros* are commercially exploited as live food in aquaculture [101].

Microalgal mass cultivation systems are found in a wide array of shapes and sizes. The design and variation are only limited by the imagination of the designer and the physical limitations of the structure. Despite the structural variations, the cultivation systems have one common important function: to facilitate optimal and economically sustainable algal biomass production by exposing the largest fraction of the microalgal cells to optimal production conditions. No optimal design exists today and there is no gold standard for photoautotrophic microalgae cultivation. Photobioreactors can be open or closed systems, both of which comes with their advantages and disadvantages [94]. Among the open reactors you find artificial ponds, tanks and raceways [91]. All these have relatively low production and maintenance cost and facilitate the use of direct sunlight. Nonetheless, the risk of uncontrollable environment and contamination by other species, both microalgal and bacterial, is high. Closed reactors are often formed as tubes or flat panels made of glass or plastic. Tubular photobioreactors have a high production cost, and are difficult to clean, though there is low risk of contamination. Ultimately, the choice of photobioreactor must be based on the requirements of the microalgae to be cultivated, and their preferred growth conditions [102].

The list of factors to consider before choosing a cultivation system is long: the biology of the producing species, energy input, water availability, nutrient, climate, light, temperature, the target product and environmental impact [91]. The last factor to be mentioned is the carbon source. All photoautotrophic organisms rely on CO_2 as a carbon source. To achieve a viable production, addition of CO_2 above the atmospheric concentration is essential. CO_2 is one of the major costs of diatom cultivation, but can potentially be solved by industrial partnership [99].

Emissions of CO_2 has for many years been the culprit of the climate change we see today. Industry, fossil fuel, travel and so much of what we depend on in our daily lives emit large amounts of CO_2. Various measures have been proposed to reduce the amount of CO_2 emissions. In Norway alone greenhouse gas emissions were 50.3 million tons in 2019, 84 % of that being CO_2 [103]. The Norwegian government has for several years worked towards a solution for carbon capture and storage (CCS), where CO_2 emissions are captured and stored in a safe manner, often in geological reservoirs below the ground or beneath the seafloor [104]. Another way of handling CO_2, which has received somewhat less attention, is carbon capture and utilization (CCU). This is a process where CO_2 is regarded as a resource, rather than waste, which can be recycled and converted into more valuable products [105]. Captured CO_2 can be utilized in chemical industry for production of methanol or urea for fertilization, it can be mineralized into solid carbon, the food industry can use it for carbonization of beer, or it can be used in production of various plastic materials. CCU can also be done by utilizing primary producers for CO_2 sequestration. Diatoms can be of substantial help in such CO_2 mitigation strategies. Emissions from point sources can be used as a carbon source in microalgae cultivation thus removing CO_2 from the Earth's atmosphere [102]. Mass cultivation of marine diatoms using CO_2 directly from a point source is one method of CCU where one exploits the natural ability of photosynthetic organisms to capture CO_2 and transforming it to valuable nutritious biomass. By connecting high-emission industries with diatom mass cultivation plants we can facilitate for a CCU symbiosis and the production of renewable marine biomass [106].

1.5 Marine biodiscovery

There is no doubt that the ocean has had an attraction on mankind since the beginning of time. The unknown, the inaccessibility and the vastness of the oceans has triggered the curiosity for new discoveries. Life began in the oceans, and out of our 36 known animal phyla, 34 are found in the ocean (15 being exclusively marine) while only 17 are found on land [107]. This great diversity has been an inspiration for scientists in the search for new bioactive compounds.

Although not nearly as widespread as terrestrial plant medicine, the use of marine species for medicinal purposes started long before we had access to new submersible technology. *Chondrus crispus* and *Mastocarpus stellatus* (red macroalgae) were used to cure symptoms of chest infections, while the red algae *Porphyra umbilicalis* as part of the diet were found to have an effect against cancer [108, 109]. After the development of technology for diving and submarines and ROVs for deep diving, we have been able to conduct a more methodical search for new and interesting chemistry among the marine biodiversity.

More than half of the drugs on the market today are Natural Products (NPs), or synthetic and semi-synthetic compounds based on NPs [110]. Common for the NPs is that they originate from secondary metabolites. Primary metabolites are the essential for the producing organism for growth, development and reproduction. In contrast, secondary metabolites are not essential, but produced in given situations and conditions in order to enhance their chances in competitions with other. This could for example be toxin production to repel grazers and predators [111, 112]. Almost all NPs in clinical use are from terrestrial sources, but of today there are 15 drugs from marine sources [109, 113]. Among these are 10 anticancer drugs, including Yondelis® from the tunicate *Ecteinascidia turbinata* against soft tissue sarcoma and ovarian cancer (2015) [114], Halaven® based on the pharmacophore from the sponge *Halichondria okadai* against breast cancer (2010) [115], Adcetris® from *Dolabella auricularia* against Hodgkin's lymphoma and anaplastic large cell lymphoma (2011) [116], and Zepzelca™, isolated form a tunicate against metastatic small cell lung cancer [117]. The other drugs are Vira-A® with antiviral activity against Herpes Simplex virus [118] and Prialt® from the cone snail *Conus magus* used as a pain killer for patients undergoing severe chronic pain [119]. In addition, three Omega-3 fatty acids from fish are used for treatment of hypertriglyceridemia: Lovaza®, Vascepa® and Epanova® [113].

As of 2020 [109, 113, 120], 23 marine NPs were in clinical trials phase I, II or III, and the search for new drugs is constantly ongoing among researchers in the field. One might question why there is a need for new drugs, and the reasons are plentiful. Living conditions have improved in many countries, increasing our life expectancy [121]. Living longer also means increase in cases of non-communicable diseases like cardiovascular diseases, cancers, asthma and diabetes, many of which there is no cure for today. Another reason for our increased life expectancy is the possibility to treat infectious bacterial diseases. Antibiotics have been used to treat serious infections since World War II. But already by the following decade, penicillin resistance had become a problem. Since then many new antibiotics have been discovered, but

resistance has developed towards nearly all of them [122]. Former lack of regulation, non-prescription over the counter sale, extensive use in agriculture, and over use in general has increased the rate of resistance [122]. Simultaneously, antibiotic development has not been considered economically viable over the last years, and the recourses allocated to this purpose has decreased. All of this makes antibiotic resistance one of the greatest risks to human health in the future according to WHO. Apart from the non-treatable diseases we face today, new diseases can and will certainly also arise. It is inevitable not to mention the events of 2020, and the ongoing COVID-19 pandemic. The rapid global spread and occurrence of mutations of a new type of corona virus has showed us that there could be a sudden need for new treatments and new vaccines. Natural products have formerly contributed to treatments of MERS-CoV and influenza, and could be a source for drugs against viral diseases such as the one we are battling at this very moment [123].

1.5.1 The role of diatoms in marine biodiscovery

Today, there are no pharmaceuticals derived from diatoms (or microalgae in general), and they are perhaps less studied than invertebrates and bacteria. However, among the few species that are investigated, there is no lack of reported bioactivity [124-130]. The aforementioned polyunsaturated aldehydes (Figure 6) are not only teratogenic against zooplankton, but also have an anticancer activity against human lung adenocarcinoma and colon adenocarcinoma cell lines [128]. Miralto et al. [64] reported in 1999 that three aldehydes isolated from *Skeletonema costatum*, *Pseudo-nitzschia delicatissima* and *Thalassiosira rotula* had an anticancer effect towards the colon cancer cell line Caco2. Monoacylglycerides (MAGs) isolated from

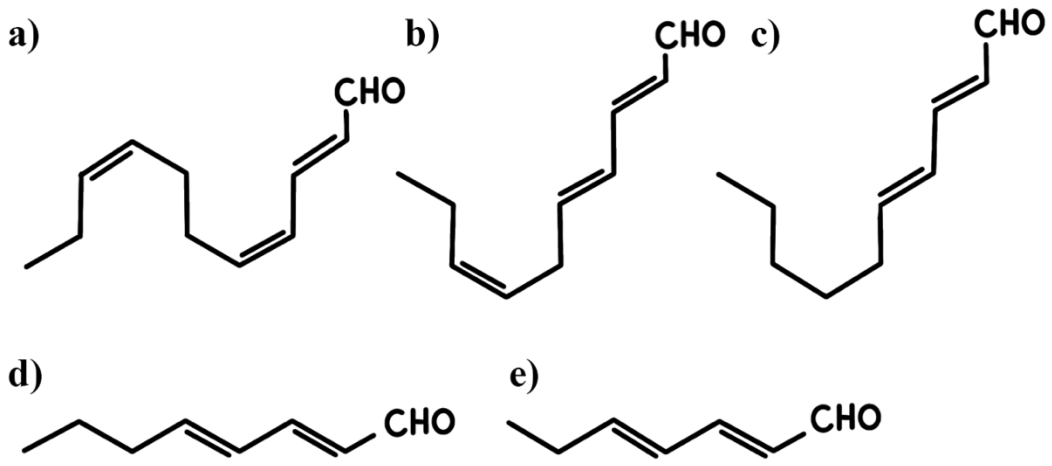

Figure 6: Diatom derived polyunsatruated aldehydes (PUAs); (a) 2-trans-4-cis-7-cis-decatrienal; (b) 2-trans-4-trans-7-cis-decatrienal; (c) 2-trans-4-trans-decadienal; (d) 2-trans-4-trans-octadienal; (e) 2-trans-4-trans heptadienal. Figure adapted from Martínez Andrade, 2018 [184].

Skeletonema marinoi have displayed anticancer effects on colon cancer and haematological cancer [131]. The MAGs tested consisted of both EPA and DHA, ω-3 PUFA with several documented beneficial health effects [124, 132, 133]. Diatoms produce a water-soluble polysaccharide called chrysolaminarin (Chrl) and can make up >50 % of the dry weight of the cell. Chrl have exhibited activity against two colon cancer cell lines (HT-29 and DLD1), but the commercial potential is yet to be developed [134, 135]. Anticancer effect has also been reported from the pigment fucoxanthin (Fx) produced by *Odonotella aurita* [136]. Fx is one of the carotenoids giving diatoms their golden-brown colour. It has also proven to be a quite interesting compound with an array of bioactivities, in addition to anticancer activity: antioxidant, anti-inflammatory, antihypertensive and antiobesity [137, 138]. A study by Lauritano et al. [139] investigated antiinflammatory activity in the benthic diatom *Cylindrotecha closterium*, and dereplication analysis showed that the activity was most likely caused by lysophosphatidylcholines and pheophorbide a, which are membrane compounds and chlorophyll *a* (Chl *a*) derivatives respectively. Apart from the bioactivity connected to specific diatom derived compounds, activity has also been reported from testing more complex chemical fractions. Fractions of extracts exhibiting anticancer, antiinflammatory, antibacterial and antibiofilm activity was found in studies screening several species of diatoms [125, 127, 129, 130]. Some diatoms species of the genus *Pseudo-nitizscha* also produce a potent neurotoxin called domoic acid (DA). A toxin responsible for a specific type of shellfish poisoning termed amnesic shellfish poisoning due to one of the symptoms being memory loss [140]. The chemical structure of DA resembles that of glutamic acid, an important neurotransmitter in the mammalian nervous system. DA acts as a potential agonist of the glutamate receptors, and can therefore have potential in the study of neurodegenerative disease [140, 141]. All examples above proves that diatoms harbour a vast pharmaceutical potential, and though not realized yet, in time it could be turned in to valuable commercial products.

Biodiscovery does not only cover the search for new drugs, but all compounds with a commercial interest. Thus, including nutraceuticals, “cosmeceuticals”, antifouling agents, enzymes or whatever bioactivity one might seek. Development of functional products is a lot faster than development of pharmaceuticals. Many companies opt for that route, as it offers lower risk and quicker potential return. Diatoms are packed with valuable nutrients: lipids (7.2-23 %), proteins (12-59 %) and carbohydrates (4.6-9.8 %) [142-144]. Diatom derived ω-3 PUFA for human consumption are already on the market. The Swedish company Simris Alg [145] has several products containing EPA from *Phaeodactylum tricornutum* sold worldwide. A valuable

marketing tool for algae derived ω-3 PUFA is that it represents a "green" alternative, or the vegan alternative to products from cod liver, krill or *Calanus* species. With the increasing focus on sustainability, organic products and a reduction of meat intake this is a valuable point for producing diatom biomass for human consumption.

Diatoms get their colour from a variety of carotenoids, among them β-carotene, fucoxanthin (Fx), and diadinoxanthin (Ddx). Fx from *P. tricornutum* is used in the dietary supplement FucoVital™ claimed to improve liver health due to antioxidative effect [146]. The antiobesity active Fx is used in a few weight-management products e.g. fücoTHIN® from Garden of Life [147]. The Fx in these products is derived from brown algae, but as diatoms has a higher content, and the access is not limited by seasonality in growth, diatoms can be a potential future source [135]. Due to the bioactivity of Fx, it can also be a promising candidate in the development of a new marine drug [136]. Apart from use in pharma- or nutraceuticals, carotenoids have potential as food supplements, food colorants or for cosmetic purposes [109].

Fossilized diatom shells form "diatomaceous earth" which has a variety of applications, ranging from filter medium in water filtration systems, abrasive agent in tooth paste, mechanical insecticide and absorbents, to dynamite [140]. The ornamentation of the diatom frustule has been scrutinized carefully, and in nanotechnology it has e.g. a possible application in production of solar panels [148]. The frustule is also suggested to have medical application. Due to the pores in the biosilica and the possibility to be modified to bind with antibodies there are studies on using diatom frustules as drug delivery systems to direct drugs directly to the target. In that way it is possible to deliver drugs known for low solubility or low uptake by oral administration [149, 150].

The potential of diatoms as a source of commercial products is without doubt great, not only in terms of their nutritional content but also bioactivity and the use of their silica shell in nanotechnology. Publications that deal with diatom physiology and ecology is abundant, but comprehensive knowledge of the production of bioactive compounds and secondary metabolites under various cultivation conditions is largely lacking. The present PhD project was carried out with this in mind, trying to take a step further on the road to a greater understanding of the biochemistry and bioactivity of *P. glacialis* and how well the species respond when introduced to new environmental conditions.

2 Materials and Methods

2.1 The carbon capture and utilization (CCU) project

This PhD was conducted as part of a carbon capture and utilization project. The project is a collaboration between the Arctic University of Norway (UiT) and one of the largest ferrosilicon producers in Norway, Finnfjord AS. The aim of the CCU project is to mass cultivate nutritious diatom biomass by sequestering CO_2 directly from the factory fume (Figure 7). Inherent of the project was also monitoring of algae growth rate, cultivation environment, chemical composition of the biomass, cytotoxicity but also to detect bioactivity that could be exploited for nutraceutical and pharmaceutical purposes. What affects the bioactivity and biochemistry of the diatoms is quite beneficial to investigate as it can aid in the research towards finding the optimal growth conditions for high quality biomass. As a part of the CCU project, the main focus of this thesis has therefore been the investigation of the bioactivity of the marine diatom *Porosira glacialis* and assessing how bioactivity can relate to the cultivation environment.

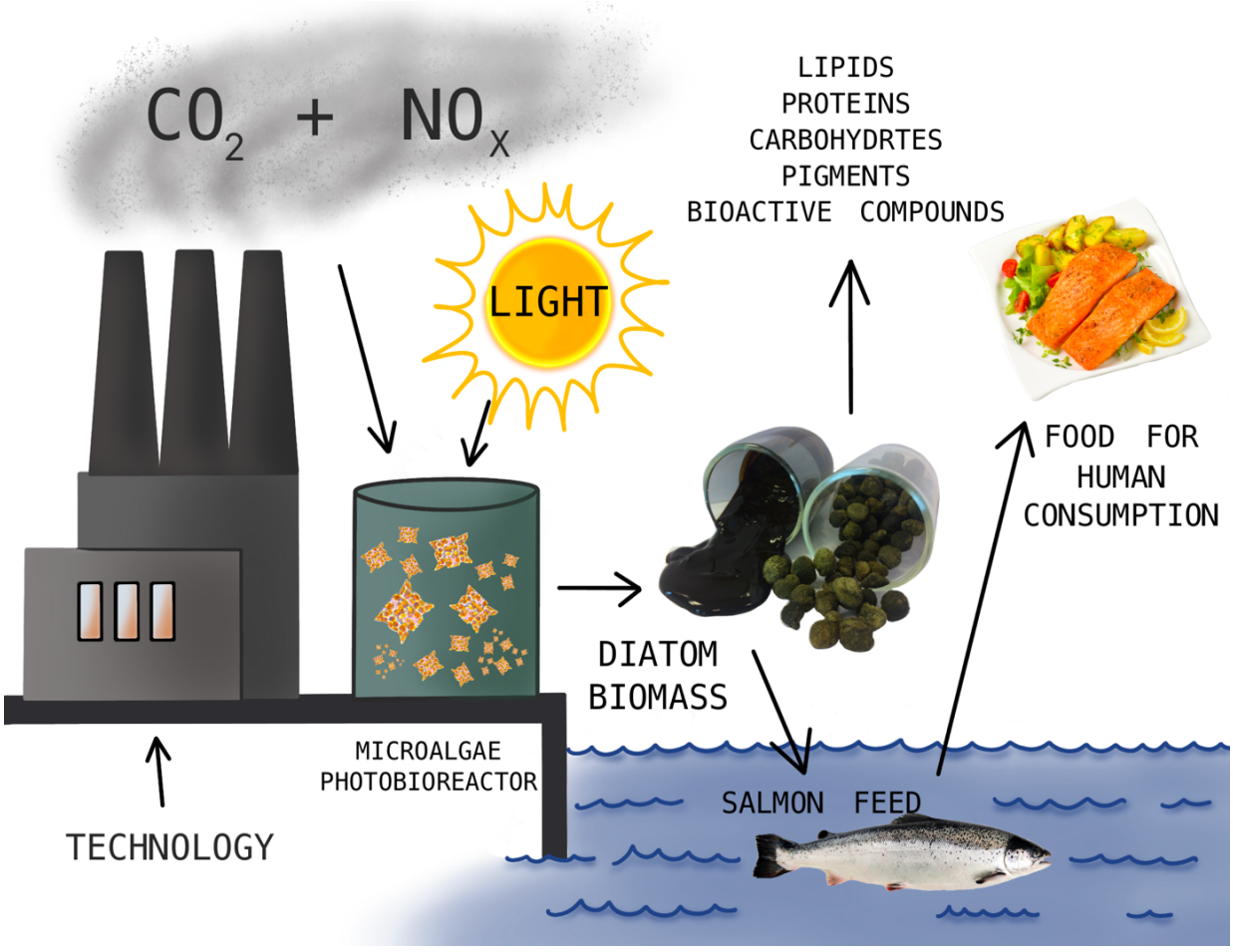

Figure 7: Overview of mass cultivation pipeline at Finnfjord AS. Figure: R. Osvik

2.2 Experimental pipeline

The experimental work included in this project was diverse and involved collection of diatom stocks during research cruises in open Arctic waters and polar ice, large-scale algae cultivation, bioactivity testing, metabolomic analysis and isolation of bioactive compounds in the laboratory. The two large-scale cultivation experiments have been based on applying stress factors, both abiotic and biotic, to the diatom monocultures of *P. glacialis* to investigate if stress would induce changes in the bioactivity or biochemistry of the diatom biomass. An overview of the cultivation and further workflow can be seen in Figure 8. This shows the process of cultivation, harvest, bioactivity testing, metabolomic analysis, and finally isolation of potential bioactive compounds in the biomass.

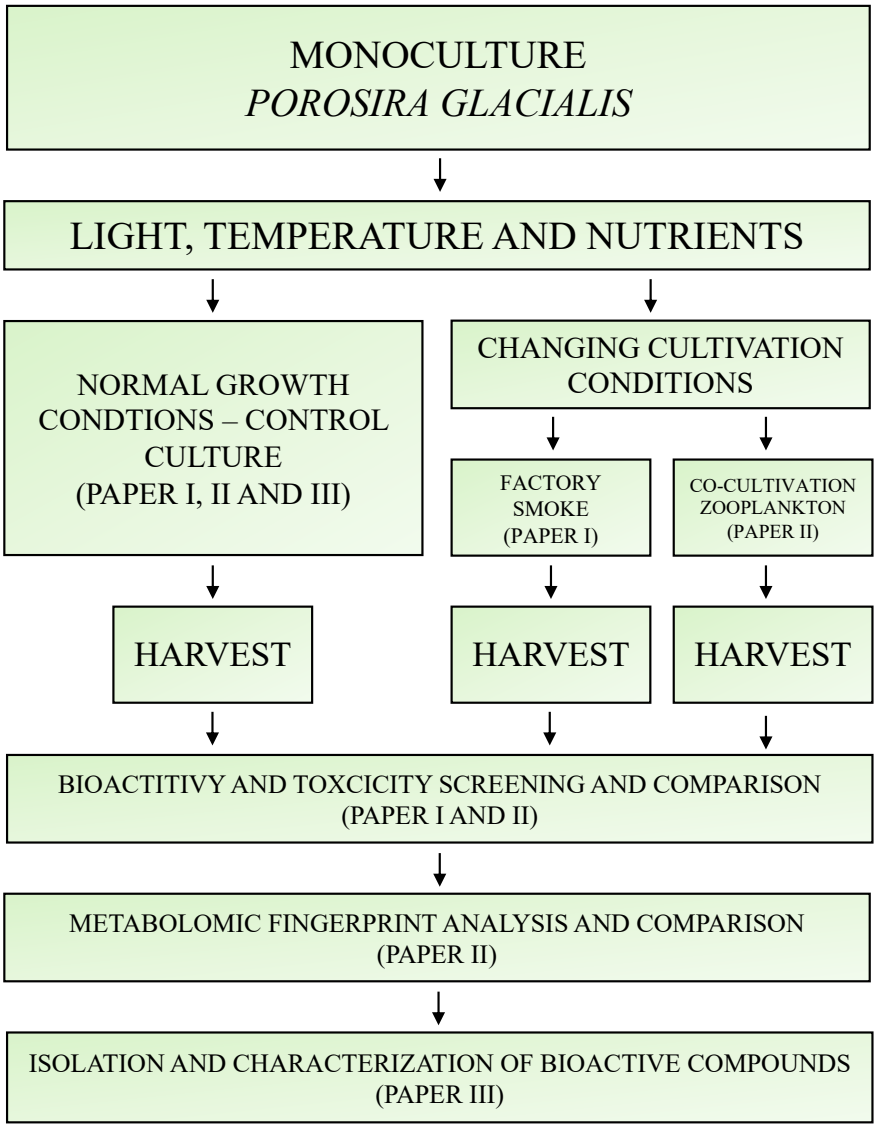

Figure 8: Schematic illustration of the workflow leading to paper I, II and III in this PhD project. Figure: R. Osvik

2.3 Species selection and monoculture maintenance

Around 16,000 species of diatoms are currently described [16]. Thus, there is a formidable number of species to choose from. Species selection is often based on specific traits, either morphological, ecological or biochemical. The microalgae research group at UiT has been working with diatoms for decades, collecting, isolating, cultivating and analysing numerous species, gaining experience with a variety of different diatoms. This groundwork has eventually led to selecting a panel of "species of interest" for more in-depth analysis. These are species that have shown promising results in terms of mass cultivation, nutrient content and possible bioactivity. The marine temperate diatom *Porosira glacialis* (Grunow) Jørgensen (strain 201) (Figure 9) is one of those species of interest and has been the subject of this PhD thesis.

The original culture 201 used throughout this project was isolated as a single cell from a water sample collected near the ice edge south east of Svalbard (76°27.54 N; 33°03.54 E) during a scientific expedition with R/V Helmer Hanssen in May 2014. Taxonomic identification of the species was done based on morphological traits of the cell and genetic characters [151, 152]. The initial identification was done using inverted light microscopy. Later the identification was confirmed by Uradnikova [152] by electron microscopy and DNA barcoding using the rbcL gene of the chloroplast as a marker. Species isolation was performed manually using elongated Pasteur pipettes and serial dilution. Frequent examination of cultures in the microscope was done to ensure there is no contamination of other phytoplankton species.

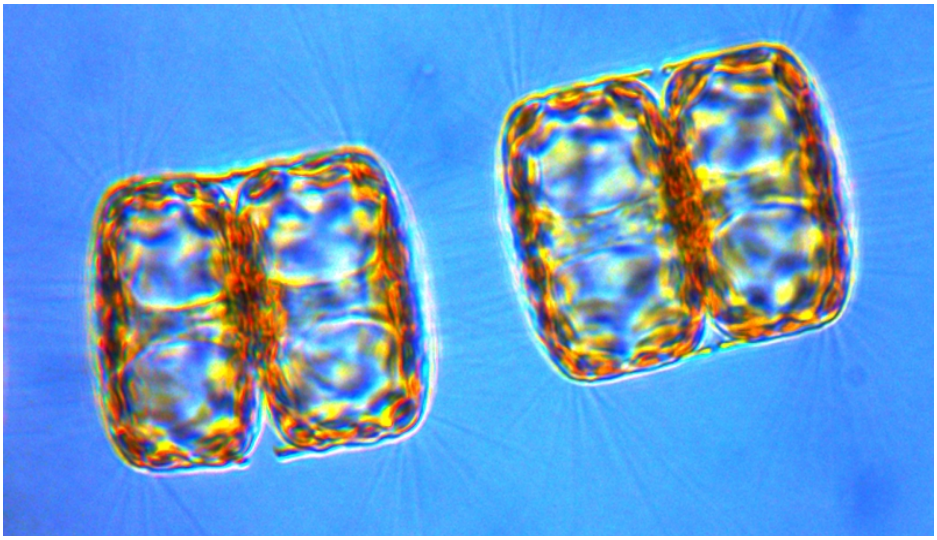

Figure 9: Light microscopy micron displaying four cells of *Porosira glacialis*. Photo: R. Ingebrigtsen

P. glacialis is a temperate species often found along the coast of Norther Norway, and along the ice edge in the Barents Sea during the spring bloom. It can handle temperatures as low as -

2 ºC [153]. It is also one of the larger diatom species, as the diameter of the centric silica frustule often exceeds 30 μm. The size gives them certain advantages in cultivation as they are easier to harvest than smaller species (Stokes law). Many diatom species such as those belonging to the genus *Chaetoceros* and *Skeletonema* are known to form long chains, *P. glacialis*, on the other hand, mostly appear as single cells but can sometimes be seen in pairs or shorter chains of 4-6 cells (Figure 9). The frustule does not have a connective thread but rather many thin setae that interlocks keeping the cells close to each other. Monocultures of *P. glacialis* strain 201 has been kept in the strain collection at the Norwegian College of Fishery Science at UiT since it was isolated in 2014.

2.4 Mass cultivation of the diatom *Porosira glacialis*

Proper environment and cultivation conditions are essential for a successful cultivation of microalgae. The conditions can be adapted to the purpose for the cultivation, maximum growth rate, optimal lipid production, pigment analysis or bioactivity testing. When performing mass cultivation, it takes place at much higher biomass concentrations than in the sea under natural bloom conditions. This calls for large focus on sufficient C for biosynthesis, i.e. CO_2 well above natural air and sea concentrations, as well as that light easily become a scarce commodity here. A change in the cultivation conditions can also lead to changes in the quality of the biomass, and the biochemistry of the diatoms, e.g. it is known that N-starvation can lead to an increase in concentration of LC-PUFA [153-155].

The biomass of *P. glacialis* for this PhD project was cultivated at a pre-industrial size test cultivation facility. The photobioreactors (PBR) used are open vertical column airlift PBRs located outside. Material for paper I and II were cultivated in two 6,000 L PBRs, one made of steel and one fiberglass. A 300,000 L fiberglass PBR were used to produce the material for paper III. The overview of the cultivation process is shown in Figure 8. All cultivation experiments started with stock cultures of *P. glacialis* strain 201 (20 L) kept in incubators at 14:10h light:dark cycle with scalar irradiance at 30 μmol quanta m^{-2} s^{-1} and 4 °C. The cultures were diluted as the concentration of cells increased, until the volume became high enough to be transferred to the larger PBRs. This was a step-by-step process from 20 L inoculum to 2 x 600 L before transferring to 6,000 L PBR and adjusting concentration of cells. The start concentration of cells L^{-1} in the monocultures was based on the type of experiment to be conducted (for details on the cultivation process for each experiment se paper I, II and III). The diatoms were grown in semi-continuous cultures; harvesting cultures at a predetermined cell

concentration and refilling with filtrated sea water and replenish with nutrients to reach the starting point. Continuous mixing of the culture to avoid settling of cells and to facilitate even nutrient distribution was done by bubbling air and/or factory smoke from the bottom of the PBRs. The cultivated biomass was harvested by draining culture from the bottom of the PBRs, directing it through a continuous centrifuge to remove all access water leaving *P. glacialis* as a thick paste. The harvest and de-watering process are easier when the cells are larger as they do not clog membranes as fast as the smaller sized species and are easier to centrifuge. Diatoms have a high concentration of polyunsaturated fatty acids making the biomass prone to oxidation; hence the samples were immediately frozen and stored at -80 °C to avoid degradation.

2.4.1 Introducing stress factors

Changing cultivation conditions by introduction of stress factors, which in turn could trigger a change in biochemistry or production of interesting secondary metabolites has been termed "One strain many compounds" (OSMAC) [156]. This method is based on the discovery that microorganisms harbour "silent gene clusters" which are not expressed under normal, and non-stressful conditions [157]. Any deviation from "normal" and optimal growth conditions may alter the biochemistry of an organism. Changes in temperature, CO_2 or pH, inorganic nutrient concentration and composition, competition or grazing stress may trigger production of secondary metabolites with attractive bioactivity.

Change in cultivation conditions were introduced in monocultures of *P. glacialis* in paper I and II. In paper I, two identical cultures of *P. glacialis* were cultivated in parallel, where one of them were supplied with excess CO_2 in the form of factory fumes. All other parameters (temperature, nutrient supply and irradiation) were kept as similar as possible and the only difference between them were the addition of factory smoke CO_2. In the second experiment (described in detail in paper II), biotic stress factors were introduced to a monoculture of *P. glacialis* in the form of grazing zooplankton. When harvested, the zooplankton was removed from the culture and only the diatom biomass retained for further analysis. A monoculture of *P. glacialis* was used for comparison. The introduction of both abiotic and biotic stress factors in the cultivation process of *P. glacialis* facilitates the possibility to see how susceptible this species is to changes in the environment.

2.5 Extraction and sample preparation

The first step of uncovering the bioactive potential of a marine organism is the extraction of the biomass. Which extraction method to use should first be based on the type of biological material as well as the nature of the desired compounds [158]. Also, often the target compound(s) are unknown, and the aim is to extract as much material as possible to be able to do a broad range of testing and chemical analysis without running out of extract. A common method to use is solid phase extractions, where the material is suspended in an organic solvent by choice. Water is removed by freeze-drying and if the material has tough cell walls, like plant material or some microalgae species it should be grinded using mortar and pastel to access the cell material. Solvents such as ethanol (EtOH) or methanol (MeOH) are common to use. The same method was applied in all experiments included in this thesis. Samples were first extracted using MilliQ-H_2O removing salt and water-soluble compounds. Subsequently a 1:1 mixture of MeOH:dicholormethane (DCM) was used to generate the organic extract. Further detailed descriptions are found in paper I, II and III.

2.6 Fractionation of extracts

Marine crude extracts are quite complex chemical mixtures of neutral, acidic, basic, lipophilic, and amphiphilic compounds [159]. Testing extracts directly in bioassays might give skewed results due to masking of bioactive compounds, thus partitioning and pre-fractionation prior to testing of the extracts is recommended. Certain groups of compounds are undesired and considered less interesting when searching for bioactive compounds. The high concentration of salt in aqueous extracts from marine organisms can lead to false results in the bioactivity testing, especially if the assay is sensitive. In addition, it can be prone to bacterial or fungal growth which may seriously affect the analysis. The organic extract on the other hand contains highly lipophilic compounds which should be removed from the extracts, as they can be difficult to handle, and their chemical properties are not desired in drug development [160]. Liquid-liquid partitioning can be used as a method to remove the bulk of unwanted materials, e.g lipids, salt, sugars and surfactants [159].

Fractionation based on the chemical properties of the compounds is a traditional way of decreasing complexity of the extracts. Flash chromatography and high-performance liquid chromatography (HPLC) are both common methods to use in the initial separation. Flash chromatography is a method of separating a larger amount of extract, in gram quantities, over

a short time [161]. This method is efficient and produce a manageable number of fractions. If the extract is separated into a too high number of fractions, one might experience that the active compounds are spread over several fractions decreasing the concentration to undetectable amounts. Flash chromatography is therefore a good method to use in the initial step of the purification of natural products. This method was used in paper I, II and III (details of method found in the papers) after a liquid-liquid partitioning of the extracts using hexane to remove the highly lipophilic compounds. In paper III, HPLC was used to re-fractionate Flash fractions that had shown activity in the first round of bioactivity testing. HPLC is also a type of column chromatography which typically handles smaller amounts of material than Flash chromatography, leading to higher degree of separation. The type of column, and column material is chosen based on how well the compounds in the extract are separated in the column, hence how well they can be collected without impurities [162]. Preparative HPLC combined with mass spectrometry is also one of the methods most often chosen for dereplication and further isolation of secondary metabolites [163, 164].

2.7 Uncovering changes in the biochemistry of *Porosira glacialis*

Changes in the biochemistry of a diatom due to changing cultivation conditions can be detected using different methods. One method is by investigating the species bioactivity and search for possible changes in the bioactivity due to the changing cultivation conditions. Although, not all changes can be detected in expressed bioactivity which makes it necessary to employ methods such as metabolomics for analysing all metabolites in the sample (paper II).

2.7.1 Bioactivity testing

When testing extracts or fractions in bioactivity assays it is possible to examine the response of whole organisms, tissues, cells or specific cellular targets. Bioassays using whole cells or organisms, where the measurement could be e.g. cell-survival, presence of biofilm or morphological changes are so-called phenotypic bioassays. Among these assays are cancer cell proliferation assays, where the measured parameter is the survival of the cells. In target-based bioassays the potential bioactive compounds are tested towards a single, specific target. This could be a gene or a molecular mechanism like e.g. enzymes, receptors or antioxidant activity [165]. In biodiscovery, bioassays are used to determine the bioactivity of the extracts, which might lead to the isolation of compounds with commercial potential. In the experiments in paper I and II, phenotypic bioactivity assays were used to uncover the potential activity of *P. glacialis*, but also to investigate whether the addition of factory smoke, or the co-cultivation with

zooplankton triggered any changes in the production of bioactive metabolites. A panel of bioactivity assays were chosen for this purpose. A cancer cell proliferation assay against human melanoma (A2058) and human colon carcinoma (HT29) cell lines, and a cytotoxicity assay towards normal lung fibroblasts (MRC5). A sea urchin embryo development toxicity assay (*Paracentrotus lividus*) was used to assess the effect on invertebrate larvae. The antibacterial effects were investigated against both Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus, Enterococcus faecalis* and *Streptococcus agalactiae* Group B), in addition to antibiofilm assay on *Staphylococcus epidermidis*. Details on the specific methods are found in papers I and II. Paper III focused on the antibiofilm activity found in the previous two studies. The bioassays used tested both activity of *P. glacialis* on the inhibition of biofilm formation and eradication of established biofilm by *Staphylococcus epidermidis*.

2.7.2 Metabolomics

Metabolomics is one of the emerging "omics" disciplines, seeking to investigate the biological mechanisms and processes of living organisms on every level: full genome sequencing (genomics), RNA transcripts and gene expression (transcriptomics) and metabolite profiles (metabolomics). Metabolomics is a method used to analyse a set of metabolites in biological samples such as cells, tissue, organs, fluids or whole organism. This is done to compare the metabolite profile or specific metabolites under optimal conditions versus non-optimal conditions, i.e. under stress, environmental impact, diseases or drug treatment [166]. When investigating the metabolome, one can use targeted metabolomics, searching for specific metabolites, and how they change in concentration or if they are present, or not. Another method, and the method used in paper II, is untargeted metabolomics, or metabolomic fingerprinting. Untargeted metabolomics aims at identifying the highest possible number of metabolites in the biological samples and compare their expression. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are common tools in metabolomic analysis, both having their advantages and drawbacks. [166] MS is more sensitive than NMR but requires more pre-fractionation and separation using e.g., HPLC or ultra high performance liquid chromatography (UPLC), compared to NMR, where samples can be analysed directly. MS is the most used technique, and the most comprehensive of the two. In many cases, the choice of analytical platform depends on what the researcher is comfortable with. Metabolomic fingerprinting with HR-MS was used in paper II to investigate changes in the metabolite

composition or concentration when *P. glacialis* was exposed to grazing pressure. Detailed description of the system used, and the data analysis is found in paper II.

2.8 Isolation and structure elucidation of bioactive compounds

2.8.1 Isolation

When interesting bioactivity is detected in extracts or fractions, the next step in the process is dereplication to investigate whether this is a compound previously found. Indication of new and interesting chemistry leads to the isolation of the specific compound. Preparative HPLC was used for the initial isolation of compounds from *P. glacialis*. Column materials were carefully selected to give optimal separation of the target compounds, making them easier to isolate from the complex extracts. The preparative HPLC was used in combination with mass spectrometry to collect compounds based on mass. Isolation of secondary metabolites is often based on testing several columns, elution gradients and selecting the right trigger method to be able to collect the desired compounds from the extracts. Collection of desired compounds can either triggered by mass, or by using timed events based in retention time. The collection method is chosen based on which eliminates possible impurities in the best possible manner. Isolation of compounds from *P. glacialis* was performed in three steps of separation and purification. Initial isolation was performed using a preparative HPLC-MS system with a 600 HPLC pump, 3100 mass spectrometer, 2996 photo diode array detector and a 2767 sample manager (Waters). The column used was Waters Xterra Prep MS C_8 10 µm, 10x250mm column as it showed best separation after the first injection. The second and third step of isolation was performed using a semi-preparative HPLC system coupled to a PDA and QDa-MS with a Sample-manager FTN-R, Quaternary-solvent-manager (HPLC-pump), isocratic solvent manager, column-oven, QDa detector, a 2998 PDA detector and a fraction manager, all ACQUITY-Arc™ components. The column used was a Waters Atlantis™ T3 3 µm, 3.0×150 mm C_{18} column. The gradient elution time was adapted based on a trial-and-error manner to adjust for best possible separation (details in paper III)

2.8.2 Structure elucidation

Fractions displaying activity and the isolated compounds from *P. glacialis* were all analysed using high resolution mass spectrometry (HR-MS). MS is a technique measuring the mass-to-charge ratio (*m/z*) of gas phase ions. A MS system consists of an ion source, a mass analyser and a detector. There are a variety of different mass analysers. In this thesis, both for

metabolomic analysis and analysis of bioactive fractions/compounds, an UPLC system coupled to a PDA Detector and a VION IMS-qTOF, using electrospray ionization (ESI) (details in paper II and III). In a time-of-flight (TOF) mass filter, the ionized molecules are accelerated by an electric field into the analyser which is a vacuum chamber. The ions are separated by *m/z* as the lighter arrive and are detected before the heavier ones [167]. It is a reliable tool for accurate mass measurement, which allows for the elemental composition of the compounds to be calculated. Nuclear magnetic resonance spectroscopy (NMR) was used in the structure elucidation of isolated compounds from *P. glacialis.* Experiments and elucidation were performed by Kine Ø. Hansen and Johan Isaksson (UiT). NMR spectroscopy is a common tool used to study the structure and bonding of molecules by using the magnetic properties of atomic nuclei. Commonly examined nuclei are ^{1}H and ^{13}C which will give information about the physical and chemical properties of a molecule [168]. To elucidate the structure of a complex compound, a combination of several NMR experiments, as well as other analytical data, are often required.

2.9 Software

A set of software packages were used to analyse and visualize all the results. All figures and plots presenting bioactivity data in paper I, II and III were made using Prism 8 for Mac (GraphPad Software Inc.). Statistical analysis in paper I used Prism 8, while in paper II statistical analysis of data was done by running chi-square tests in Excel for Mac 2020 version 16.36. The map in paper II was made using with Maps version 3.1.1. in R version 3.3.2. Metabolomics data was analysed, and statistical presentations and plots were made using EZinfo v.3.0.3.0 (Umetris ab) and Progenesis QI v.2.4 (Nonlinar Dynamics). UNIFI 1.9.4 software (Waters) was used for acquisition and analysis of HR-MS data. Figure 3 and 8 in the thesis introduction and materials and methods chapter was drawn using Procreate version 5X 5 (Savage Interactive Pty Ltd, Hobart, Tasmania) on an iPad Pro.

3 Aim of thesis

The work in this thesis has been a part of a carbon capture and utilization (CCU) project. The aim of the cultivation studies was to investigate the effects of large-scale cultivations on extracts of diatoms and to understand how the marine diatom *Porosira glacialis* responded to changes in cultivation conditions. The study was designed to assess how cultivation conditions could affect the biochemistry and bioactivity of *P. glacialis* by investigating the response to changes in both abiotic and biotic environmental factors by using the OSMAC "one strain many compounds" cultivation method. Further, another objective was to isolate and characterize possible bioactive compounds from the activity discovered in the first part of the project.

4 Summary of papers

4.1 Paper I

Bioactivity of a marine diatom (Porosira glacialis (Grunow) Jørgensen 1905) cultivated with and without factory smoke CO2.

Renate Døving Osvik, Jeanette Hammer Andersen, Hans Christian Eilertsen, Anne-Marie Geneviere, Espen Holst Hansen

Two cultures of the temperate marine diatom *Porosira glacialis* were cultivated in parallel in 6,000 L photobioreactors under the same cultivation conditions (temperature, light and nutrients), except one culture received excess CO_2 from factory smoke, while the other received pressurized air (Figure 1, paper 1). The harvested biomass was freeze dried, extracted and fractionated before being tested in a panel of bioactivity assays: antibacterial (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*. *Escherichia faecalis* and *Streptococcus agalactiae*), antibiofilm (*Staphylococcus epidermidis*), anticancer (human melanoma and human colon carcinoma), cytotoxicity (normal lung fibroblasts) and sea urchin toxicity assay (*Paracentrotus lividus*). This to assess the bioactivity of the biomass, and to reveal whether the direct addition of factory smoke would trigger changes in the bioactivity.

The study revealed bioactivity in antibiofilm assay and in both anticancer assays, but the addition of factory smoke CO_2 did not trigger any changes in the bioactivity compared to the control. There was no activity found in the cytotoxicity assay against normal lung fibroblasts. The results from the sea urchin toxicity assay confirmed previously reported diatom toxicity against sea urchin larvae development. Although toxicity was detected, the addition of factory smoke did not increase toxicity showing that the bioactivity and toxicity of *P. glacialis* is not affected by the addition of factory smoke. These results indicates that the factory smoke can be used as a safe source of CO_2 in the industrial production of diatom biomass.

4.2 Paper II

Adding zooplankton to the OSMAC toolkit: Effect of grazing stress on the metabolic profile and bioactivity of a diatom.

Renate D. Osvik, Richard A. Ingebrigtsen, Fredrika Norrbin, Jeanette Hammer Andersen, Hans Christian Eilertsen and Espen Holst Hansen

"One strain many compounds" (OSMAC) is a term in microbiology where changes in cultivation conditions is applied to trigger production of bioactive compounds. OSMAC is not widely applied in microalgae cultivation, hence the aim of this paper was to assess the effect of co-cultivation of diatoms and zooplankton.

A monoculture of *Porosira glacialis* was cultivated over a period of 5 days before half of the culture was harvested. A mixed sample of zooplankton was collected in a bay area close to the cultivation facility. The zooplankton sample was added to the *P. glacialis* culture and cultivated for 5 days, and then the diatom biomass was harvested by sieving out the zooplankton. The cultivation was repeated twice (Figure 9, paper II). The harvested biomass was freeze dried, extracted (aqueous and organic), and fractionated. The extract was analysed using LC-MS to assess the metabolomic differences between the cultivation conditions. A set of bioactivity assays (anticancer, antibacterial and antibiofilm) was run to investigate whether the presence of zooplankton changes the bioactivity of *Porosira glacialis*.

Analysis of the metabolomic profile revealed differences between the monoculture and co-culture. Markers contributing most to the differences between the samples were identified to be phaeophytin (monoculture) and carotenoids (co-culture). Bioactivity was found in all bioassays performed. Co-cultivation had no effect on the antibacterial activity. The cytotoxicity assay on normal lung fibroblasts showed elevated toxicity by the co-culture extract, thus revealing a possible higher toxicity as a result of grazing pressure.

4.3 Paper III

Antibiofilm activity of compounds isolated from temperate diatom *Porosira glacialis*

Renate Døving Osvik, Kine Østnes Hansen, Richard Andre Ingebrigtsen, Jeanette Hammer Andersen, Espen Holst Hansen

The temperate diatom *Porosira glacialis* has revealed antibiofilm activity in several studies, which motivated an in-depth investigating of the activity with the aim of isolating compound responsible for the activity. *P. glacialis* was cultivated in a 300 000 L vertical column airlift photobioreactor. The biomass was extracted using DCM:MeOH, fractionated using Flash-fractionation and preparative HPLC. Flash, and HPLC fractions were tested for inhibition of biofilm formation and eradication of established biofilm by *Staphylococcus epidermidis.* In addition, HPLC-fractions were tested for antibacterial activity in a growth inhibition assay.

Two flash fractions were found to inhibit biofilm formation. These two were further fractionated using preparative HPLC and tested for inhibition of biofilm formation and for eradication of established biofilm. Out of 80 fractions tested, 6 fractions inhibited formation of biofilm wile 3 fractions were able to remove established biofilm. Inactive and active fractions were analysed using HR-MS to find compounds unique to the active fractions. This investigation resulted in the isolation of 6 compounds found in or in relation to the active fractions. Out of the 6 compounds, three were evaluated to be pure enough for NMR analysis, and two of them resulted in compound structures. All 6 collected compounds were tested for inhibition of biofilm formation. Two compounds were able to inhibit biofilm formation: methyl 3-hydroxyoctadecanoate, a pheophorbide like compound, both known compounds. A third compounds showed activity, though no structure was obtained. This study is the first to report on antibiofilm compounds isolated from *P. glacialis* as well as the first report on antibiofilm activity by the isolated compounds.

5 General discussion

Increasing CO_2 emissions are one of the main forces driving the anthropogenic climate changes we are experiencing today. The rate of global warming will increase rapidly unless we reduce emissions and make attempts to remove CO_2 from the atmosphere [169]. Carbon capture and storage strategies (CCS) has received much more focus and investment by the Norwegian government than other strategies to reduce CO_2 emissions [170]. Carbon capture and utilization (CCU) using photoautotrophic microalgae to convert CO_2 to organic biomass is emerging as a feasible CO_2 sequestration strategy [99, 105, 171-173]. We know that marine diatoms are responsible for at least 25 % of the inorganic carbon fixed in the ocean annually, which makes them natural candidates for biological CCU [3, 5]. Connecting large-scale cultivation plants to industries with high CO_2 emissions can lead to promising CCU symbiosis, resulting in a reduction of CO_2 emissions and production of biomass for commercial applications. Diatom biomass has a variety of applications based on their nutrient content. *P. glacialis* is rich in LC-PUFAs which is in-demand in the Norwegian aquaculture industry, making it a potential source of sustainable feed ingredient production [153, 155]. In addition, *P. glacialis* could also be a potential source of other interesting compounds with commercial applications based on the discovery of bioactivity in this study.

5.1 Large-scale cultivation

In large-scale production of diatoms for carbon capture, the CO_2 must be added to the algae cultures in the most feasible and economically viable way. One of the strategies is to add CO_2-rich industrial emissions directly from point sources to the PBRs, skipping any extra purification process of the factory smoke. Due to laws and regulations regarding pollution and air quality, companies are required to purify all emissions (smoke and water) to ensure that harmful substances are set within limit values [174]. Nevertheless, the factory smoke still contains large amounts of CO_2, in addition to a substantial amount of NOx, SO_2 and trace metals. In addition, an industrial environment is never completely clean, hence dust and particles might mix with the microalgae culture when cultivating in open PBRs. The direct use of smoke in this manner might affect the diatoms growth rate, physiology, toxicity and biochemistry due to changing cultivation conditions. A large-scale cultivation study thus is necessary to investigate the response of the diatom. Our cultivation facility enables mass cultivation of marine diatoms, using factory smoke as a source of CO_2. The facility is under constant development and upscaling. Since the spring of 2015 the PBRs have expanded in

volume from 6,000 L to 300,000 L. Large-scale experiments with marine diatoms provides an excellent opportunity to investigate growth requirements, growth rates and biochemistry of the biomass. All the biomass of *P. glacialis* used in this project has been cultivated at the large-scale facility in order to reflect the "real life" conditions of commercial production, not only the optimal laboratory conditions. When cultivating diatoms outdoors, using open cylindrical PBRs there are challenges that needs to be taken into consideration regarding the consequences they might have for further investigation of the biomass. Such challenges can be due to climate e.g. temperature (air and ocean), precipitation and variation in natural irradiation, or contamination by bacteria, fungi or other species of microalgae. Keeping a large-scale culture axenic is near impossible. Sustaining a sterile culture requires closed systems, filtration and maintenance which is difficult even in small volumes in the laboratory. On a large scale, this will be far too expensive and laborious to be economically viable. However, as discussed in the introduction the bacteria present usually don't cause any harm to the diatom culture, and some of them are even beneficial [31, 175]. Heavy bacterial contamination is easy to detect in the microscope, as the concentration in bacteria increase and the diatom culture looks unhealthy (as shown in Figure 10). During exponential growth, the diatom culture looks healthy (Figure 10, left side) and there are few dead cells in the culture. When the number of broken/dead cells is low there is less organic carbon available for bacterial growth, hence the bacterial concentration is often low and under control. Some diatom species have antibacterial activity which was also found during the present study [176]. Such activity may be one of the reasons why its observed that the diatoms keep the concentration of bacteria on a non-harmful level in the culture. It's important to keep in mind that we work with organisms that, in nature, have a

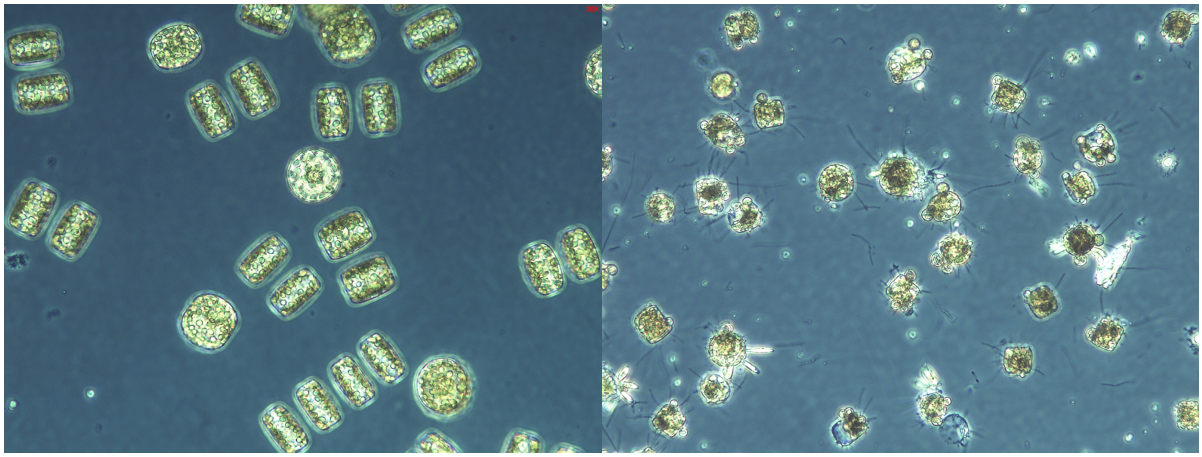

Figure 10: *P. glacialis* in culture. Healthy monoculture to the left, contaminated culture on the right. Contamination caused by flagellates, bacteria and possible fungi (chytrid). Photo: Gunilla K. Eriksen

close relationship with other planktonic organisms. Diatoms have shown to depend on some bacterial species, thus non-axenic cultures should be accepted as long as the diatoms are healthy [44, 45].

Searching through literature on the bioactivity of marine diatoms reveal that the majority have been focusing on species cultivated in small batches under laboratory conditions [101, 177-179]. Cultivation in small volumes (<100 L) in the laboratory renders full control of the applied conditions by using temperature regulated incubators, artificial lighting and high-quality nutrient supply, e.g. Guillard's F/2 marine water enrichment solution. The sea water used for cultivation can be autoclaved or one can use artificial sea water, and the air inlet can be filtrated to exclude contamination of the monocultures keeping them axenic, or close to axenic. These conditions are perfect for curating monoculture collections, and for conducting small scale experiments in the initial phase of the biodiscovery process. However, in order to produce diatom biomass for commercial applications, scaling up from the laboratory to large-scale cultivation is needed [100]. When scaling up to large-scale cultivation systems certain changes in infrastructure must be implemented to increase production yield, at the same time as the control over the cultivation conditions is maintained [101, 177-179]. It is impossible to maintain laboratory conditions in large scale, outdoor PBRs, but is a trade-off made to keep up with viable production while keeping expenses and workload at a manageable level.

Diatoms are known to alter their bioactivity and biochemistry according to changes in environmental conditions: temperature [180], irradiation [129], nutrient supply [125], and presence of other planktonic organisms [86]. Some changes can be desired, such as increased LC-PUFA concentrations, while other changes are unwanted e.g. toxin production. Cultivated diatom biomass to be used in food, feed or other applications for human consumption must be safe and of high quality. All variations and unwanted changes in the biomass which may arise from a changed environment must be uncovered. Scaling up production to a commercial scale will inevitably result in other cultivation conditions than those found in the lab [100]. Hence, it is highly important to acquire information on the robustness of the algae to be cultured for commercial purposes, in this case *P. glacialis.* Few papers are published on the process of scaling up to a level of commercial production. Often, industrial partners are included, and results can be protected by intellectual property rights, resulting in less publications [100].

5.2 Species selection

Choosing the right strain for mass cultivation is key, but how does one choose among thousands of species? There are numerous factors to consider when choosing the right species or strain; growth-rate, desired end-product, productivity, harvest and extraction [181]. To find the optimal species for large-scale cultivation, one must assess the species ability of high production under the conditions that can be provided at the cultivation facility. Optimal growth temperature can sometimes be reflected in where you find the species in the ocean, whether its tropical, temperate or Arctic regions. *P. glacialis* is described as a cold-water species. It is often found during the spring bloom in Northern Norway and is a common species to find in the ice edge bloom in the Barents Sea and Arctic oceans, the latter region being the collection site of this specific strain. Needless to say, it grows well at low temperatures making it a perfect candidate for large-scale production in colder areas. Experience with *P. glacialis* both in the laboratory and in the field has shown us that it grows fast, has a wide tolerance of growth temperatures and is easy to harvest due to its cell size [153]. The cultivation experiments in paper I, II and III were all conducted during winter (December – March). The air temperatures being - 12°C on certain days, and the water temperature was below zero during the cultivation. Yet, *P. glacialis* reached exponential growth (Fig. 2 in paper I and Fig. 1 in paper II) and showed no signs of struggling based on the visual control of the cultures. This adaption to low temperature makes it possible to cultivate through the changing seasons in Northern Norway. Most large-scale production facilities are located in areas with warm climate and with little seasonal variations. Several of the traditional species for large-scale production have an optimal growth temperature between 20 and 40 °C. Hence, it might seem counter intuitive to produce microalgae above the Arctic Circle where the sun is below the horizon for two months every winter, and to aim for year-round production. Still, the closeness to the ocean, industry providing CO_2 and large areas of otherwise non-arable land, are all factors contributing to a viable production of diatoms in Northern Norway [90]. A study by Matsumoto et al. suggested using different species for different seasons for production of biomass to overcome the struggle of seasonal changes [98]. This is of course a possible solution, although finding species tough enough to withstand variations the seasonal variation, will improve and simplify the production. For cultivation in Nordic countries it is natural to search for species found naturally on higher latitudes [90, 182]. Using cold-adapted species, like *P. glacialis*, will increase the likeliness of success for year-round cultivation using one species, as the optimal conditions differ from the traditional species cultivated on lower latitudes. Although how a

species acts under optimal conditions is one thing, it is also important to understand the versatility of a species, and how it reacts to upscaling and less optimal conditions.

5.3 Changing cultivation conditions

The cultivation studies in paper I and II were designed to investigate how *P. glacialis* responds to changing abiotic and biotic factors in the environment. This was done with the aim of further understanding how *P. glacialis* can be treated in large-scale cultivation in terms of cultivation conditions, and how the species react if the conditions change. To investigate this, a method used in biodiscovery termed OSMAC "One strain many compounds" was applied [156]. The method is well-regarded and widely used in the search for secondary metabolites in terrestrial prokaryotes and fungi, although the use is increasing in marine biodiscovery [157]. In general, diatoms are poorly represented among the studies on bioactivity and marine natural products. The OSMAC approach have not been used extensively to look for new activity in diatoms, but some studies have looked at their chemical response to changing environments. Some of these studies have been conducted at Marbio – UiT [125, 129, 180]. Those studies have shown that temperature, light intensity and nutrient availability can change the bioactivity of diatoms. With those results in mind, it was natural to hypothesize that diatoms might change their bioactivity when cultivated using direct addition of factory smoke in the CCU project. Adding factory smoke to the monoculture, changes the abiotic factors in the environment (paper I). The next to study was to find out whether biotic factors, in the shape of zooplankton, could alter *P. glacialis* bioactivity and metabolite expression (paper II).

5.3.1 Toxicity and bioactivity profiling

All samples of *P. glacialis* tested (paper I, II and III) were bioactive, which could be relevant for commercial application. In paper I, two cultures of *P. glacialis* were cultivated in parallel, keeping all cultivation parameters similar (temperature, nutrient supply and irradiation intensity). The only difference between the two were the direct addition of factory smoke as a source of CO_2 in one. Pressurized air was used in the other to secure similar mixing by air bubbles. In paper II, the concept was similar although the change in cultivation conditions was not based on changing the abiotic factors, but rather by introducing a change in the biotic factor by co-cultivating *P. glacialis* with zooplankton. The samples harvested from both studies were tested in a panel of bioactivity assays to assess the bioactivity of *P. glacialis* and how the species responds to abiotic and biotic changes. Previous bioactivity studies that included *P. glacialis* has shown activity against human melanoma (A2058) [129], which was confirmed by the

results in paper I and II. Anticancer activity towards human colon carcinoma (HT29) was found in both studies (Figure 11). Anticancer activity in other diatom species have been found in several studies [124, 125, 127, 128, 183]. In studies where dereplication of active fractions has taken place, PAHs has been shown to be the active compound, although these have not been detected in any of the active fractions analysed during the present study [128]. A few diatom species display antibacterial activity [48], and there are studies showing that diatoms synthesize some fatty acids with antibacterial effect e.g. EPA [47, 184]. Antibacterial activity was found against *Streptococcus agalactiae* (paper I and II) and *Enterococcus faecalis* and *Staphylococcus aureus* (paper III). The extracts also inhibited formation of biofilm of *Staphylococcus epidermidis* (Figure 11). A literature search has revealed that this is the first evidence of antibacterial activity by *P. glacialis*.

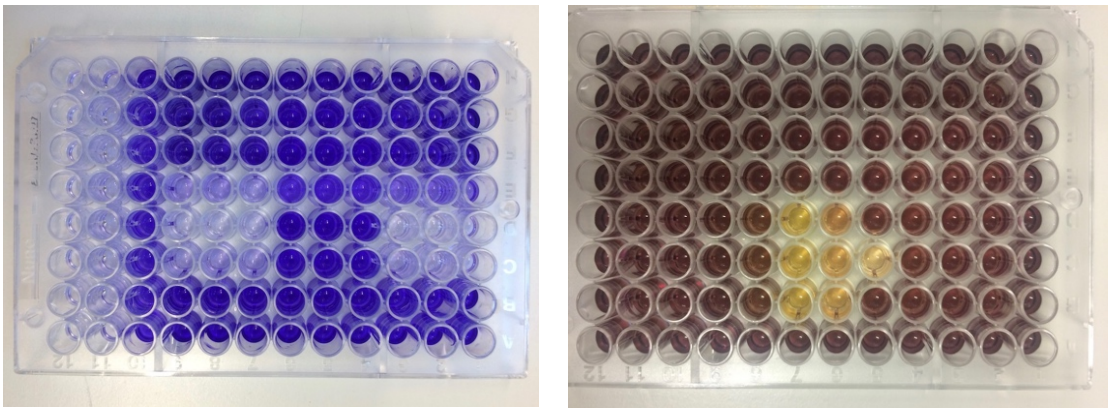

Figure 11: 96-well Nunc plates shows results from antibiofilm(a) and anti-cancer (b) bioassays. The clear wells in (a) and the yellow-coloured wells in (b) shows fractions in which biofilm formation and cancer cell growth has been inhibited, respectively.

Apart from the bioactivity, the toxicity of the samples was tested to investigate whether the changing cultivation triggered production of toxic compounds. In the cultivation study using factory smoke (paper I), two phenotypic toxicity assays were chosen: a cell viability assay using normal lung fibroblasts (MRC5) and sea urchin embryo development assay (*Paracentrotus lividus*). There was no activity towards the lung fibroblast, while the sea urchin development assay revealed toxicity. Considering all published papers showing teratogenic effect of diatoms on zooplankton species, and previously found specific toxicity towards *P. lividus* sea urchins these results were not surprising [57, 59, 68, 70, 71, 74, 80, 185-188]. In the co-cultivation study (paper II), toxicity was tested using normal lung fibroblasts, and here toxicity was detected in the organic fractions.

In terms of the response of *P. glacialis* to changing the abiotic and biotic cultivation conditions we can see that the direct addition of factory smoke as a source of CO_2 has no obvious effect on the bioactivity of the biomass. The factory smoke does not trigger any toxicity towards human cells, as the results from the toxicity assays show the same pattern in both cultivation conditions. Neither does it seem to alter the bioactivity of the samples since the antibacterial and antibiofilm activity is preserved. Although there are compounds present that lead to developmental failure in *P. lividus*, there is no increased toxicity when *P. glacialis* is cultivated using factory smoke as a source of CO_2. These results show that *P. glacialis* is quite resistant to these abiotic changes in the environment, and that it does not trigger an elevation in cytotoxicity. The presence of grazing zooplankton on the other hand had an effect on the toxicity of *P. glacialis*. When co-cultivated with zooplankton, the biomass showed increased toxicity against normal lung fibroblasts (paper II, Figure 4). Studies have shown that chemical cues released by copepods, termed copepodamides, influence diatoms. Copepodamides can induce morphological changes, such as chain-reduction in *S. marinoi,* as well as increased production of domoic acid [86, 189]. As the neither the zooplankton, nor the exudates in the cultivation medium was analysed, we cannot say whether copepodamides were present, but a thought. Analysis using UPLC-HR-MS was performed on the active fractions, and identification of prominent peaks in the chromatogram revealed signals corresponding to the primary metabolites: chlorophyll derivatives, carotenoids and phosphocholines, which are all known to have bioactivity [156, 157, 190, 191]. This can be part of an explanation of the increased toxicity, although finding obvious differences between the active and non-active fractions proved to be difficult, as the majority of the compounds seemed to be present in both samples.

Comparing the studies in paper I and II it is obvious that the toxicity towards MRC5 has changed in general, as there was no detected toxicity in paper I. In a study by Ingebrigtsen et al. [130] extracts of *P. glacialis* was tested against MRC5, and there was no toxicity. An explanation is that the cytotoxicity is connected to the aforementioned primary metabolites which are present under all cultivation conditions, but in varying concentrations making the chemical analysis more difficult to interpret. These compounds are not normally cytotoxic, but at high concentrations i.e. in organic extracts they might have an effect and non-specific bioactivity against the normal lung fibroblasts. Nonetheless, the variations in toxicity, shows that there is a need to monitor the toxicity of diatoms when cultivated for commercial production to ensure a safe and high-quality product.

It also shows us that changing the biotic factors can be used as a method to trigger a biochemical response in *P. glacialis*, enabling OSMAC to be a workable tool in diatom biodiscovery. Secondary metabolites are not always present in the cell. Full genome sequencing has over the last two decades revealed "silent" gene clusters to be possible producers of secondary metabolites in microorganisms. These gene clusters can produce secondary metabolites when the organism is exposed to stressing factors e.g. nutrient limitation or competition, but are left silent when growth conditions are optimal [156, 157, 190, 191]. Secondary metabolites are often produced by organisms to enhance their chances in competition with other. Plants produce secondary metabolites as a defence against herbivores, or as colourful pigments to attract pollinators [111]. Bacteria produce antimicrobial compounds in the presence of competing species [192]. Interactions with the surrounding abiotic and biotic factors shape organisms behaviour in terms of chemical ecology and which secondary metabolites they produce [111]. Many of these compounds have complex chemical structures, demanding energy from the producing organism. Without the presence of competition or environmental challenges, there might be no need using an unnecessary amount of energy. The production of secondary metabolites is therefore rendered silent, until needed. This again shows that laboratory conditions optimal for high growth rates, are not always the best for discovering novel secondary metabolites.

5.3.2 The metabolomic fingerprint

The current study and other published studies reveal that diatoms display a variety of interesting bioactivities: anticancer, antidiabetic, anti-inflammatory, antibacterial, antibiofilm [47, 124, 129, 134, 139, 193-195]. Nonetheless, still a lot of information related to the chemical profiles of diatoms are missing [196]. Using untargeted metabolomics can be a way of obtaining such information and can be a valuable tool in the search for bioactive compounds. A metabolic fingerprint is a method where you do an analysis of the metabolites present in a sample at a given time, under the given conditions present when the sample was taken [197]. This method can be used to investigate the metabolic profile of an organism under what is considered normal growth conditions, versus deviating conditions. In paper II we investigated the metabolite profile of *P. glacialis* cultivated in monoculture, and then in co-culture with zooplankton.

When analysing the metabolomics data, it was evident that there were differences in the metabolomic fingerprint between the two cultivation conditions. The markers contributing most to the difference between the monoculture and the co-culture were attempted identified (S-plot,

Figure 7, paper II). Identification revealed that the markers contributing most to the co-culture samples were carotenoids based on the elemental composition. The two most contributing markers elemental compositions coincide with halocynthiaxanthin ($C_{40}H_{54}O_4$) and halocynthiaxanthin acetate ($C_{42}H_{56}O_5$). Halocynthiaxanthin is known as a metabolite of fucoxanthin, isolated from sea squirts (*Halocynthia roretzi*). [198, 199] Fucoxanthin is a well-known part of the diatom pigment profile, and a compound with a variety of bioactivities. [200-202] The markers in the monoculture samples were identified as chlorophyll derivates: a pheophorbide like structure ($C_{36}H_{38}N_4O_6$) and pheophorbide b ($C_{33}H_{34}N_4O_3$). These identifications correspond to the prominent peak identified in the active fractions (chlorophyll derivates, carotenoids and phosphocholines) and could add to the possibility that the bioactivity in the cell viability assays is caused by these compounds). In higher plants, carotenoids are well-known antioxidants, and has shown to increase due to light-induced stress. Though there are no results backing this up, maybe it is possible that diatoms increase the concentration of antioxidants i.e., carotenoids in response to grazing-induced stress.

It is also known that diatoms release metabolites into the environment. Some of these metabolites are known as infochemicals, or allelochemicals for communication between species, or influencing other planktonic species [200-202]. A study investigating the exometabolomic is often termed the metabolic footprint. Barfosky et al. [203] came up with a method to perform a metabolic profiling of exudates from *Skeletonema marinoi* and *Thalassiosira pseudonana*. The study found that the patterns of excreted metabolites varied significantly during the different growth phases. It is plausible to suggest that diatoms might also alter the exometabolism in response to environmental changes. This was not analysed during this PhD project, as the focus was on analysis of harvested diatom biomass. Though, it would be interesting to analyse exudates in the cultivation medium of *P. glacialis* in the search for secondary metabolites and novel bioactive compounds. This could be done by applying the method of Barofsky et al. [203] to investigate the exometabolomic of *P. glacialis*. This would also be useful information in the current CCU project, to obtain as much information as possible on the species of interest, but also to add information to the OSMAC study performed in paper II.

The metabolic fingerprint analysis in paper II was performed on the same crude extract from *P. glacialis* that was used for Flash-fractionation and further bioactivity testing. The reason for this was that the results from the bioactivity assays and the metabolic fingerprint analysis could be compared to see whether the same changes could be seen using both methods. The use of

solvents, the handling of the material, and the freezing temperature will influence the samples. It is therefore important to decide on the method in advance and carry out the analysis based on the choices made. An important aspect, as stated previously, a large outdoor culture will never be axenic. Contamination due to other algal species is less likely due to the filtration of the water filtration. Bacteria on the other hand, will always be present. Although most bacteria in the sea water will be washed out during the harvesting process, there will still be some prokaryotes left in the biomass. The concentration of bacteria compared to *P. glacialis* in the analysed biomass is therefore low, but it must be considered.

There is no doubt that metabolomics is a powerful method in discovering the rich, and yet unexplored source of metabolites, of which can harbour interesting commercial applications. Metabolomics can uncover changes in biochemistry not detected by bioactivity and toxicity assays. Although together form a helpful tool to understand the biochemical responses that might occur in diatoms when the abiotic and biotic environmental factors change. Using this to acquire new information on the species to be cultivated can improve large-scale cultivation systems and understanding how to treat the cultivated species for safe production of high-quality biomass.

5.4 Antibiofilm activity

The antibiofilm activity was observed in all samples tested throughout the cultivation studies in paper I and II. This observation triggered an interest in further investigations of this activity (paper III). The antibacterial and antibiofilm activity is interesting as it is the first evidence of such activity in *P. glacialis*. Also, due the potential for commercial applications of such compounds and the benefits they bring to mass cultivation of diatoms. The fact that the antibiofilm activity was present in all three cultivation studies is valuable information. These results substantiate the claim that antibiofilm activity is ever-present in *P. glacialis*, regardless of the environmental changes, both abiotic and biotic, introduced during this study. Extracts of *P. glacialis* cultivated in the 300,000 L PBR was used for isolation of compounds with antibiofilm activity. The isolation was performed in several steps using preparative HPLC to remove all possible impurities, to finally end up with pure substances that could be analysed by NMR for structure elucidation (details on method in paper III).

A total of 6 compounds were targeted for isolation, three of them were evaluated as pure enough for NMR analysis, and two of them were pure enough for structural elucidation. The

compounds isolated was a hydroxy fatty acid methyl ester; methyl 3-hydroxyoctadecanoate (Figure 12) and a pheophorbide-like compound, both of which have previously been described [204, 205]. The antibiofilm assay revealed that both compounds showed some degree of inhibition of biofilm formation by *S. epidermidis* compared to the control. A study by

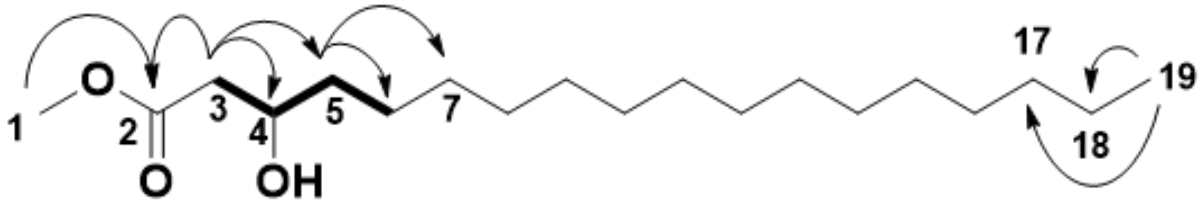

Figure 12: Structure of methyl 3-hydroxyoctadecanoate obtained by HMBC (arrow) and COSY (bold) NMR.

Ingebrigtsen et. al (unpublished manuscript) [206] and a study by Lauritano et al. [125] reported antibiofilm activity in the diatom species *Bacteriosira bathyomhala, Cylindrotheca Closterium, Thalassiosira antarctica var. borealis* and *Leptocylindrus danicus.* This activity was observed in more complex fractions and not from isolated compounds. Antibacterial activity has previously been found in free fatty acids and EPA isolated from diatoms [47, 48]. Diatoms are known to have a high content of fatty acids, which can be a possible explanation for the previous found activity in extracts of several diatom species. Regarding the pheophorbide-like compound, such break-down products of chlorophyll are known to have diverse and somewhat unspecific bioactivities such as antiviral, antiinflammatory and cytotoxic activities, but antibiofilm activity has not previously been reported [206-208].

Bacterial biofilms can grow on virtually any surface, and are known to cause an array of issues ranging from hospital acquired infections [209-211] to marine biofouling on ship hulls [212]. The biofilm is a polymeric matrix made up of proteins and polysaccharides wherein the bacteria are embedded. The matrix traps nutrients and water, and facilitates interspecies communication and horizontal gene transfer. At the same time, it protects the bacteria by suppressing the antibiotic penetration, making the biofilm highly persistent to treatment [213-215]. Biofouling can also cause significant problems in PBRs [216]. Microalgae and bacteria adhering to the walls of the PBR reduce the solar radiation in the culture and increase the release of DOC from the biofilm which is a substrate for bacterial growth. This makes the cultivation conditions unfavourable, leading to culture crashes which results in loss in yield, need for maintenance and lost income [179, 216]. This in most problematic in glass tubular or flat panel reactors which rely heavily on irradiation from the outside of the reactor. The PBRs used in this study were designed so that light sources could be submerged into the culture. Thus, biofouling could

cause growth on the lights and other submerged equipment (sensors). In addition, the polymeric matrix could be a nutrient base for possible harmful bacteria causing harm. Production of antibiofilm and antibacterial compounds can therefore be a great advantage for a species used in mass cultivation. Our observation and experience with large-scale cultivation of *P. glacialis* has shown a surprisingly low formation of visible biofilm inside the PBRs. It has been quite remarkable that after several months of continuous cultivation, there were almost no growth on the walls of the PBR. Whether the lack of visible biofilm on the inside of the PBR is due to *P. glacialis* antibiofilm activity that we have found in the lab is not known at this stage. Regardless, we see it as a highly desired quality in species to be considered for mass cultivation.

In terms of further investigation and possible commercialization of the antibiofilm agent(s), it is definitely a key point that the activity is present in *P. glacialis* at different cultivation conditions. Limited supply of material can sometimes be a prominent problem as secondary metabolites are often present in the organism in miniscule concentrations, a phenomenon known as the 'supply issue' [217]. An advantage with mass cultivation of microalgae is the potential to eliminate this supply issue. Earlier biodiscovery studies conducted on diatoms at Marbio, UiT have shown that cultivation of diatoms in laboratory scale (100 – 600 L) has yielded just enough biomass to run a limited selection of bioassays. The opportunity to run large-scale cultivation give us access to larger quantities of biomass. Large-scale cultivation facilitates bioactivity testing and chemical analysis without the worry that our extracts would not suffice. Our results combined with a renewable and sustainable production of *P. glacialis,* lays a proper foundation for further work towards commercialization of the biomass.

5.5 Future perspectives

When searching through literature on diatoms secondary metabolites, it is evident that they produce compound which are excreted into the environment [203]. As this study focused on compounds extracted from the collected biomass, it would be interesting to do a deeper investigation of what is present in the cultivation media. The exudates are highly interesting in terms of the antibiofilm activity found in this study. Experience with cultivation of *P. glacialis* has shown us that, contrary to many other microalgae used in large-scale cultivation, the cultures are not prone to growth of biofilm in the PBRs. This might be due to the discovered antibiofilm activity in the biomass, but it could also be due to possible exudates. Such a study could be performed both by absorbing compounds from the media using e.g. resin and by submerging a wide range of materials in the diatom culture to investigate biofilm formation.

This, in addition to a further investigation of other potentially bioactive compounds would lay a great foundation for possible commercial applications.

Morphology is an aspect not included in the studies presented in this thesis. It has been mentioned that copepod exudates, copepodamides, influence diatom morphology. The chain length of *Skeletonema marinoi* have shown to decrease when exposed to copepodamides. Other studies have shown that both certain strains of bacteria, and temperature can affect the morphology of diatoms [218, 219]. A morphology study of *P. glacialis* in response to co-cultivation with zooplankton or other changing environmental factors would be valuable.

The OSMAC method was used as a tool to investigate whether it is possible to tigger production of bioactive metabolites in *P. glacialis.* Based on the results found, revealing elevated toxicity against human cell lines when co-cultivated with zooplankton, it would be tempting to investigate this further. It would be interesting to repeat the study, using other species of zooplankton, as well as higher concentrations of grazers to investigate the mechanism behind the increased toxicity. The use of OSMAC in diatom cultivation is something that should be explored further to uncover more information on the chemical ecology of diatoms and their potential for commercial mass cultivation and application.

The future of commercial production of diatoms in CCU seems bright. A large-scale cultivation for sustainable production of marine ingredients for the Norwegian aquaculture industry is definitely a step in the right direction in terms of reaching the UN sustainability goals (Figure 13). By cultivation of marine phytoplankton for fish feed arable land can be freed and the pressure on wild fish stocks could be reduced. Production of marine lipids and proteins the omega-3 content in salmon could be increased and the overall sustainability of farmed fish would be improved. The infrastructure used in the CCU project can be implemented and used at other point sources for CO_2 emissions resulting in innovations and new technologies leaving behind a lower carbon footprint.

Figure 13: UN sustainability goals connected to the CCU project.

6 Concluding remarks

For large-scale production of marine diatoms with the aim of CO_2 sequestration from point sources, it has been fundamental to investigate how the diatoms responds to large-scale cultivation conditions. The presented study aimed to uncover bioactivity related to extracts of *P. glacialis* and at the same time investigate how the species responded to environmental changes in terms of bioactivity and metabolic expression. Both bioactivity and cytotoxicity were found in the cultivation studies. Although, it was discovered that the direct addition of factory smoke does not elevate the cytotoxicity, and at the same time the beneficial antibacterial and antibiofilm activity is preserved. These results are very positive for the future of diatom CCU for production of marine food or feed ingredients. The OSMAC study revealed that the cytotoxicity of *P. glacialis* towards human cell lines increased when co-cultivated with zooplankton. These results are interesting and shows that OSMAC could be useful tool in the future to uncover interesting bioactive compounds. Furthermore, the antibiofilm activity was studied in depth and we managed to isolate compounds most likely to be responsible for the activity. This was the first evidence of antibiofilm activity in *P. glacialis*. Antibiofilm compounds in diatoms used in large-scale cultivation is highly beneficial as it can prevent biofilm formation and fouling in the PBRs decreasing the amount of maintenance needed.

Large-scale production of marine diatoms demands an in-depth knowledge on chemical ecology of the cultivated species. The results found during this study have shown us that *P. glacialis* is a robust species with many good qualities that make it a good candidate for biological CCU and production of valuable marine biomass. The antibiofilm activity found opens up for further research in this area and antibiofilm compounds can be important for treatment of infections and prevent formation on medical devices. In addition, it can be valuable for treatment of biofilm in large-scale microalgae cultivation. The cultivation studies conducted during the work with this thesis has provided a new insight into the biochemistry of how diatoms respond to large-scale cultivation and environmental changes, though there is still a lot to uncover.

7 References

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Paper I

Bioactivity of a Marine Diatom (*Porosira glacialis* [Grunow] Jörgensen 1905) Cultivated With and Without Factory Smoke CO_2

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Abstract

Using industrial emissions as a strategy for CO_2 sequestration through carbon capture and utilization (CCU) in cultivation of microalgae can potentially change cultivation factors such as pH, nutrient availability and presence of trace metals, which could alter the growth and metabolism of the microalgae. It is therefore important to investigate whether such changes in culturing conditions can lead to changes in the diatoms metabolism, such as production of unwanted toxic compounds or by reduction of the diatoms' natural ability to control the growth of competing microorganisms (e.g., by decreasing the production of antibacterial compounds). The cold-water marine diatom Porosira glacialis *was cultivated in two, 6,000-L photobioreactors in an industrial setting; one culture had the direct addition of factory smoke, and to the other fresh air was added. The biomass was extracted and screened for toxicity in viability assays against human cells (cancer and normal lung fibroblasts) and development of sea urchin larvae (*Paracentrotus lividus*). Bioactivity was tested in two bacterial assays: growth inhibition assay and anti-biofilm assay. The results confirm earlier reports on the presence of toxic compounds against human cell lines and* P. lividus *larvae, but no elevated toxicity could be detected using factory smoke. Anti-biofilm activity was present in both cultures. This indicates that the natural toxic properties of the microalgae do not increase by adding factory smoke, and that we keep the beneficial ability of the microalga to suppress growth of bacteria. These are key elements in a successful, industrial-scale cultivation as the product is safe and at the same time the monocultures are not being contaminated by competing organisms.*

Keywords: microalgae, biotechnology, bioactivity, toxicity, bioprospecting

Introduction

Marine diatoms are of great importance as primary producers in highly productive temperate and Arctic ocean areas, as well as in southern upwellings.[1–3] Diatom biomass contains an array of valuable nutrients and bioactive compounds with commercial potential, including marine long chained polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA);[4,5] amino acids and ß-carotene with cosmetic applications;[6,7] carotenoids with applications in food/feed industry and as pharmaceuticals;[8,9] oil with potential for biofuel production;[10,11] and as feed ingredients in aquaculture.[6] In addition, typically for microorganisms, diatoms have high growth rates (they double by binary fission), making them promising candidates for mass cultivation and production of valuable marine biomass.[12] Despite this, the global production volume of cultivated microalgae is small, i.e., less than 20,000 tons.[13] This is due to technological limitations with respect to scalability and economic sustainability of the currently available cultivation technology.[14] The photobioreactor types used today are quite diverse, ranging from small-scale closed tubes to large open ponds.[15,16] Many systems are complex constructions and prone to growth of unwanted bacterial or algal biofilms on the surfaces of the bioreactors.[17] Development of economically viable cultivation systems with stable and optimal biological environments are therefore important if mass production of microalgae shall develop further.

Mass cultivation of microalgae at high densities requires addition of CO_2 at concentrations higher than what is naturally present in air and sea. One ''low-cost'' way to overcome this issue is to boost production by injecting factory smoke with elevated CO_2 concentrations to the cultures.[18] Fossil CO_2 emissions is one of the factors contributing to climate change, and reductions in CO_2 emissions is regarded as one of the main approaches to reduce these problems. Several CO_2-mitigation strategies have been evaluated over the years, such as carbon capture and storage (CCS), e.g., aiming to store CO_2 in geological reservoirs;[19] or carbon capture and utilization (CCU).[20] Diatoms are promising candidates for biological CCU because of their high growth rates and hence their ability to fixate large amounts of CO_2 through photosynthesis.[14,18,21] Since diatom biomass contains valuable marine long chained polyunsaturated fatty acids such as EPA and DHA,[4,5] it can therefore be considered a potential sustainable source for feed ingredients for the aquaculture industry.[14,22] This facilitates production of valuable commercial products coupled to reduced emissions of greenhouse gases through CCU.

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Cultivation of photosynthetic microalgae has been practiced for more than 100 years, and the use of microalgae to capture CO_2, also from factory smoke, has been investigated over recent decades.[23] Industrial activities might have a negative impact on human health and the surrounding environment through emissions of factory smoke and dust-containing climate gases and pollutants such as heavy metals. If CCU through the production of diatom biomass is to become a successful industrial process, it is important to investigate possible metabolic changes in the microalgae triggered by industrial smoke that might affect the quality of the product.

Diatoms produce an array of low molecular weight secondary metabolites, some of which are known to deter grazing zooplankton and other organisms competing in the same ecological niche.[24–28] These secondary metabolites have developed as mechanisms to cope with the constant pressure of grazers, bacteria and viruses that can be found in their aquatic habitats.[29] Oxylipins, especially polyunsaturated aldehydes (PUAs), have been studied due to their teratogenic effect against echinoderms and copepods.[30–33]

Previous studies have shown that, when grown under different and often extreme conditions with respect to temperature,[34] illumination,[35] and growth media,[36] marine diatoms may alter they biochemistry, leading to changes in growth rates, bioactivity and toxicity. Use of CO_2 directly from factory smoke changes the cultivation conditions, and it is important to investigate whether the this could lead to an increase in production of toxic compounds or alter the production of beneficial bioactive compounds naturally present.

The study was designed to investigate whether the direct use of factory smoke in the cultivation process could trigger changes in diatom biochemistry and bioactivity. A set of viability assays was chosen to screen for toxicity. Viability assay were carried out for three human cells lines (human colon carcinoma, human melanoma and normal lung fibroblasts) and development of echinoderm larvae (*Paracentrotus lividus)*. The bioactivity assessment of the biomass was done using a bacterial growth inhibition assay against *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Streptococcus agalactiae*, and anti-biofilm assays agianst biofilm formation by *Staphylococcus epidermidis*.

Materials and Methods

LARGE-SCALE CULTIVATION EXPERIMENTS

A stock culture of *P. glacialis* was started from cells collected near the ice edge southeast of Spitzbergen during a research cruise with the Norwegian research vessel Helmer Hanssen in spring 2014. Isolation was done under a microscope (Primo Vert, Zeiss, Oberkochen, Germany) using a manual capillary pipette, and single cells were transferred to a 24-well plate (Nunc) with autoclaved seawater. Taxonomical identification was done based on morphological and genetic characters.[37,38] Non-axenic monocultures of this diatom have since been maintained in culture in Guillard's f/10 marine water enrichment solution (Sigma-Aldrich), at the Norwegian College of Fishery Science in Tromsø, Norway.

Cultivation of *P. glacialis* was done at an industrial test cultivation facility in Northern Norway. The cultivation is aimed at utilizing factory smoke CO_2 (factory smoke composition can be found in *Supplementary Table S1*) in photosynthesis to produce nutritious marine biomass to be applied as e.g., food and aquaculture feed, i.e., CCU.

Cultivation was performed in parallel in two open 6,000-L vertical column photobioreactors, and an overview of the cultivation process is presented in *Fig. 1*. Both reactors were cylindrical with a diameter of 1.7 m and height of 2.5 m. One was made of steel and the other of fiberglass (the difference in material did not affect the growth of the diatoms). Seawater was pumped from 25 m depth and filtered through a series of 4 filters with 1 μm pore size (Pall Water, New York, NY, USA) and germicidal UV-lamps (Nordisk Vannteknik, Stockholm, Sweden). Inorganic plant nutrients, i.e., Substral™ (Scotts Company, Nordics A/S, Glostruk, Denmark) was added to a concentration of 0.25 mL L^{-1} (substral content found in *Supplementary Table 2*). Silicate solution prepared from Na_2O_3-$Si \times 9H_20$ (Sigma-Aldrich, St. Louis, MO, USA) was added to a concentration of 12.3 μmol L^{-1}. Artificial daylight white LED-illumination submerged in the culture was used as illumination in both cultures (mean intensity ca. 45 μmol photons m^{-2} s^{-1} in the middle of the reactor) at 24 h photoperiod. Factory smoke was added daily in intervals (2–4 hr) throughout the culivation period. The addition of factory smoke was controlled by pH measurements as the pH value decreases with the increase of dissolved CO_2 in the sea water. The addtion of smoke was stopped before the pH dropped below pH 6.5. pH was measured manually using a handheld pH-meter (WTW MultiLine®Multi 3630 IDS) and controlled every 15 min. The smoke was added directly from the factory outlet pipes using a compressor

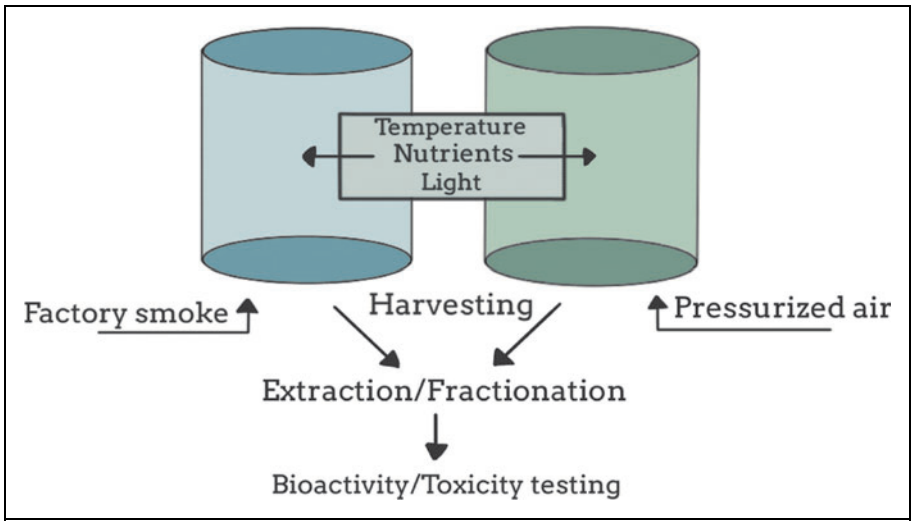

Fig. 1. Overview of cultivation experiment workflow. Color images are available online.

(6 bar/ 201 L min^{-1}) and dispersed into one of the cultures through a system that facilitates mixing by bubbling from the bottom of the reactor. Manually controlled valves were adjusted to accommodate an appropriate amount of bubbling that would not damage the cells under excessive mechanical stress. The same system was used in the second cultivation tank, but with addition of pressurized air instead of factory smoke.

Both cultures were inspected daily to check for contamination and state of the cells (color, shape, chromatophore arrangement) to make sure both cultures remained healthy. Biomass concentration was monitored daily by counting of cells in an inverted microscope[39] and by measuring the concentration of the photosynthetic pigment chlorophyll *a*. Chlorophyll *a* was measured using the method of Holm and Riemann[40] with ethanol (96%, Sigma Aldrich) as extractant and quantified using a Turner Designs fluorometer (TD 700, Sunnyvale, CA, USA). Temperature and pH were monitored throughout the cultivation period.

Cultures were harvested when the number of cells reached between 5.2–5.9 million cells L^{-1} to avoid differences in cultivation conditions due to cell concentrations. Desired concentration was reached after 4–6 days and 3,000 L of both cultures were harvested using a continuous centrifuge (Algae Centrifuge, Sacramento, CA, USA). After centrifugation, the biomass was frozen and stored at −20°C until further use. To continue cultivation, 3,000 L of filtrated water was added to both cultures, in addition to nutrients and silicate solution in the same concentration, as stated earlier. Cultivation then continued for 4–6 days before 3,000 L of each culture was again harvested and stored at −20 °C.

SAMPLE PREPARATION AND FLASH FRACTIONATION

Samples were freeze-dried (Heto PowerDry PL9000, Thermo Fisher Scientific, Waltham, MA, USA), crushed and extracted using a protocol developed for marine plants and animals.[41] The samples were first extracted overnight using Milli-Q H_2O (Millipore, Burlington, MA, USA) at 4°C and subsequently centrifuged (4,600 rpm). The supernatant was freeze-dried and ground into a fine powder. The remaining pellet was freeze-dried and re-extracted three times (3 x 4 h in room temperature) with a 1:1 mixture of methanol and dichloromethane (vol:vol) (Sigma Aldrich). The organic extract was evaporated under reduced pressure (Laborata 4002, Heidolph, Schwabach, Germany). Dried aqueous and organic extracts were stored at −20°C until further use.

The extracts were Flash fractionated using a Biotage SP4 Flash chromatography system using self-packed Biotage columns (Uppsala, Sweden) with 6.5 g of Diaion® HP-20SS resin. Approximately 1.5 g of the organic extract was resuspended in hexane (40 mL g^{-1} sample). The hexane solution was transferred to a separating funnel, a 90% aqueous MeOH (30 mL x 2) was added, and the solution was mixed carefully. After separation, the lower liquid phase (MeOH) was transferred to a round-bottomed flask and 2 g of Diaion HP-20SS resin was added and dried under reduced pressure. For the preparation of aqueous extracts, 1.5 g were transferred to two 13-mm glass tubes, to which was added 4 mL of 90% MeOH, 1.5 g of Diaion® HP-20ss resin and 1 mL of Milli-Q H_2O, before the mixture was dried under reduced pressure. Fractionation was done in two steps: first with a gradient of 5–100% MeOH and MilliQ-H_20 at a flow rate of 12 mL min^{-1} over 32 min, and then a MeOH:acetone gradient ending at 100% acetone, flow rate 12 mL min^{-1} over 18 min. The eluent was collected into 8 separate fractions and dried under reduced pressure before dissolving in 100% DMSO to a concentration of 40 mg mL^{-1}.

BIOACTIVITY AND TOXICITY SCREENING

Viability assay human cells. All Flash fractions were tested against two cancer cell lines: A2058 Human melanoma (LGC Standards ATCC CRL-11147, Teddington, Middlesex, UK) and HT29 Human colon carcinoma (LGC Standards ATCC HTB-38), and against normal lung fibroblast MRC5 (LGC Standards ATCC CCL-171). Assays were performed on 96-well plates (Nunc, Roskilde, Denmark). 2,000 cells were seeded into each well (4,000 cells per well for MRC5 cell line). Roswell Park Memorial Institute 1640 cell medium with 10% fetal bovine serum and 10 mg mL^{-1} gentamicin was used as a cell growth medium, and the seeded plates were incubated at 37°C in 5% CO_2 for 24 h. After the incubation period, the culture medium was replaced (50 μL) and the cells were then exposed to the Flash fractions (50 μL) giving a total volume of 100 μL in each well. All fractions were tested in triplicate. Culture medium and Triton X-100 were used as a negative and positive controls, respectively. Plates were then incubated for another 72 h, before adding 10 μL of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) and incubated for another 1 h with the reagent. Absorbance was read using a DTX 880 Multimode Detector at 485 nm, and results were calculated as % cell survival compared to the aforementioned positive and negative controls.

Sea urchin development assay. Toxicity assays on sea urchin larvae were performed at the Observatoire Oceanologique in Banuyls sur Mer, France. Mature *Paracentrotus lividus* (Lamarck, 1816) sea urchins were collected in the Mediterranean Sea near Banyuls sur Mer. Spawning was induced by shaking the sea urchins and thereafter placing them with the gonopore facing down on top of a beaker with seawater. Sperm was released in long viscous threads that could be collected with a pipette and transferred to a petri dish to be stored at high concentration. The egg suspension was filtered through a 120-μm mesh nylon filter to get rid of feces and debris. The experiment was performed in a temperature-controlled room at 18°C. Eggs were left to sediment and washed twice with filtered seawater (0.22 μm, Millipore). The density of eggs was counted under a microscope and diluted to 700 eggs mL^{-1}. Sperm was diluted by transferring 10 μL of concentrated sperm to 1 mL seawater and from this dilution 1 μL per 1 mL was added to the solution of eggs. One minute after fertilization, the eggs were checked for the presence of a fertilization membrane. The assay was performed in 24-well plates (Nunc) and 1 mL of fertilized eggs were transferred to a prepared plate with extracts. All extracts were tested in duplicates. Plates were controlled after 2 h for inhibition of first cell division and left at 18°C for development.

Pictures of each well were taken 24, 48 and 72 h after fertilization. Pictures were taken of sub-samples of each well fixated with 4% formaldehyde. Analysis of results was done based on visual examination of larvae in living samples, and the pictures taken after 48h.

Bacterial growth inhibition assay. All flash fractions were tested for antimicrobial activity in a growth inhibition assays against 5 bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Streptococcus agalactiae* (ATCC 12386). Colonies of each bacterial strain were transferred from blood agar plates and inoculated for at 37°C for 24 h in 8 mL growth media; *S. aureus*, *E. coli* and *P. aeruginosa* in Mueller-Hinton media (MH, Becton Dickinson, Franklin Lakes, NJ, USA) and *E. faecalis* and *S. agalactiae* in brain heart infusion media (BHI, Sigma-Aldrich). After 24 h, 2 mL was transferred to 25 mL of new growth medium, incubated until log-phase was reached, and diluted 1:1,000 to adjust the density. The bacterial solution was then transferred to 96-well microtiter plates, 50 μL in each well, and the fractions were added to the plate to a total volume of 100 μL per well. All fractions were tested in duplicates. Plates were then incubated at 37°C overnight, and growth was measured, first by visual examination, and then absorbance at 600 nm was measured in a VICTOR 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA).

Inhibition of biofilm formation. The biofilm-forming bacterial strain *Staphylococcus epidermidis* (ATCC – 35984) was used in this assay. Colonies of *S. epidermidis* were seeded from blood agar plates in Tryptic Soy Broth enrichment media (TBS, Merck, Burlington, MA, USA) and incubated overnight at 37°C, before being diluted 1:100 in TBS with 1% glucose. The assay was performed in 96-well plates, and 50 μL bacterial suspension was transferred to each well, while 50 μL of fractions were added in triplicates. The non-biofilm producing strain *Staphylococcus haemolyticus* (Clinical isolate 8-7A) was used as a negative control, to which was added a 50-μL bacterial suspension and 50 μL dH_2O. The blank control consisted of 50 μL TBS and 50 μL dH_2O. Plates were incubated overnight at 37°C. The bacterial suspension was then removed by carefully turning the plates upside down and washing with MQ-H_2O before the biofilm was fixated at 55°C for 1 h. A solution of 0.1% crystal violet was then added to each well for 5 min and removed by washing the plates twice with MilliQ-H_2O. The plates were thereafter dried at 55°C and visually examined for the formation of biofilm and then resuspended in 70 μL EtOH before reading the absorbance in a VICTOR 1420 Multilabel Counter (Perkin Elmer) at 600nm.

Removal of established biofilm. S. epidermidis was grown overnight in TBS at 37°C and diluted 1:100 in TBS with 1% glucose. The bacterial culture (50 μL) and dH_2O (50 μL) was then transferred to a 96-well plate. *S. haemolyticus* was used as a negative control, and TBS (1% glucose) and dH_2O (1:1) as the blank. Plates were incubated at 37°C for 24 h. Enrichment media was removed by gently turning the plate upside down, and the plates were washed 2 x with PBS. Fresh TBS (1% glucose) was added in addition to the fractions (50 μL) in triplicates; prior to that the plates were incubated for 24 h at 37°C. Fixation, coloring and reading of absorbance was done following the same method as in the inhibition of biofilm formation assay.

STATISTICAL ANALYSIS

Statistical differences of samples cultivated with and without factory smoke for the viability assays were determined by Student's t-test using the program Prism 7 for Mac OS X V7.0e (GraphPad Software Inc.). Data were considered significant when at least p was <0.05. All figures were made using the same software.

Results and Discussion

Mass cultivation of marine microalgae using industrial factory smoke as a source of CO_2 can be a sustainable and feasible way to produce nutritious biomass to be applied as food and/or feed. However, a relevant question is if the use of factory smoke changes the metabolism of the diatoms and could trigger them to produce previously unknown toxic substances that can be deleterious to marine organisms and humans. The present study was therefore designed to enable investigation of possible changes in biological effects of the extracts of *P. glacialis* after exposure to factory smoke. As it is known that certain growth conditions such as temperature, light and nutrient availability can affect the biochemistry of diatoms, it was important to keep all growth conditions as similar as possible, except for the addition of factory smoke.

Cell counts showed an overall increase in biomass from the first day until the day of harvest, with growth rates between 0.11–0.21 doublings day^{-1} in the four cultures (*Fig. 2a*). The high number of cells in culture NS2 on day two is most likely due to an error in counting,[42] since the chl *a* value (*Fig. 2b*) showed a concentration corresponding to a lower cell density. *P. glacialis* is a large diatom (25–50 μm diameter), and the cell densities were equivalent to 0.1–0.15 g L^{-1} biomass when harvested. Addition of CO_2 is used to enhance growth rates and densities in mass cultivation, but in the present experiments optimization of cultivation conditions was not a priority. The effect of added CO_2 on growth rates is usually observed at higher cell densities when the atmospheric and ocean concentration of CO_2 becomes a growth-limiting factor. We did observe that the growth of *P. glacialis* was not affected negatively by the factory smoke added directly to the culture.

Parameters such as pH and temperature were closely monitored (*Fig. 3*). During the first cultivation (NS1 and S1), the temperature ranged from –0.2 to 3.6°C, while during the second cultivation period (NS2 and S2), the temperature ranged from 2.2 to 3.8°C (*Fig. 3a*). The variation in temperature was due to the outdoor location of the cultivation facility, i.e., the air and sea water temperatures had an impact on the cultivation temperature. Note that cultures grown in parallel had similar temperatures throughout the period. *P. glacialis* is a northern temperate/Arctic species with a wide, but not much varying temperature range, with maximum growth at ca. 12°C and still

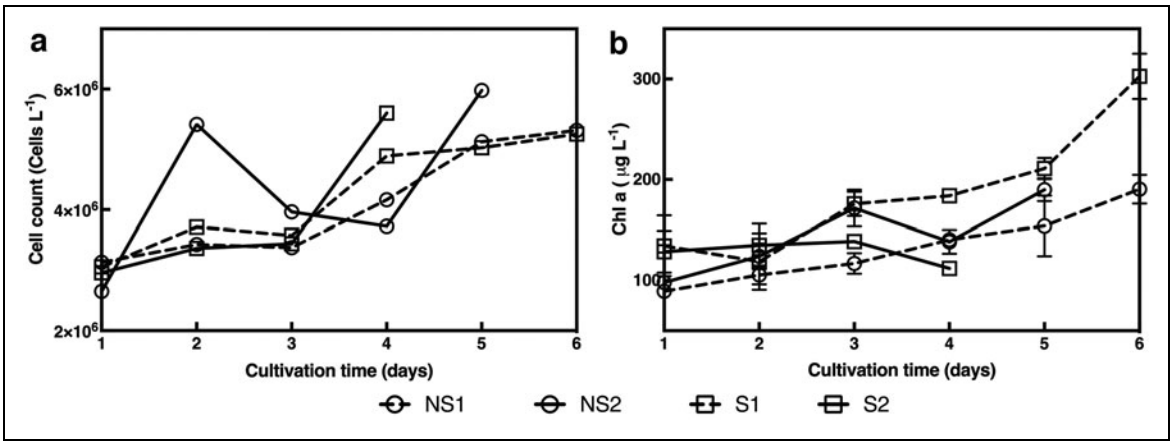

Fig. 2. Cell numbers in **(a)** cells L^{-1} and **(b)** chlorophyll a concentration ($\mu g\ L^{-1}$) vs. time for the four diatom cultures of P. glacialis; two cultures grown without factory smoke (NS1 and NS2), and two cultures grown with factory smoke (S1 and S2). Chl a represents values of three replicates.

positive growth even at sub-zero water temperatures. In a study by Svenning et al.,[5] a growth rate of 0.17 doublings day^{-1} was logged at −2°C and 0.41 at 12°C in 100-L Plexiglas columns, while results from the temperature range between 2 and 12°C were not statistically different. Gilstad and Sakshaug[43] observed a maximum growth rate at light saturation of 0.2 to 0.4 doublings day^{-1} in small-scale experiments (20 mL) at temperatures around 0°C. Hence the growth rates measured in the present study were in the same range as the two aforementioned studies, and the observed discrepancies could possibly be explained by differences in reactor size, even if all experiments reported the same (low) light saturation (50–75 mmol photons $m^{-2}\ s^{-1}$) and no inorganic nutrient scarcity.

Daily pH measurements (*Fig. 3b*) were made, once a day in cultures NS1 and NS2 and twice a day in cultures S1 and S2 (before and after addition of factory smoke). Cultures without addition of smoke had a stable pH ranging from 8.03–8.23 throughout the cultivation periods. Fluctuations in the pH values in culture S1 and S2 were due to the addition of factory smoke containing CO_2, which dissolves in the seawater and lowers the pH; the average values before and after the addition of smoke were 7.95 and 7.28 respectively.

BIOACTIVITY AND TOXICITY ASSAYS

The aqueous and organic crude extracts were each fractionated into eight fractions using Flash chromatography to reduce the chemical complexity of the extracts. The biological characterization of the extracts was done by testing all fractions in a selection of toxicity and bioactivity assays: viability of human cancer cells and normal lung fibroblasts; an in vivo sea urchin development assay and two bacterial assays; a growth inhibition assay; and an anti-biofilm assay. In all assays, only results from organic fractions are shown, as there was no activity to report from the aqueous fractions.

Viability assay. Cell viability assays were performed on three different cell lines (*Fig. 4*): human melanoma, human colon carcinoma, and normal lung fibroblasts. All the organic fractions,

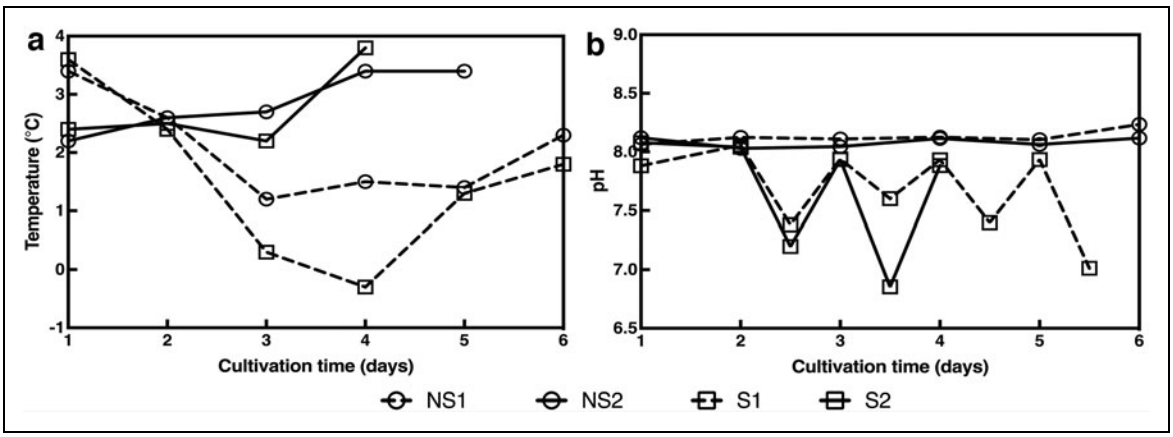

Fig. 3. (a) Temperature (°C) and **(b)** pH measurements of samples NS1, NS2, S1 and S2 during cultivation of *P. glacialis*. Daily measurement was taken in cultures NS1 and NS2. pH in cultures S1 and S2 were measured twice each day, before and after addition of factory smoke.

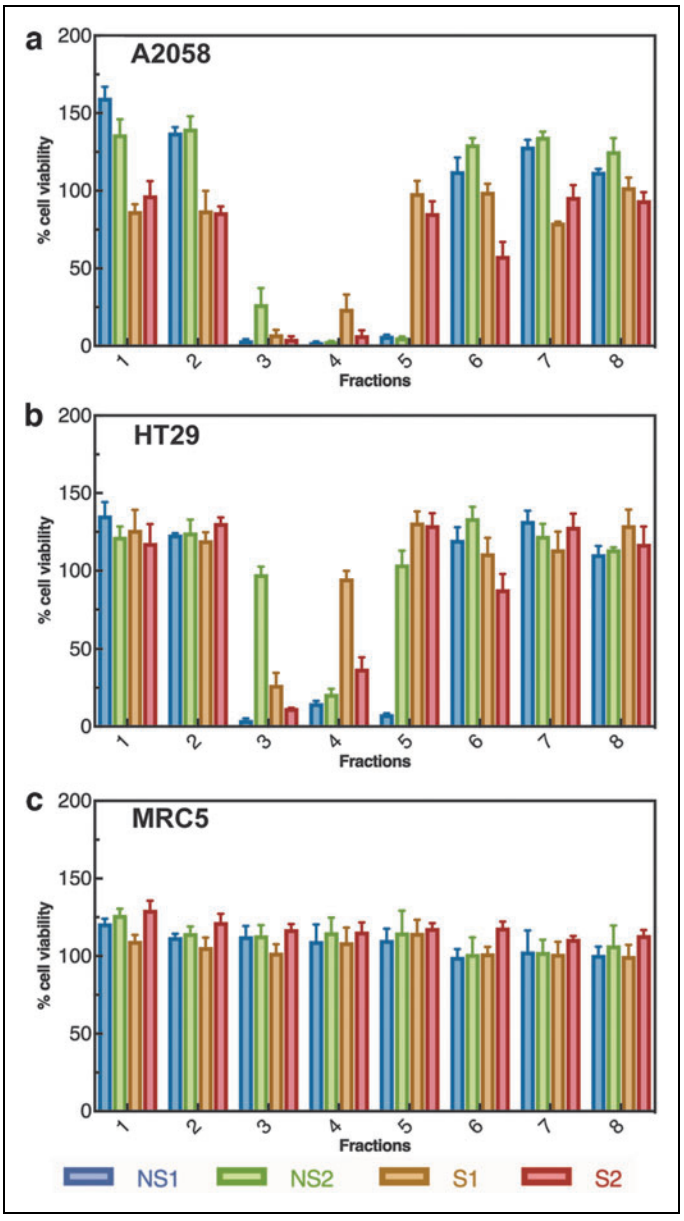

Fig. 4. Activity profiles of all fractions in samples NS1 (blue), NS2 (green), S1(yellow) and S2 (red) tested at 100 μg ml^{-1} against **(a)** A2058 human melanoma, **(b)** HT29 human colon carcinoma and **(c)** MRC5 normal lung fibroblasts. Fractions with cell viability lower than 50% is considered active. Data represents mean of 3 replicates. Color images are available online.

64 in total, were tested at 100 μg mL^{-1}. *Figure 4* shows the toxicity profiles of all four samples (i.e., NS1, NS2, S1 and S2). Toxicity screening of the diatom fractions showed that there were anti-proliferative effects against the two human cancer cell lines human melanoma (A2058) and human colon carcinoma (HT29), and all activity was found in fractions 3, 4, and 5. Activity profiles showed a few differences between the treatments; fraction 3 in NS2 and fraction 4 of S1 were not active against human colon carcinoma (*Fig. 4b*), and only fraction 5 of samples cultivated without smoke (NS1 and NS2) were active against human melanoma (*Fig. 4a*). For the remaining fractions, the activity seems to be similar for the two cultivation treatments, without any clear differences. Statistical analysis of results from the bioassays with the two cancer cell lines revealed no statistical significance between the two treatments (Student's t-test $p > 0.05$). An assay was also performed on normal lung fibroblasts (MRC5) to assess toxicity against normal human cells. The assay indicated no reduction in cell survival for any of the tested fractions from all four samples (*Fig. 4c*). The results indicate that there was no toxicity in the extracts against normal human lung fibroblasts, and no differences between the two cultivation treatments, showing that direct addition of factory smoke during the cultivation of *P. glacialis* did not lead to any elevated toxicity in the extracted biomass.

Anti-cancer activity has been reported in extracts of several marine diatom species.[28,44–46] Sansone et al.[47] found that diatom-derived PUAs had activity against two cancer cell lines but no activity against normal human cell lines. Studies on bioactivity in *P. glacialis* are scarce, with less than a handful of publications on this species.[35,48] A study of bioactivity in cold water diatoms, including *P. glacialis*, was done in our research group by Ingebrigtsen et al.,[35] which found anti-cancer activity against human melanoma cells. The present study confirms these results and show that the activity was still present when *P. glacialis* was cultivated in large scale. We also found that large-scale cultivation using factory smoke does not directly trigger any increase in toxicity against human cell lines.

Sea urchin development assay. A sea urchin development assay was conducted to assess the impact of the extracts on larvae of *Paracentrotus lividus.* The results revealed that the organic extracts of *P. glacialis* had toxic effects against *P. lividus* larvae. The toxicity assay was carried out in two stages. First, flash fractions were screened at a concentration of 100 μg mL^{-1} to investigate which fractions were active or not, followed by a dose-response assay of the three most active fractions. The results from the initial assay of the organic flash fractions from NS1 after 48 h are shown in the micrographs in *Fig. 5*. The toxicity of the fractions of all four samples were similar, therefore the results from fraction 1–8 of NS1 (*Fig. 5*) are representative for all four samples (NS1/2 and S1/2). When referring to the fractions, the same results apply to all four samples. In fractions 1 and 8, the larvae were fully developed except for a few minor abnormalities, similar to the controls, and were therefore not regarded as active. Larvae treated with fraction 2 and 3 reached the pluteus stage of development, but with deformations, showing that there was some toxicity in these fractions, but not high enough to regard the fractions as active. Fraction 4 of the organic extracts inhibited the initial cell division at 100 μg mL^{-1}, and fraction 5–7 arrested cell division before reaching early blastula, after approximately 10 cell divisions. Fractions 4–7 had a clear toxic effect on the sea urchin larvae. Based on the results of the initial screening, a dose-response assay was performed with the concentrations 100, 75, 50, 25 and 5 μg mL^{-1} on fraction 4, 5 and 6 from all four samples.

The activity profile for fractions 4, 5 and 6 from all four samples tested can be seen in *Fig. 6*, showing the percentage of

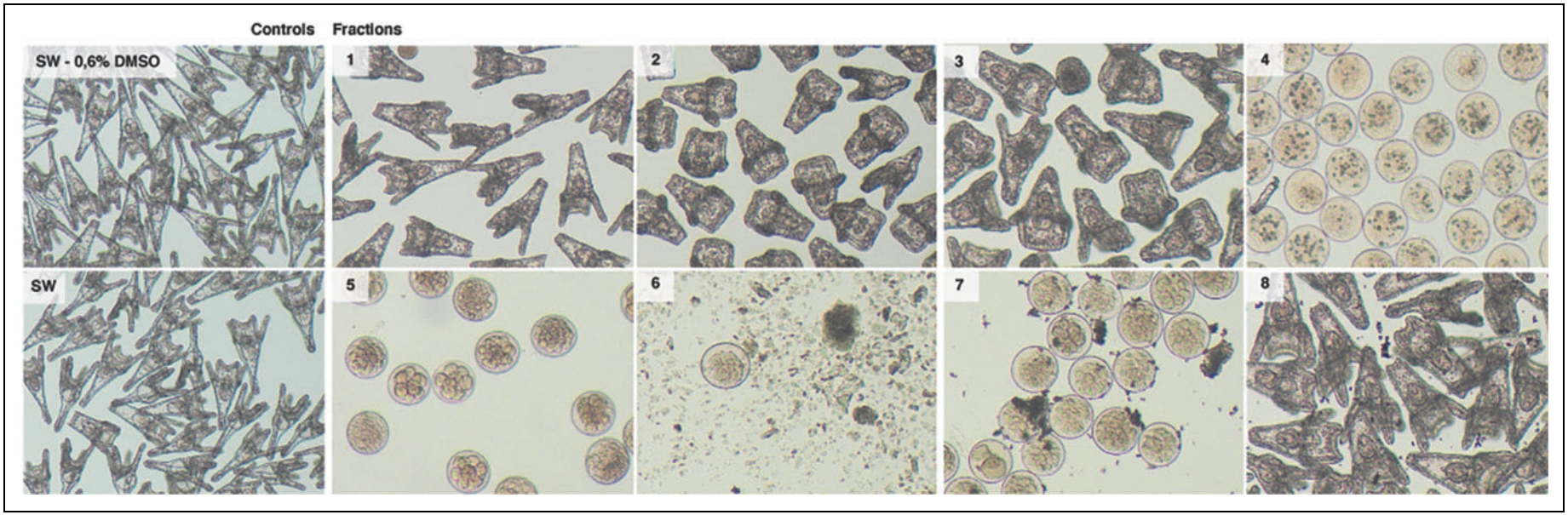

Fig. 5. Micrographs of sea urchin larvae (*P. lividus*) after being treated with Flash fractions 1–8 of NS1 at 100 μg mL^{-1} for 48 h (4X magnification). Controls are filtrated seawater (SW) and seawater with 0.6% DMSO. Color images are available online.

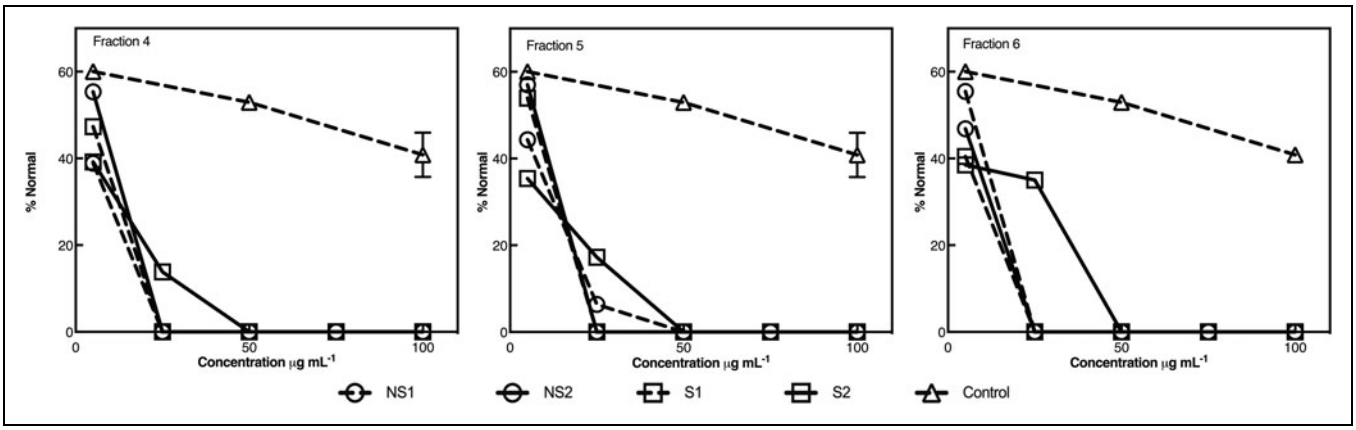

Fig. 6. Activity profiles from sea urchin toxicity assay of fraction 4, 5 and 6 in sample NS1, NS2, S1 and S2. Activity is measured in % normal developed larvae after 48 h of exposure to fractions at 100, 75, 50, 25 and 5 μg mL^{-1}. All malformed and dead larvae are counted as not normal. Data represents mean of two replicates.

normally developed sea urchin larvae for each of the treatment concentrations. There were no normal development in treatments at 50 μg mL^{-1} and above. Treatments at 25 μg mL^{-1} also showed severe effects on the sea urchin larvae that experienced no normal development. At 5 μg mL^{-1}, the survival was 35–55%, while the control had 60% survival. The overall activity of all the samples and fractions were almost identical when comparing the two cultivation treatments. This shows that the industrial smoke used in the cultivation neither increased nor decreased the toxicity against *P. lividus* larvae, and that the compound(s) responsible for the effect was present in both cultures (NS1 + 2 and S1 + 2).

Effects of diatom extracts on echinoderms and copepods have been well-studied through several experiments where they have shown teratogenic effects,[32,49–52] and PUAs have been shown to be the one of the culprits.[52,53] A previous study by Gudimova et al.[54] where both fertilized eggs and larvae (4- and 6-armed development stages) of the sea urchin *Paracentrotus droebachiensis* were exposed to living cells of *P. glacialis* showed that the living diatoms had an anti-proliferative activity on the fertilized eggs, and that the larvae avoided grazing on the diatoms. No previous studies on the effect of extracts from *P. glacialis* have been found, making this the first one to document its effect on *P. lividus* larvae development.

Antibacterial growth inhibition assay. Open photobioreactors for mass cultivation of marine algae are challenging, if not impossible, to keep free of bacterial contamination. Whereas several bacterial strains are important symbionts to the diatoms, some bacteria can cause problems and lead to a decrease in growth and fitness of the algae.[29,55,56] Several diatom species have shown antibacterial effect,[29] and the diatoms natural ability to prevent bacterial growth is important to consider in a mass-cultivation scenario. To test the antibacterial effect of *P. glacialis,* the extracted biomass was tested against a selection of bacterial strains, both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*, *E. faecalis* and *S. agalactiae*). All fractions were tested at a concentration of 100 μg mL^{-1} and a fraction was regarded as active against the bacteria when absorbance at 600 nm was <0.05.

Results of the assay revealed that fractions from all samples had activity against the Gram-positive bacteria *S. agalactiae.* All four samples showed similar activity, where fractions 4 and 5 were active, except in sample S2 where only fraction 4 was active. As there was no clear difference in activity between the two cultivation treatments, it seemed as though cultivation with factory smoke had no effect on the antibacterial activity of *P. glacialis*. Anti-bacterial effects of diatoms in general are poorly documented, and there is only one study on *P. glacialis* performed by Ingebrigtsen et al.,[35] and no activity was found there. The results show that *P. glacialis* had anti-bacterial effect against *S. agalactiae.*

Inhibition of biofilm formation. The formation of bacterial biofilm, and subsequent build-up of fouling by algae, is one of the issues in mass cultivation of diatoms and other microalgae.[17] Bioactivity that inhibits formation of bacterial biofilms could therefore be a useful trait in diatoms for mass cultivation. *P. glacialis* showed activity against formation of biofilm by *S. epidermidis*. This bacteria is considered a common cause of hospital infections,[57,58] and is a sensitive assay when screening for compounds that can inhibit biofilm formation. The screening was done at a concentration of 100 μg mL^{-1}, and the results are shown in *Fig. 7*. Fractions were regarded as active when absorption was <0.25 at 600 nm. As can be seen from *Fig. 7*, there is little that separates the two sample sets from each other in terms of activity. Fraction 3 of all four samples were active, and except for NS2, fraction 4 is active in the remaining three samples.

These results show that, just like in the growth inhibition assay, factory smoke seems to have no apparent effect on the biofilm activity of the extracted biomass. What is interesting with these results is the anti-biofilm activity itself; only a few studies have been conducted on anti-biofilm activity in diatoms, except for research on biofilm formed by the diatoms

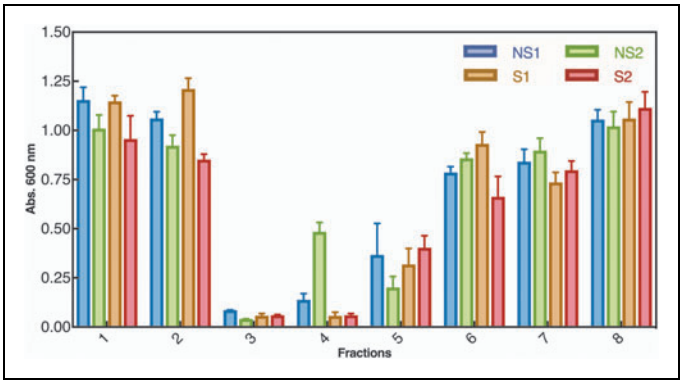

Fig. 7. Activity profile of Flash-fractions from NS1 (blue), NS2 (green), S1 (yellow) and S2 (red) showing inhibition of bacterial biofilm produced by *S. epidermidis* at 100 μg mL^{-1}. Fractions are characterized as active when absorbance at 600 nm <0.25. Data represents means of 3 replicates. Color images are available online.

themselves, usually by pennate diatoms.[59,60] The study by Lauritano et al.[36] showed anti-biofilm activity in the diatoms *Leptocylindrus danicus* and *Leptocylindrus aporus* against biofilm formation by *S. epidermidis*. The present study is the first to report anti-biofilm activity in extracts from a marine cold-water centric diatom. Activity against biofilm formation can be promising, in particular for combating hospital infections related to medical devices, but also for the development of novel anti-fouling compounds for marine industries. During the *P. glacialis* cultivation period of two weeks, there was no formation of visible biofilms on the walls of the bioreactor or on the equipment that were continuously submerged in the diatom cultures. Biofilm formation of bacteria in sea water can establish quite fast—about one week on plastic[61]—and it is likely that the same applies to fiberglass and metal bioreactors like those used in this study. Earlier experiments (not published) in the same bioreactors showed no formation of biofilm after a period of four months. The absence of biofilms is promising for the prospects of mass cultivation of microalgae. Growth on the surfaces of bioreactors leads to reduction of light efficiency in tube or plate reactors, higher maintenance costs as the tanks and equipment need frequent cleaning, and lowered quality of culture conditions due to accumulation of bacteria in the biofilm.[17] The anti-biofilm activity combined with the observed absence of growth on reactor walls suggests that there are active anti-biofilm compounds produced by *P. glacialis,* and that the natural ability to inhibit formation of bacterial biofilm is not changed by adding factory smoke during cultivation.

The results indicate that the direct use of factory smoke as a source for CO_2 when mass cultivating *P. glacialis*, does not change the positive biological effects of extracts of the biomass, and does not lead to increased toxicity against neither human cells nor sea urchin larvae. In addition, valuable bioactivity such as inhibition of biofilm formation is preserved. Anti-biofilm compounds in the biomass can have positive effects in cultivation as it leads to less fouling of photobioreactors and can be useful in the search for new anti-fouling compounds.

Conclusion

Mass cultivation of marine diatoms demands amounts of CO_2 above atmospheric concentrations. The direct use of factory smoke as a source of CO_2 could therefore be a possibility to ensure sustainable production of biomass. Such cultivation of diatoms would also be a means of decreasing CO_2 emissions through carbon capture and utilization. As the use of factory smoke CO_2 changes the cultivation conditions, a thorough mapping of the changes in the biological effects of the biomass is important to ensure safe production, avoid deleterious effects of harmful compounds, and comply with existing food/feed regulations.

The results of the present study confirm the well-known fact that diatoms produce metabolites that may act toxic, but the toxicity does not increase when factory smoke is added in the cultivation process. It is therefore possible to mass cultivate the marine diatom *P. glacialis* using factory smoke as a direct source of CO_2 without affecting and increasing the biomass toxicity, and at the same time preserving valuable anti-bacterial and anti-biofilm activity in the biomass. The research was based on the biological effects observed through a set of bioassays, and future chemical analysis should be performed to investigate the biochemistry of the biomass to analyze possible changes and to identify possible interesting compounds responsible for the anti-biofilm activity.

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Author Disclosure Statement

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Bioactivity of a marine diatom (*Porosira glacialis* (Grunow) Jörgensen 1905) cultivated with and without factory smoke CO_2.

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Supplementary Information

Table 1 Composisition of factory smoke used in cultivation experiment.

Factory smoke composition		
Component	Content ratio (volume %)	Content ratio (weight %)
O_2	13.2 – 16.9	14.6 – 18.6
N_2	72.2 – 75.3	69.7 – 72.7
CO_2	4.5 – 8.1	6.9 – 12.3
H_2O	2.1 – 0.4	1.3 – 0.8
SiO_2 (silicon dioxide dust)	0.2 – 0.4	0.5 – 0.8

Table 2 Content of Substral plant nutrients

Substral	
Component	Contents ($mg\ L^{-1}$)
Nitrate	33.0
Ammonium	27.0
Phosporus	13.0
Potassium	50.0
Trace elements (Cu, Fe, Mn, Mo, Zn)	Trace amounts

Paper II

marine drugs

MDPI

Article

Adding Zooplankton to the OSMAC Toolkit: Effect of Grazing Stress on the Metabolic Profile and Bioactivity of a Diatom

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Abstract: "One strain many compounds" (OSMAC) based approaches have been widely used in the search for bioactive compounds. Introducing stress factors like nutrient limitation, UV-light or cocultivation with competing organisms has successfully been used in prokaryote cultivation. It is known that diatom physiology is affected by changed cultivation conditions such as temperature, nutrient concentration and light conditions. Cocultivation, though, is less explored. Hence, we wanted to investigate whether grazing pressure can affect the metabolome of the marine diatom *Porosira glacialis*, and if the stress reaction could be detected as changes in bioactivity. *P. glacialis* cultures were mass cultivated in large volume bioreactor (6000 L), first as a monoculture and then as a coculture with live zooplankton. Extracts of the diatom biomass were screened in a selection of bioactivity assays: inhibition of biofilm formation, antibacterial and cell viability assay on human cells. Bioactivity was found in all bioassays performed. The viability assay towards normal lung fibroblasts revealed that *P. glacialis* had higher bioactivity when cocultivated with zooplankton than in monoculture. Cocultivation with diatoms had no noticeable effect on the activity against biofilm formation or bacterial growth. The metabolic profiles were analyzed showing the differences in diatom metabolomes between the two culture conditions. The experiment demonstrates that grazing stress affects the biochemistry of *P. glacialis* and thus represents a potential tool in the OSMAC toolkit.

Keywords: diatoms; microalgae; biotechnology; biodiscovery; OSMAC; cultivation

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1. Introduction

The number of investigations of bioactive compounds from marine microorganisms has increased over the last few decades [1]. This is a natural development, since access and expeditions to the various parts of the water that covers 70% of the earth's surface have increased during the recent years. These diverse aquatic biotopes are inhabited by millions of species of microorganisms, both prokaryotic and eukaryotic. Using high throughput DNA sequencing, it has been found that there are a number of biosynthetic gene clusters (BGCs) that are linked to the production of secondary metabolites. These are not necessarily expressed when the microorganisms are cultured under laboratory conditions [2]. This realization eventually led to a concept termed "one strain many compounds" (OSMAC) [3], stating that many microorganisms have the potential to produce a broad range of secondary metabolites, but that only a few are synthesized under specific growth conditions. It is therefore possible to alter growth conditions such as nutrient concentration,

physical conditions, trace elements and the presence of other species through cocultivation to induce the production of a wider range of secondary metabolites. The OSMAC approach has led to the discovery of novel bioactive compounds. Uchoa et al. found that a strain of *Aspergillus niger* produced a novel furan ester derivative with bioactivity against a colon carcinoma cell line (HCT-116), when cultivated in MPDB (malt peptide dextrose broth medium) [4]. Cocultivation of two *Aspergillus* sp. strains and the marine fungi *Avicennia marina* led to the isolation of a new alkaloid with antibacterial activity against *E. coli* [5]. These examples and several others show us that OSMAC has become an important tool in the field of natural product biodiscovery [2,5–9].

One of the most diverse groups of marine microorganisms are the diatoms [10,11], which are a largely unexplored source of chemical diversity. Most OSMAC cultivation experiments have involved prokaryotes, and there are only a few studies involving marine microalgae. A study by Lauritano et al. [12] showed that nitrogen concentration influenced anticancer and antibacterial properties of the diatom *Skeletonema marinoi*. A study by Ingebrigtsen et al. [13] demonstrated that the bioactivity of five diatom species changed with different light and temperature regimes, e.g., *S. marinoi* only showed anticancer activity when cultivated at high temperature. Studies like these show that the OSMAC approach can be used on diatoms as well as bacteria.

Changing cultivation conditions for microorganisms is a way of mimicking natural cultivation conditions in the laboratory. In nature, microorganisms like diatoms coexist with a plethora of other organisms. There is a constant competition between species competing for the same recourses, as well as pressure from predators. The concentration of diatoms in the ocean fluctuates throughout the year. During the annual spring bloom in Northern Temperate and Arctic waters the diatoms dominate the phytoplankton community, reaching concentrations up to 6–10 $\times$ 10^6 cells L^{-1} [14]. The diatom bloom is followed by an increase in the number of grazing mesozooplankton such as copepod species [15,16], and microzooplankton such as heterotrophic protozoans, dinoflagellates and flagellates. Diatoms have for many decades been regarded as the main food source for many zooplankton species and are highly important for the transfer of nutrients up the food chain [17,18]. This relationship has been challenged after several studies found possible toxic effects on copepods feeding on diatoms [19–28]. These studies have investigated the effect of diatom species such as *Phaeodactylun tricornutum, Pseudo-nitzschia delicatissima, Thalassiosira gravia and Thalassiosira rotula,* and about 10 other species. Studies have revealed reduced hatching success in copepods fed a diet of *T. rotula*, and during a bloom of *Skeletonema costatum* and *P. delicatissima* [29,30]. Chaudron et al. [31] found that an increasing proportion of the diatoms *P. tricornutum* and *T. rotula* in the diet led to a reduction in hatching success. Diatoms in the diet can have an insidious effect, i.e., affecting the offspring rather than the consumer itself, and Poulet et al. [32] found teratogenic effects on nauplii larvae leading to fatal abnormalities. The compounds that have been deemed culprits in many of these studies are oxylipins, especially polyunsaturated aldehydes (PUAs) [30]. PUAs are not synthesized inside the cells, but are rather produced enzymatically by lipoxygenase/hydroperoxide lyase from polyunsaturated long chained fatty acids seconds after the rupture of cell membranes due to feeding [27,33]. On the other hand, studies have shown that copepods release chemical signals and cues, often polar lipids called copepodamides, that can trigger a response in microalgae [34,35]. Copepodamides have been shown to affect the morphological features of *Skeletonema marinoi,* such as a decrease in chain lengths [36,37]. Other reported effects on diatoms have been an induced production of the toxin domoic acid by *Pseudo-nitzschia* [38].

Based on the aforementioned studies it is therefore reasonable to think that diatoms can be affected by stress and thus alter their biochemistry, and we hypothesized that diatoms could change their metabolite expression in response to grazing pressure. Metabolomics can be used to investigate the diatoms at a specific time under specific conditions, e.g., when exposed to grazing pressure, in order to reveal changes in the metabolome [39,40]. Further, we hypothesized that such changes in metabolic expression could be detected as a change or an increase in bioactivity in cell-based bioassays; bacterial growth inhi-

bition assays, the inhibition of biofilm formation and the viability of human cell lines, and that grazing pressure could trigger the production of compounds leading to higher bioactivity. Inducing stress in diatom cultures by cocultivation with zooplankton could thus be a potential new "tool" in the OSMAC toolkit, i.e., a method to discover new bioactive metabolites.

In this study we cultivated the marine temperate diatom *Porosira glacialis* (Grunow) Jörgensen 1905 in a large photobioreactor (PBR), first as a monoculture and then in coculture with zooplankton from a nearby costal bay. More specifically the aim of the study was to investigate whether the presence of a small but varied grazer population could alter the *P. glacialis*' biochemistry and bioactivity.

2. Results

2.1. Cultivation of P. glacialis and Cocultivation with Zooplankton

Four nonaxenic batch cultures were cultivated during the experiment: two monocultures abbreviated Pg1 and Pg2 and the two coculture samples abbreviated PgZ1 and PgZ2. All were closely monitored to keep track of whether the cells were healthy and that we had no contamination of unwanted organisms in the culture tanks. All cultures were in good growth condition throughout the cultivation period (Figure 1). Before harvest of the coculture, 10 × 1 L of the culture was transferred to clear flasks to enumerate the number of zooplankters per liter of culture. The concentrations of animals were 1.5 individuals L^{-1} for PgZ1 and 1.0 L^{-1} for PgZ2. The growth data of diatoms based on cell counts (Figure 1) showed that all cultures, except for PgZ2 (growth rate 0.1 doubling day^{-1}), had a steady increase in cell numbers (growth rates 0.29–0.33 doublings day^{-1}), and were all harvested during the exponential growth phase when the cell numbers had reached 12–15,000,000 cells L^{-1}. The second coculture, PgZ2, obviously had a lag phase, but had started the exponential phase when it was harvested after four days. Harvest at a lower cell count was done due to time limitations on the experiment. See Supplementary Material for data on growth rates, temperature, pH, and nutrient concentrations (Figures S2–S6).

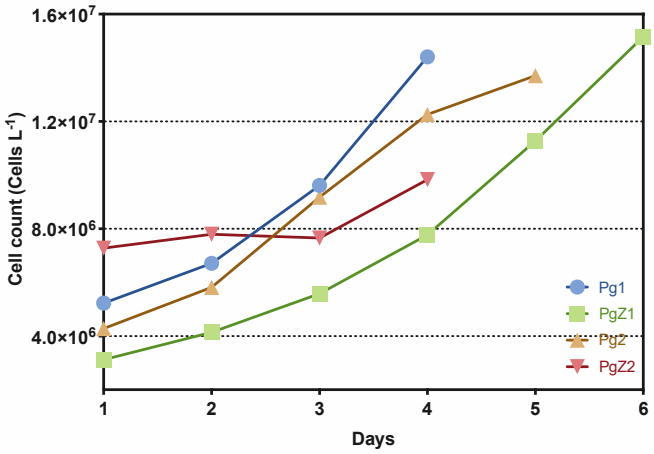

Figure 1. Cell count of *P. glacialis* in cells L-1 vs. time (days) for all four cultures (monocultures Pg1 and Pg2, and cocultures PgZ1 and PgZ2).

Taxonomic analysis of zooplankton species was done based on morphological traits. Both zooplankton batches were dominated by two genera: the cyclopoid copepod *Oithona* sp. and the calanoid copepod *Pseudocalanus* sp. (Table 1). PgZ1 had a higher diversity than PgZ2, and more species could therefore be identified. A few specimens of *Oithona* sp. from the cocultures were collected and investigated using a microscope to see whether they had fed on *P. glacialis* during the cultivation period. The micrographs in Figure 2 shows *P. glacialis* in the stomach (a and b), as well as in the fecal pellets (c) of *Oithona* sp.

Table 1. Species identification and composition (%) of both zooplankton batches. Details on zooplankton quantity can be found in Table S1.

Species	Quantity (%)	
	PgZ1	**PgZ2**
Acartia longiremis	10.0	-
Calanus finmarchicus	2.6	-
Calanus nauplius	-	2.3
Centopages typicus	1.3	2.3
Metridia longa	0.3	-
Microcalanus sp.	2.4	-
Oithona sp.	65.1	53.5
Pseudocalanus acuspes/sp.	16.4	39.5
Temora longa	0.2	-
Calanoida, uid juvenile	1.0	-
Paraeuchaeta norwegica	-	2.3
Bryozoa/cyphonautes	0.8	-

(a) **(b)** **(c)**

Figure 2. Micrographs (**a**) and (**b**) showing two *Oithona* sp. specimens which have *P. glacialis* biomass in the stomach. Micrograph (**c**) shows fecal pellets containing *P. glacialis*.

2.2. Extraction and Fractionation

From the four cultures (Pg1, Pg2, PgZ1 and PgZ2) organic and aqueous extracts were made. As the crude extracts are complex chemical mixtures, they were separated using flash-chromatography. Each of the 4 samples was fractionated into 8 flash-fractions giving 64 fractions in total. All fractions were tested in all bioassays.

2.3. Inhibition of Biofilm Formation

All flash fractions (organic and aqueous) were tested for inhibition of biofilm formation and Figure 3 shows the bioactivity profiles of all flash fractions tested in the biofilm assay. The initial screening was performed using a concentration of 50 μg mL^{-1} and revealed 13 active fractions from a total of 64 tested. Out of the total number of 13 active fractions (absorption below 0.25 at 600 mn), 9 were organic and 4 from aqueous extracts. The activity profiles revealed a similar pattern for all fractions and samples tested, where the most activity was found in fractions 3, 4 and 5 for the organic samples (Figure 3a), and in fractions 4 and 5 for the aqueous samples (Figure 3b). Fraction 5 was the only active fraction from both organic and aqueous extracts. Based on the similarity in the activity profile of all samples (both aqueous and organic), there was no clear difference between the activity in fractions from monocultures or cocultures. These results show that grazing stress did not affect the activity on inhibition of biofilm formation.

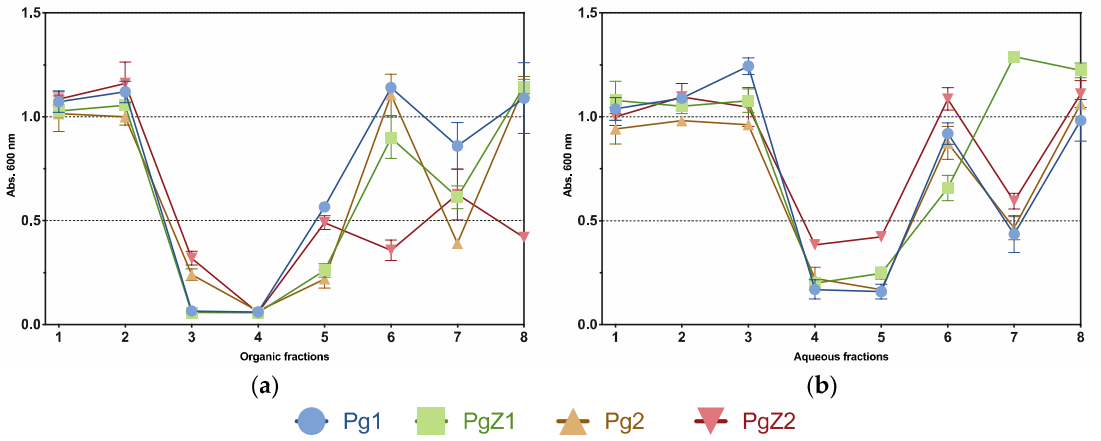

Figure 3. Activity profiles of all samples from biofilm inhibition assay, organic flash fractions from all four samples are shown in (**a**), while (**b**) shows the aqueous flash-fractions. All tested at 50 µg mL^{-1}. All fractions with absorption readings below 0.25 at 600 nm are regarded as active.

2.4. Cell Viability

All samples, organic and aqueous, were tested for activity against the three human cell lines: HT29 human colon carcinoma, A2058 human melanoma, and MRC5 normal lung fibroblasts in a cell viability assay at 50 µg mL^{-1} (Figure 4). As the figure shows, activity was found against all three cell lines. Towards the HT29 colon carcinoma cells, the activity profile of the fractions from the different cultures were similar; the only difference was observed for Pg1, where both organic and aqueous fraction 5 were active (Figure 4a,b). Towards the melanoma cell line A2058, only organic PgZ2 fraction 6 showed activity (Figure 4c). No difference in the activity between the two cultivation conditions for the other fractions was detected. The bioactivity profile against the normal cell line MRC5 revealed a difference between monoculture and coculture samples; organic fraction 6 of the PgZ1 and 2 were active against the cells, while Pg1 and 2 were not (Figure 4e). This fraction also showed activity against the A2058 for one of the cocultures (PgZ2) as described above. The bioactivity profile against HT29 shows that organic fraction 6 of PgZ1 and 2 also had some effect on human colon carcinoma compared to Pg1 and 2.

2.5. Bacterial Growth Inhibition Assay

All samples were tested in a growth inhibition assay against the five bacterial strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia faecalis* (ATCC 29212) and *Streptococcus agalactiae* (ATCC 12386) at 50 µg mL^{-1}. The results in Figure 5 showed that fraction 5 (both organic and aqueous) from all four samples was active against one of the strains, *Streptococcus agalactiae*. There was no activity against the other strains, and no difference in activity of the two cultivation treatments. This showed that grazing pressure had no obvious effect on antibacterial activity of *P. glacialis*.

2.6. Metabolic Profile

Crude extracts of all samples (organic and aqueous) were analyzed using UHPLC and HR-MS to obtain metabolic profiles of the extracts. The UHPLC-HR-MS chromatograms of the extracts were very complex, and in order to identify differences between the different samples, we analyzed the data in a metabolomics workflow. The scores' plots (Figure 6) are based on the collected markers (i.e., combinations of a mass and a retention time) from all samples. The monoculture and coculture samples were well separated both for the organic samples (Figure 6a) and the aqueous extracts (Figure 6b), showing that there are variations in the metabolic profiles of the two cultivation conditions. All monoculture samples clustered together, meaning that there was little variation within the group. The two

different coculture samples (i.e., PgZ1 and PgZ2) grouped separately for both the organic and aqueous extracts, indicating that there were some differences in metabolic profiles between the two cultivations. However, as we did not observe any significant differences in bioactivity between these two cocultivations, we assume that the difference is due to variations in cultivation temperature, as sample PgZ2 was cultivated at lower average temperature than the others.

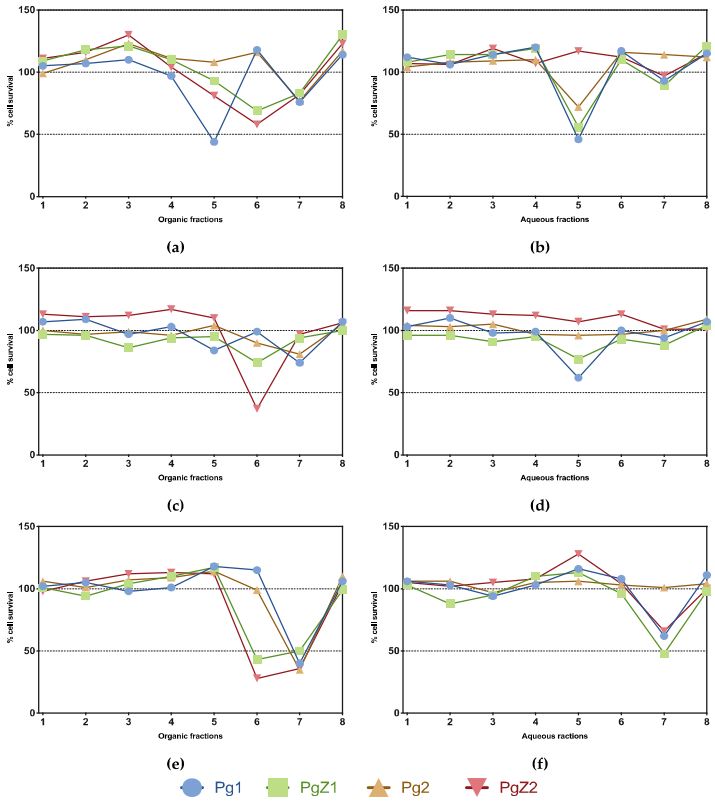

Figure 4. Overview of activity of all samples in cell viability assay against human colon carcinoma (HT29) (**a**,**b**), human melanoma (A2058) (**c**,**d**) and normal lung fibroblasts (MRC5) (**e**,**f**) at 50 µg mL^{-1}. Fractions were regarded as active when cell viability was measured below 50%.

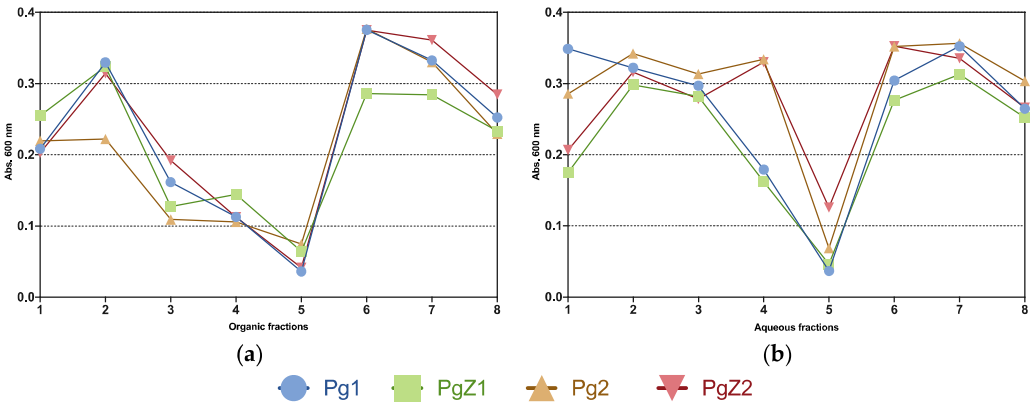

Figure 5. Overview of activity of all four samples against *Streptococcus agalactiae*. Organic fractions in (**a**) and aqueous fractions in (**b**). All tested at 50 µg mL^{-1}. Fractions regarded as active with absorbance readings below 0.05 at 600 nm.

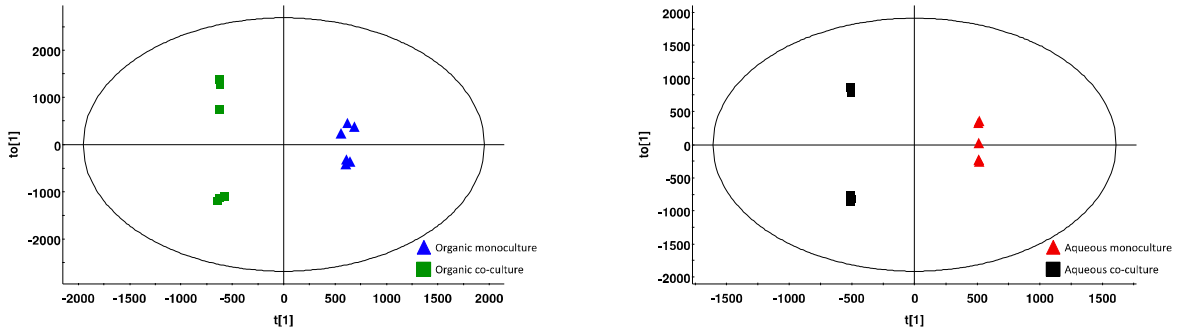

Figure 6. Scores plot of metabolic profiles. Plot (**a**) is based on collected markers from organic samples from monocultures and cocultures, and (**b**) are based on collected markers from aqueous extracts of monocultures and cocultures.

To reveal differences between the metabolic profiles, the organic extracts of both cocultures (PgZ1 and 2) and monocultures (Pg1 and 2) were compared in a s-plot (Figure 7). The x-axis of the plot denotes the contribution of the marker to the differences between the samples, and the y-axis denotes the confidence in the contribution. The markers in the left lower corner were characteristic for the coculture samples, while the markers in the upper right corner were characteristic for the monoculture samples. The 5 markers from each sample set contributing the most to the differences were attempted identified by using the MS analysis software. The markers where a matching compound was identified by the Dictionary of Marine Natural Products or MarinLit are marked in red in Figure 7. Elemental composition calculations of the markers were done using HR-MS data in ES+.

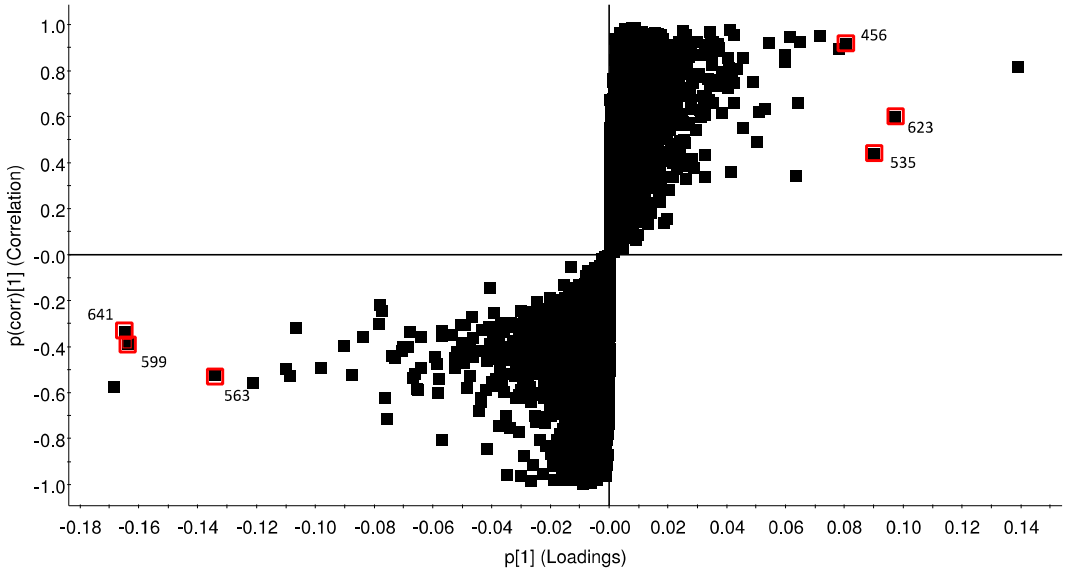

Figure 7. S-plot of organic samples of cocultures: PgZ1 and 2 (=−1, bottom left) and monocultures: Pg1 and 2 (= 1, top right) The x axis denotes the contribution of the markers to the differences, and the y-axis shows the confidence in the contribution. Markers highlighted in red are those where a matching compound was identified.

The two markers in the coculture samples *m/z* 641.4193 and *m/z* 599.4088 were identified as the carotenoids halocynthiaxanthin acetate ($C_{42}H_{56}O_5$) and halocynthiaxanthin ($C_{40}H_{54}O_4$), respectively. Both compounds were previously isolated from marine sources [41]. A third carotenoid with *m/z* 563.388 was identified as clathriaxanthin ($C_{40}H_{50}O_2$), which has been isolated from marine sponges [42,43]. Two of the markers in the monoculture samples were identified as chlorophyll derivatives. The marker *m/z*

623.2855 ($C_{36}H_{38}N_4O_6$) was suggested to be a pheophorbidelike structure and marker *m/z* 535.2706 ($C_{33}H_{34}N_4O_3$) as pyropheophorbide b. The compound with *m/z* 456.2789 and elemental composition $C_{27}H_{37}NO_5$ corresponded with a compound earlier found in marine derived fungi [44].

The active fraction 6 of PgZ1 and PgZ2 were analyzed using UPLC-HR-MS to search for compounds responsible for the activity. The chromatograms of the active fractions were compared to the nonactive fraction 6 of Pg1 and Pg2 to search for possible differences. No apparent differences were found in the chromatograms with respect to the presence of the more prominent metabolites, but there were numerous cases where the signal strength of compounds varied between the active and the inactive fractions. The max fold change of the total number of compounds found in the metabolomics dataset (of all four samples) showed that 4972/14280 compounds had a noticeable upregulation (fold change cut off > 5). Due to the high number of compounds with differences in abundances, it was not possible to attribute the differences in bioactivity to any specific metabolite. However, the manual dereplication resulted in finding signals corresponding to degradation products of chlorophyll such as pheophorbide and membrane components such as phosphocholines among the prominent metabolites.

3. Discussion

The aim of the present study was to investigate if the presence of a small but varied grazer population could alter the *P. glacialis* biochemistry and bioactivity, i.e., to evaluate if zooplankton grazing could be applied as a tool to induce the production of bioactive compounds.

Extracts of biomass cultivated with and without the presence of zooplankton were tested in three different bioactivity assays: human cancer cell viability, bacterial growth inhibition and inhibition of bacterial biofilm formation. The screening of the flash-fractions was done at a concentration of 50 μg mL^{-1}, allowing comparisons of activity between fractions and assays. Fractions that were active at 50 μg mL^{-1} were retested at different concentrations after serial dilutions to evaluate dose–response effects. Using the same test concentrations of all the fractions in the initial screening allowed us to assess possible differences between the different cultivation conditions. All samples showed activity in all performed assays, confirming the bioactivity previously found in *P. glacialis* by Ingebrigtsen et al. [13].

As diatoms are known to change their bioactivity and metabolic profile in response to changing cultivation conditions such as light, temperature and nutrients [12,13,45] we aimed to minimize variations in cultivation conditions in order to prevent cultures from being influenced by changes other than the presence of the grazing zooplankton. Our experiment was carried out in a 6000 L vertical column mesocosm bioreactor at a cultivation facility located outdoors. Being located outdoors, the temperature of the cultures was dependent on the air and water temperature of the season, but as the experiment was conducted during winter the temperature was relatively stable, i.e., average cultivation temperatures were 4.3–5.4 °C during the cultivation of cultures Pg1, PgZ1 and Pg2. Only one culture, PgZ2, had a lower average temperature (1.0 °C). Temperature change has been shown to influence the antioxidant activity of *P. glacialis* but did not have any effect on anticancer activity [13].

Several calanoid copepods are known to enter hibernation and nonfeeding (diapause) during winter when the concentrations of microalga in the water column are negligible [46]. As our experiment was performed during winter, it was uncertain how our added zooplankton sample would react to the transition from dark, clear water to a dense diatom culture with 24 h illumination. Our investigation of the copepods under the microscope showed that they were still alive at the end of each cultivation period. Visual inspection of all the investigated specimens also confirmed that they were feeding on the diatoms. Furthermore, the fecal pellets found in the same samples contained partly digested *P. glacialis*. Previous studies have revealed that the small copepod *Oithona* sp., one of the dominating species in the samples, remains active during winter, feeding on what they find [47–49].

We thus concluded that the zooplankton were actively grazing on the diatoms. Regarding the total effect of the presence of grazing zooplankton on *P. glacialis* we also need to consider the concentration of zooplankton in the culture. At the peak of zooplankton abundances during the spring bloom, the number of copepods can be > 100 L^{-1} [38,50]. Such high concentrations constitute a major grazing pressure on the diatoms and other microalgae. Our experiment was performed with a concentration of 1–1.5 specimens per liter of culture, which in comparison is very low, especially when the diatom concentrations are considered. However, copepods can scare many more prey than they consume [37,51]. In addition, it is important to consider that the zooplankton was added to a monoculture of *P. glacialis* which had been growing without competition from any other species of phytoplankton or zooplankton for a long period of time. Although our samples were sieved, a higher concentration of zooplankton could have resulted in the extraction of metabolites from zooplankton, and not *P. glacialis* as was our aim. As we managed to keep conditions similar between treatments, and at an early stage of the experiment obtained evidence that the zooplankton were grazing on *P. glacialis*, we expect that the differences we observed were due to the presence of zooplankton.

The results from the bioactivity testing against bacteria revealed no significant difference in the bioactivity of the two cultivation conditions ($p > 0.05$ for all samples, statistical analysis in Table S2). The inhibition of biofilm formation assay revealed 14 active fractions in total, 9 from monoculture samples vs. 4 from coculture samples. As the results of the assay were based on the amount of biofilm present after 24 h of exposure to the flash-fractions, it cannot be excluded that the activity was due to growth inhibition of the bacteria itself rather than inhibition of biofilm formation. This can only be ruled out by a growth inhibition assay, which should be performed if the results of the biofilm activity assay are to be studied further. There were more active fractions from the monoculture samples, but the activity profile of the tested fractions showed similar patterns in all samples tested. These similarities could indicate that the difference in activity was due the concentration of active component in the fractions, and not due to different components in the fractions from the two different cultivation treatments. Bioactivity against the formation of bacterial biofilm in diatom extracts has been investigated to a lesser extent, and there are few studies showing the biofilm activity of marine diatoms [12]. Such activity can be a strategy by the diatoms to prevent bacterial growth on the surface of the diatom, in its phycosphere or immediate surroundings, but the stress of grazing pressure has no apparent effect on *P. glacialis*' ability to inhibit the formation of biofilm. Large scale microalgal cultures will always contain bacteria. Bacteria are in fact often beneficial in diatom cultivation, and necessary to provide certain compounds needed by the diatoms, such as vitamin B_{12}, iron and other trace elements [52]. The production of antibacterial compounds by the diatoms might not be beneficial when the bacteria present are not parasitic or in any way harmful for the diatoms. The presence of grazers such as copepods, on the other hand, might induce stress reactions in the diatoms that could lead to the synthesis of secondary metabolites with activity that could also have antibacterial effects. Our experiment showed no significant difference in antibacterial effect between the two cultivation conditions, and the total number of active fractions in the growth inhibition assay was low (5/64).

All samples were screened in a viability test against two cancer cell lines, human colon carcinoma and human melanoma, and activity was found against both cell lines. Cytotoxic activity in extracts of marine diatoms is known from previous studies [12,13], but the compounds responsible in those studies has not been identified. Sansone et al. [53] found anticancer activity against A549 (ATCC CCL185) human lung adenocarcinoma and COLO 205 (ATCC CCL-222) colon adenocarcinoma when exposing the cell lines to the diatom-derived PUAs 2-*trans*, 4-*trans*-decadienal, 2-*trans*,4-*trans*-octadienal and 2-*trans*,4-*trans*-heptadienal, all of which had anticancer activity, and no activity against the normal lung/brunch epithelial BEAS-2B cell line. Although activity was observed against both cancer cell lines, the activity profile showed no significant difference between the cultivation treatments, indicating that cocultivation with zooplankton had no obvious effect on the

anticancer activity of *P. glacialis*. The viability assay was also conducted against normal lung fibroblasts (MRC5). This viability assay revealed a difference between the two cultivation treatments. Organic fraction 6 of PgZ1 and 2 were active and led to a decrease in cell viability, while fraction 6 of Pg1 and 2 had no effect on the cells. The same fraction also showed activity against A2058 for PgZ2. This shows that the presence of zooplankton induced the production of compound(s) that are toxic against normal lung fibroblasts. In a study by Ingebrigtsen et al. [54] field samples of microalgae and zooplankton from a spring bloom in the Barents Sea were screened for bioactivity and compared to a monoculture of *P. glacialis*. In the study, field samples composed of only microalgae, only zooplankton and the two combined were all active against normal lung fibroblasts, while the cultivated monoculture of *P. glacialis* was not active in the same assay. These results compared to those from the current study might suggest that diatoms under stressful conditions produce compounds that are toxic to normal human cell lines. The active fractions were analyzed using UPLC-HR-MS to search for possible differences that could reveal which compounds were responsible for the activity in fraction 6 of PgZ1 and PgZ2. Dereplication of some prominent peaks of the chromatograms revealed signals corresponding to chlorophyll derivatives such as pheophorbide and membrane components such as phosphocholines. Pheophorbidelike compounds has previously been shown to have anticancer and cytotoxic properties [55]. In the metabolomic analysis of the extracts, several carotenoid compounds were found to contribute most to the differences in the cocultivated samples. Carotenoids such as fucoxanthin have shown anticancer activity in previous studies both in diatoms and green algae [56–58]. Analysis of the chromatograms revealed no apparent differences in metabolite composition, but rather a difference in the concentration of the metabolites, based on the signal strength of the peaks in the chromatogram. The fractions are highly chemically complex, and pin-pointing a culprit responsible for the specific cytotoxicity is difficult. However, based on evidence found in the LC-MS analysis of active fractions and metabolomics samples, the cytotoxicity might be attributed to chlorophyll degradation products or carotenoid compounds.

Grazing copepods are known to influence diatom morphology [36,59] and biochemistry [38]. Amato et al. [60] investigated the metabolomic and transcriptomic changes in *Skeletonema marinoi* after cocultivation with *Calanus finmarchicus* and *Centropages typicus* and found that there was an activation of stress response, and a change in lipid and nitrogen metabolism of the diatoms. Our analysis of the metabolomic data and metabolic profiles of the organic extracts show a difference between the samples from the monocultures and the cocultures, but this difference might not be detectable as a change in the bioactivity of the samples. Initial attempts to identify the compounds contributing the most differences were done. In the monoculture samples the markers were identified as a possible alkaloid and two pheophorbidelike compounds. Pheophorbide has been linked to grazing, as it is a pigment often found in fecal pellets of copepods due to the degradation of chlorophyll in the stomach [61]. In addition to phaeophytin a, pheophorbide is a known degradation product of chlorophyll a that we expected to be present in our samples [62]. Analysis of the stress response by Amato et al. [60] showed the downregulation of chlorophyll binding proteins when the diatom *Skeletonema marinoi* was exposed to copepod grazing. The compounds contributing most to the difference in the coculture sample set were identified as carotenoids. Carotenoids are one of the most abundant groups of pigments in nature, and play important roles in many physiological functions [63]. In addition to being color compounds and accessory pigments in the photosynthesis, they are also known as antioxidants and UV protecting molecules [64,65]. Former studies have shown that algae and higher plants increased the production of carotenoids in response to stresses such as UV light associated damage, nutrient depletion, pH and temperature [65–68]. Although no studies were found relating grazing stress to the production of carotenoids it is plausible that this may have a connection.

In conclusion, the present study reveals that grazing zooplankton have an effect on the temperate diatom *P. glacilias*, and that the effect can be seen in both the metabolic profile and the expressed bioactivity.

4. Materials and Methods

4.1. Mass Cultivation of Porosira Glacialis

Two nonaxenic batch cultures of *P. glacialis* were cultivated at an outdoor mass cultivation facility, in a 6000 L glass fiber vertical column open photobioreactor. As irradiance from natural light is scarce and the solar angle is low during winter in Northern Norway (69°13′0″ N, 18°5′10″ E), the cultures were illuminated using LED light (500–700 W) at photoperiod 24 h to provide consistent light conditions. Seawater used in the cultivations was from an inlet at 25 m depth filtrated through a series of filters; 5 µm particle filter (Azud, Murcia, Spain), 1 µm filter cartridge (Eaton, Dublin, Ireland) and a UV unit (450 mJ cm^{-1} at 12 m^3 t^{-1}) (ULTRAAQUA A/S, Aalborg, Demark). Silicate solution and inorganic nutrients (N, P, Mg, K, S and Fe) were added in order to allow the microalgae to grow at nutrient replete conditions. Daily measurements of nutrient concentrations (NO_3^-, SiO_2 and PO_4^{3-}), temperature (°C) and pH were done to monitor cultivation conditions. In addition, biomass concentrations were inferred by cell counts [69] and chlorophyll concentration measurements (raw fluorescence). Daily visual examination was performed to assess culture health using an inverted microscope (Zeiss Axiovert A1, Carl Zeiss Microscopy GmbH, Jena, Germany). Diatom biomass was harvested after 5 days of growth using a continuous centrifuge (Evodos 10, Evodos B.V., Raamsdonksveer, The Netherlands), frozen immediately after harvest and kept at −80 °C until further use. After the first harvest the diatom culture was diluted and prepared for the zooplankton challenge experiment.

4.2. Zooplankton Collection

Samples of zooplankton were collected near the shore in Finnfjordbotn in Northern Norway (Figure 8) using a WP2 plankton net (180 µm) with a detachable cod-end. The net was towed at ca. 1 knots towing speed just below the surface at the side of a Polarcircle 560 Work boat. Each WP-2 haul lasted for about 20–30 min before the net catch was emptied into sample flasks. The transfer was done quickly due to low air temperature (ca. −13 °C) and risk of sea water freezing. All net samples were pooled and stored at approximate seawater temperature at the sampling site, i.e., 2 °C in an insulated container onboard until use (2–4 h). Larger species, such as ctenophores were removed from the sample. Subsamples were preserved in 96% ethanol and stored for taxonomic analysis.

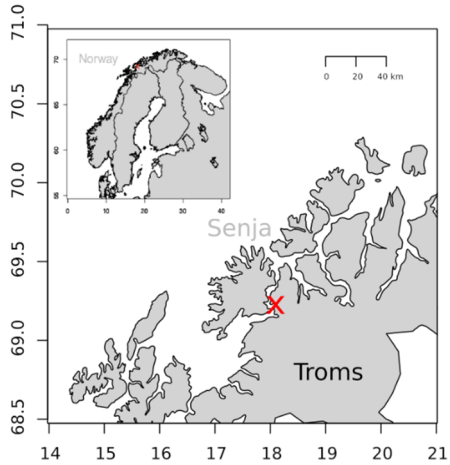

Figure 8. The map shows the area where the zooplankton samples for the cocultivation experiment were collected. The map was generated using the package "Maps" version 3.1.1. in R version 3.3.2.

4.3. Cocultivaion

The collected zooplankton sample was transferred to the 6000 L open photobioreactor containing the *P. glacialis* monoculture. *P. glacialis* was cocultivated with the field zooplankton sample. The other cultivation parameters and daily measurements were the same as for the monoculture. As a survival control, flasks (2 L) with the coculture of *P. glacialis* and the zooplankton sample were kept in a cultivation incubator in the lab at 4 °C with a photoperiod of 14:10 (light:dark) to monitor if the zooplankton survived in the dense culture. Feeding/no feeding was monitored by inspecting zooplankton gut content after 2 days, using an inverted microscope at 100× magnification (Zeiss Axiovert A1, Carl Zeiss Microscopy GmbH, Germany). Figure 9 shows an overview of the cultivation and cocultivation pipeline. For each cocultivation new zooplankton bulk samples were collected from a nearby bay area. The harvest of the coculture was done using a continuous centrifuge (Evodos 10, Evodos B.V., The Netherlands), but the culture was filtered through a plankton net (180 µm) prior to the centrifugation to make sure that zooplankton were kept from entering the centrifuge. Harvested biomass was frozen immediately and kept at −80 °C until use.

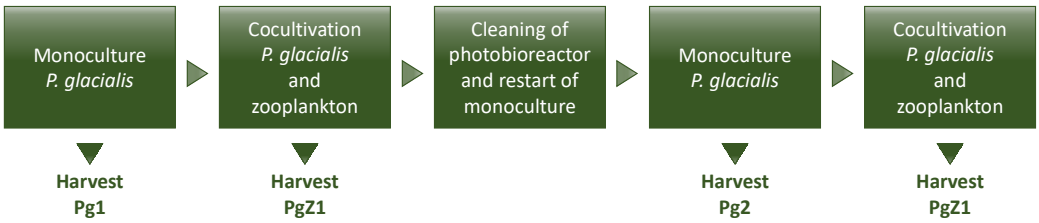

Figure 9. Overview of experimental workflow.

4.4. Extraction and Flash-Fractionation

All diatom biomass for bioactivity testing was freeze-dried, ground into a fine powder using mortar and pestle, and extracted overnight using MilliQ-H_2O. It was then centrifuged at 4600 rpm and 4 °C for 30 min, and the supernatant was kept. The pellet was resuspended in MQ-H_2O and extracted a second time for 30–60 min, and centrifuged. The supernatant was frozen at −80 °C, then freeze-dried and ground into a fine powder before being frozen at −20 °C. The extracted pellet was freeze-dried before being extracted overnight using a 1:1 mixture (vol:vol) of methanol (Sigma-Aldrich, St. Louis, MO, USA) and dichloromethane (Merck, Darmstadt, Germany). The extract was filtered, and the pellet was extracted a second time. The combined organic extract was dried under reduced pressure using a rotavapor (Laborata 4002, Heidolph Instruments GmbH, Schwaback, Germany).

Aqueous and organic extracts were fractionated using a flash purification system (Biotage HPFC SP4, Biotage®, Uppsala, Sweden). Organic extracts were prepared by suspending 1.5 g of extract in Hexane (40 mL g^{-1}) in a separation funnel. MeOH (90%) was added (30 mL × 2), the solution mixed carefully, and the lower liquid phase (MeOH) was transferred to a round flask prefilled with 2 g of Dianon HP-20SS resin (Supelco, Bellefonte PA, USA) and dried under reduced pressure. Aqueous extracts were prepared by mixing 2 × 0.75 g of dried extract with 4 mL 90% MeOH, 1.5 g Dianon HP-20SS resin and 1 mL MilliQ-H_2O, before being dried at reduced pressure.

Columns for flash-fractionation were prepared by washing 6.5 g of resin with MeOH for 20 min. MeOH was then exchanged with MQ-H_2O, and the material was transferred to a flash column (Biotage® SNAP, Uppsala, Sweden). The column was equilibrated with 5 % MeOH, and the dried extract was loaded to the column. Flash fractionation of the sample was done in two steps; first with a gradient of 5–100% MeOH and MilliQ-H_2O at a flow rate of 12 mL min^{-1} for 32 min, and then a MeOH:acetone (Merck, Germany) gradient ending at 100% acetone, flow rate 12 mL min^{-1} over 18 min. The eluent was collected into eight fractions and dried under reduced pressure. Fractions were dissolved in 100% DMSO to a concentration of 40 mg mL^{-1}. Deep well screening plates were prepared by

adding 25 µL (i.e.,1 mg dry material) of each fraction and stored at −20 °C until screening. Fractions were resuspended in 975 µL dH_2O right before screening.

4.5. Biofilm Assay

All flash-fractionated samples were tested for inhibition of biofilm formation using the bacterial strain *Staphylococcus epidermidis* (ATCC-35984) which is known to form biofilm. Bacterial colonies were transformed from blood agar to a liquid Tryptic Soy Broth enrichment media (TBS, Merck, Germany) and incubated at 37 °C overnight, and then diluted 1:100 in TBS + 1% glucose. The assay was performed in 96-well microtiter plates at a concentration of 100 µg/mL, and all fractions were tested in triplicates. 50 µL of each fraction and 50 µL of bacterial suspension was added to each well. The nonbiofilm forming bacteria *Staphylococcus haemolyticus* was used as negative control, *S. epidermidis* + dH_2O as a positive control and TBS (1% glucose) and dH_20 as blank. Plates were incubated at 37 °C overnight. The bacterial suspension was removed carefully, and the wells were washed using MQ-H_2O. To fixate the biofilm, the plates were stored at 55 °C for an hour. Then the biofilm was stained using 70 µL of 0.1% crystal violet (Merck, Germany) for 5 min. The liquid was removed by repeated washing with MQ-H_20, dried at 55 °C, and resuspended using 70% EtOH. Absorbance was read in a VICTOR 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA) at 600 nm.

4.6. Growth Inhibtion Assay

Antibacterial activity was investigated in a growth inhibition assay. A panel of five different bacterial strains were used, and both Gram negative and positive bacteria were included. Colonies of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were transferred from blood agar plates an inoculated at 37 °C for 24 h in Mueller–Hinton growth media (MH, Becton Dickinson Company, Franklin Lakes, NJ, USA), and *Escherichia faecalis* (ATCC 29212) and *Streptococcus agalactiae* (ATCC 12386) in brain heart infusion media (BHI, Sigma-Aldrich, Germany). After incubation 2 mL of each suspension was transferred to 25 mL of fresh growth medium and incubated until log-phase, before being diluted to 1:1000 to adjust cell density. The assay was performed in 96-well titer plates at 100 µg mL^{-1}. 50 µL of each fraction and 50 µL of bacterial suspension was added to each well. All fractions were tested in duplicates. Growth medium + dH_2O was used as negative control, and gentamicin was used as positive control. The plates were incubated at 37 °C overnight and absorbance was measured in a VICTOR 1420 Multilabel Counter at 600 nm.

4.7. Cell Viability Assay

The flash fractions were tested against two cancer cell lines: human melanoma (A2058, LGC Standards ATCC-CRL-11147) and human colon carcinoma (HT29, LGC Standards ATCC HTB-38), and for comparison, against normal lung fibroblasts (MRC5, LGC Standards ATCC CCL-171). 2000 cells were seeded into each well of a 96-well micro titer plate (4000 cells/well for MRC5 cell line) in Roswell Park Memorial Institute 1640 cell medium (RPMI) with 10% fetal bovine serum added 10 mg mL gentamicin and incubated at 37 °C and 5% CO2 for 2 h. After incubation the medium was replaced with 50 µL per well and the cells were exposed to 100 µg mL^{-1} of flash fractions for 72 h. All fractions were tested in triplicate. Culture medium was used as a negative control, while 10% (*v/v*) DMSO (Sigma-Aldrich, Germany) was used as the positive control. At the end of incubation, 10 µL of CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added and incubated for 1 h. Then the absorbance was read at 485 nm using a DTX 880 Multimode Detector. Results were calculated as % cell survival, and survival below 50% was counted as active.

4.8. UHPLC-ESI-HR-MS Analysis and Data Processing for Metabolomic Profile

The UPLC-HR-MS analysis was done using a Waters Acquity I-class UPLC system (Waters, Milford, MA, USA) coupled to a PDA Detector and a VION IMS-qTOF, using electrospray ionization (ESI) in positive mode. Wavelengths from 190–500 nanometers were detected. VION IMS-qTOF conditions for UPLC-HR-MS analysis; capillary voltage (0.80 kV), cone gas (50 L h^{-1}), desolvation temperature (350 °C), desolvation gas (800 L h^{-1}), source temperature (120 °C) and acquisition range was *m/z* 50–2000. Chromatographic separation was performed with a BEH C18 1.7 µm (2.1 × 100 mm) column (Waters, Milford, MA, USA) maintained at 40 °C. Data from the analysis of flash fractions were processed using UNIFI 1.9.4 software (Waters). Selected peaks were dereplicated using MarinLit, ChemSpider, and Dictionary of Natural products, as well as extensive literature searches. Statistical analysis of metabolomics data was done using EZinfo v.3.0.3.0 (Umetris ab) and Progenesis QI v.2.4 (Nonlinar Dynamics) software for analysis of LC-MS data. Scores plot and s-plot were made using EZinfo.

4.9. Statistics and Software

Statistical analysis of data was done by running chi-square tests in Excel for Mac 2020 version 16.36. Figures were made using Prism 8 for Mac (GraphPad Software Inc.) and in Rstudio version 1.2.1335 [70]. Map was made with Maps version 3.1.1. in R version 3.3.2. [71].

5. Conclusions

Investigation of the metabolic profiles from the monocultures and the cocultures revealed metabolomic differences indicating that stress from grazing affected, e.g., the production of carotenoids in *P. glacialis*. Cocultivation with zooplankton also induced the production of compounds with cytotoxic activity towards normal lung fibroblasts. Using cocultivation to enhance or trigger the production of bioactive compounds in bacteria is quite common and this method can also be used in microalgae biodiscovery. Future OSMAC studies on diatom and zooplankton cocultivation should include cultivation also using alternative species of diatoms and zooplankton, as well as different concentrations of grazers and cultivation at other times of the year. Full genome sequencing of diatoms has revealed that their large genomes most likely harbor "silent" gene clusters. It is therefore reasonable to believe that diatoms have a greater potential to produce secondary metabolites than what emerges in "static" laboratory conditions and cultivation in monoculture.

Supplementary Materials: The following are available online at https://www.mdpi.com/1660-3397/19/2/87/s1, Figure S1: Temperature and salinity profiles of zooplankton sampling sites; Figure S2: Daily temperature measurements; Figure S3: Daily pH measurements; Figure S4: Nitrate measurements; Figure S5: Silicate measurements; Figure S6: Phosphate measurements; Table S1: Zooplankton composition of bulk zooplankton samples; Table S2: Statistical analysis of bioactivity.

Author Contributions: Conceptualization, R.D.O., R.A.I., H.C.E., E.H.H.; methodology, R.D.O., R.A.I., E.H.H., J.H.A.; data analysis, R.D.O., R.A.I., M.F.N., E.H.H.; writing—original draft preparation R.D.O.; writing—review and editing, all authors.; visualization, R.D.O., R.A.I. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Adding zooplankton to the OSMAC toolbox: Effect of grazing stress on the metabolic profile and bioactivity of a diatom.

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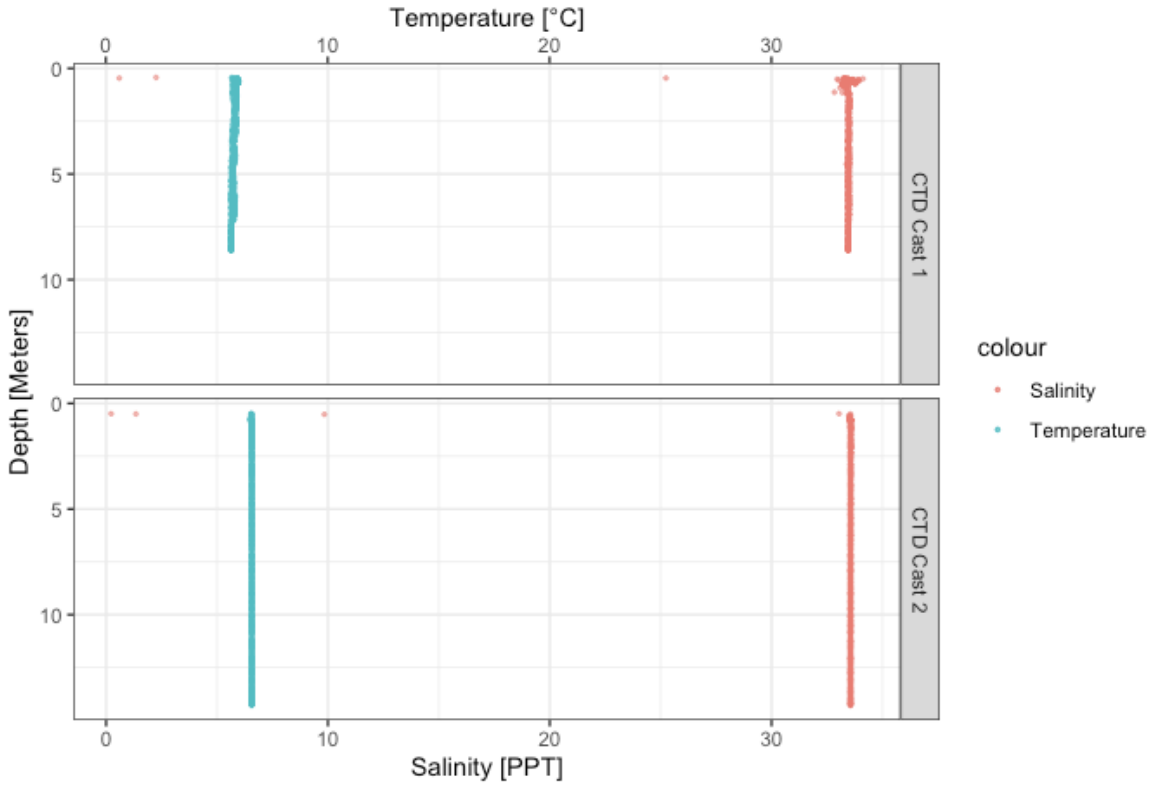
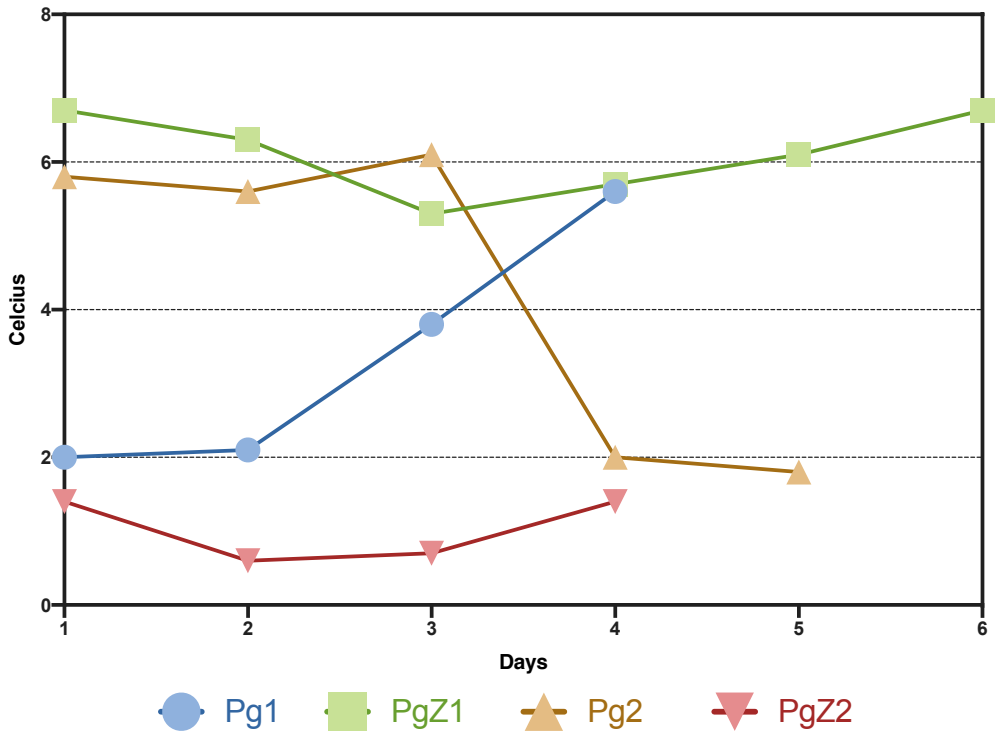
Supplemental Information Table of Contents



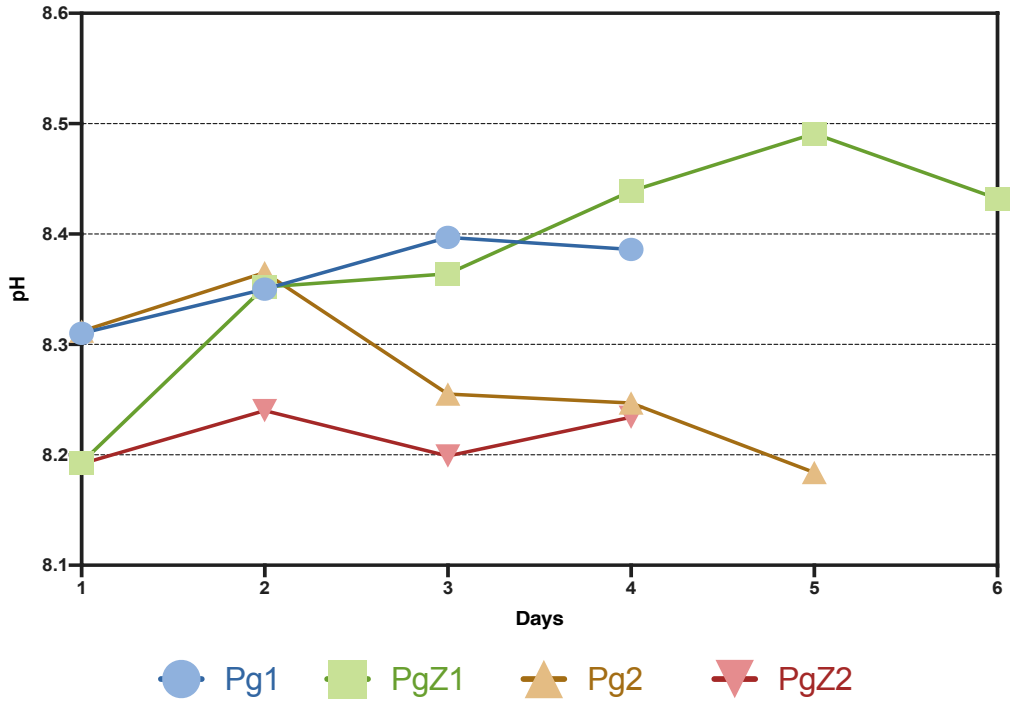
Supplementary Figure S1. Panel showing temperature and salinity profiles from the two sample sites where we collected zooplankton. The lower x-axis shows salinity as PPT with red dots and the upper x – axis shows temperature in degrees Celsius (°C) with blue dots, while the y-axis is depth in meters. CTD casts data were taken from surface to 50 cm above bottom and back to surface. Samples sites were similar, and the water column was well-mixed at both sites.

Supplementary Table S1: Zooplankton composition of bulk zooplankton samples. Zooplankton sub-samples were collected before zooplankton were added to culture of P. glacialis and stored in 96% EtOH.

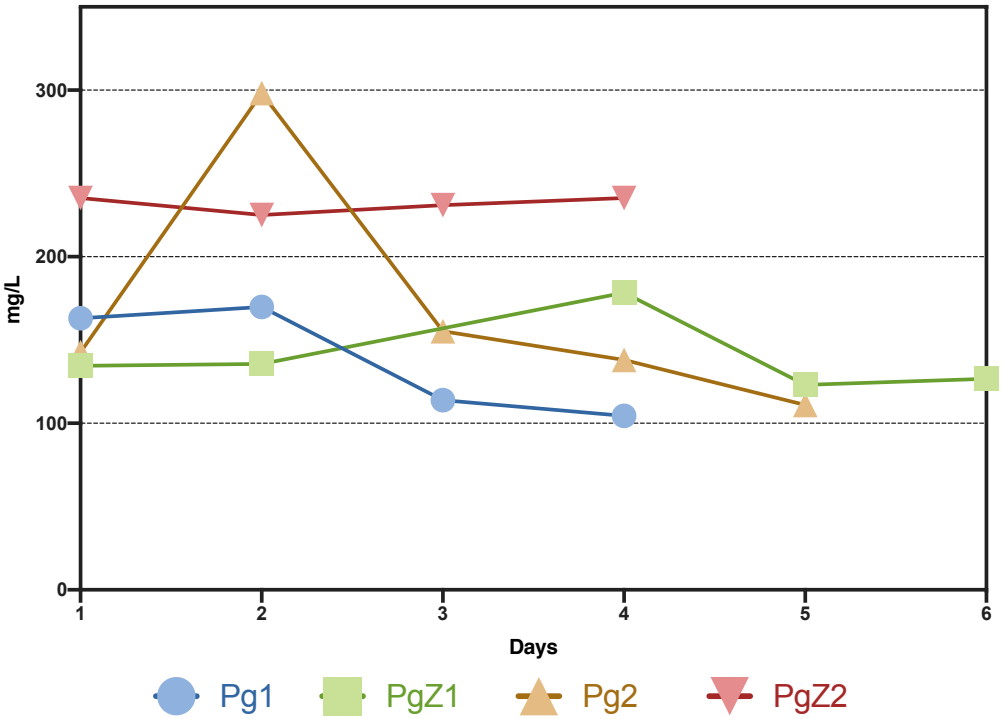
Species	**Samples 1 (PgZ1)**			**Samples 2 (PgZ2)**		
	Count	%	**Comment**	**Count**	%	**Comment**
Acartia longiremis	62	9,98	mostly adult female			
Calanus finmarchicus	16	2,58				
Calanus nauplius				1	2,33	
Centopages typicus	8	1,29	copepodites	1	2,33	
Metridia longa	2	0,32				
Microcalanus sp	15	2,42	copepod/adults			
Oithina sp.	404	65,06	most stages	23	53,49	
Pseudocalanus acuspes/sp	102	16,43	copepod/adults	17	39,53	5 females
Temora longa	1	0,16	copepodite			
Calanoida, uid juvenile	6	0,97	mostly adult females	1	2,33	
Paraeuchaeta						
Bryozoa/cyphonautes	5	0,81				
Total	621	100		43	100	



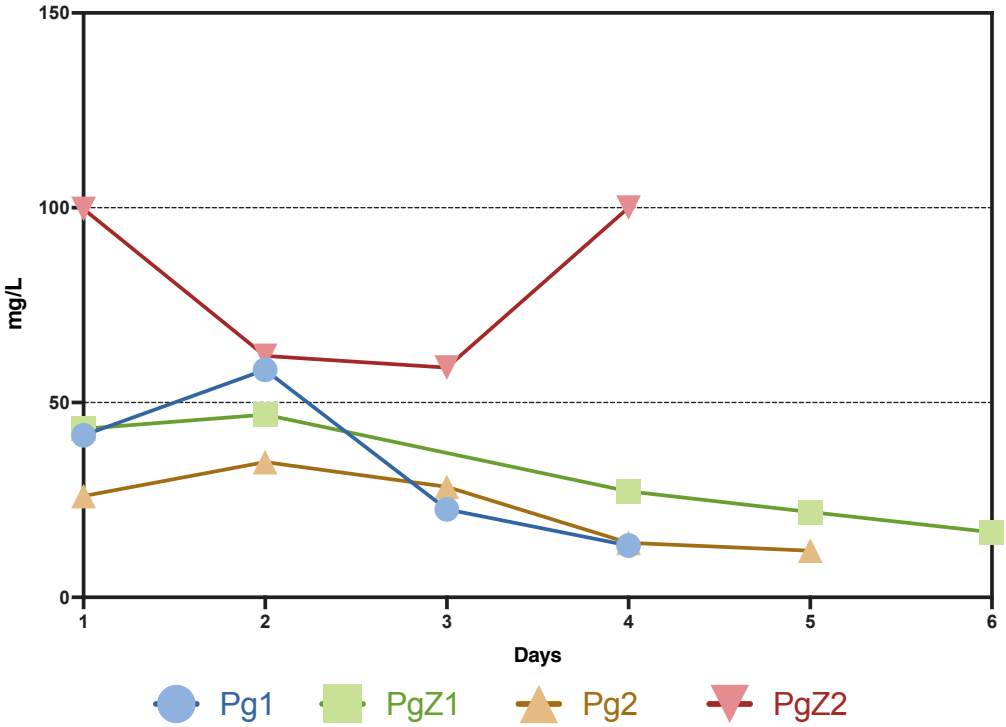
Supplementary Figure S2: Daily temperature measurements (in °C). The temperature was measured daily just beneath the surface of the culture.



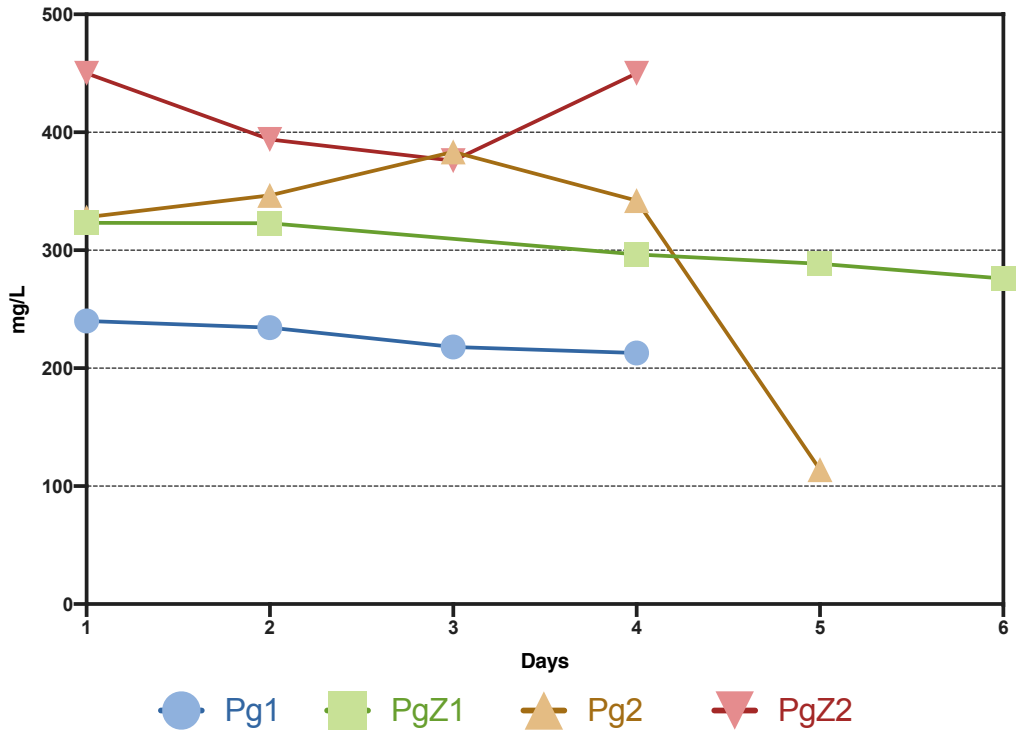
Supplementary Figure S3: Daily pH measurements. The pH was measured daily in all cultures using a pH meter just below the surface of the culture.



Supplementary Figure S4: Daily nitrate measurements of all cultures (in mg/L). Nitrate concentration was measured using Spectroquant® Nitrate test in seawater (Merck KGaA, 64271 Damstadt, Germany)



Supplementary Figure S5: Daily silicate measurements of all cultures (in mg/L). Silicate concentration was measured using Spectroquant® Silicate (Silicic Acid) Test (Merck KGaA, 64271 Damstadt, Germany)



Supplementary Figure S6: Daily phosphate measurements of all cultures (in mg/L). Phosphate concentration was measured using Spectroquant® Phosphate Test (o-phosphate) (Merck KGaA, 64271 Damstadt, Germany)

Supplementary Table S2. Chi-square table showing p-values of the total number of active Flash - fractions versus inactive flash fractions between the different treatments.

Sample Content	**Pg1**	**Pg2**	**PgZ1**	**PgZ2**
Pg1	-	0.426	0.426	0.273
Pg2	0.426	-	1.000	0.426
PgZ1	0.426	0.759	-	0.759
PgZ2	0.273	0.426	0.759	-

Paper III

Compounds isolated from the marine diatom *Porosira glacialis* inhibits formation of biofilm by *Staphylococcus epidermidis*

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Abstract

Bacterial biofilms are produced in order to protect the cells against antibacterial treatment, to facilitate interspecies communication and horizontal gene transfer, in addition to make nutrients readily available for the bacteria embedded in the biofilm. Biofilms are formed on nearly any possible surface making it a widespread issue, causing problems such as both infections and biofouling on installations in the aquatic environment. It is therefore desired to find effective treatments for removal of biofilm. Marine diatoms live in close relationship with bacteria. Though some diatom species are known to form biofilm themselves, previous studies of marine diatoms have reported inhibition of biofilm formation by bacteria. In the present study the anti-biofilm activity towards *Staphylococcus epidermidis* by the marine temperate diatom *Porosira glacialis* was investigated. Bioassay guided isolation resulted in isolation of two compounds that were able to inhibit biofilm formation: methyl 3-hydroxyoctadecanoate and a pheophorbide like compound. This is the first report on anti-biofilm compounds isolated from *P. glacialis*.

Introduction

Bacterial biofilms are sessile prokaryotic communities encapsulated in a three-dimensional exopolymer matrix in a moist environment. The matrix can consist of a variety of different

biopolymers (i.e. polysaccharides, proteins, lipids, teichoic acid and nucleic acids) known as extracellular polymeric substances, and is synthesized by the bacteria themselves [1, 2]. Formation of biofilm has a wide range of advantageous functions for the bacteria within. The biofilm facilities adhesion, it creates cohesion which in turn creates opportunity for transfer of genetic information as well as intracellular communication. In addition, it serves as a trap for nutrients, provides protection against desiccation and protects the bacteria against exposure to antibacterial compounds, grazing and host immune systems [1, 3-7]. The formation of the biofilm is a multi-step process starting with bacterial attachment to a surface. Once the bacteria are attached they start synthesizing and excreting EPS, divide and eventually form a mature biofilm which can be seen as a slimy layer covering moist or submerged surfaces [7, 8]. The mature biofilm can detach and spread to form biofilm elsewhere.

Biofilm formation can occur on nearly any submerged surface where bacteria are present, and it is suggested that more than 90% of bacteria naturally exist within biofilms [9-11]. Biofilm forming bacteria can cause issues in a range of areas from nosocomial infections to biofouling on ship hulls and other installations submerged in the aquatic environment [12, 13]. Nosocomial infections are often caused by bacteria forming biofilm on medical implants such as artificial hip joint, breast implant and catheters [13-15]. Biofouling in the marine environment start with the formation of marine biofilm bacteria and microalgae, before settlement of larger species such as macroalgae and mussels. Such growth can cause problems for e.g. ships by increasing frictional drag thus leading to increased fuel consumption, as well as increasing cost related to painting, cleaning and general maintenance [16, 17] The variety of issues caused by biofilm forming bacteria illustrates a need for new and effective treatments.

Bacterial strains that are usually sensitive to antibiotics can increase their susceptibility when embedded in a biofilm. Compared to free-living bacteria, being protected by a biofilm, bacteria can be up to 1000 times more resistant towards traditional antibiotic treatment [18]. One example is a β-lactamase negative strain of *Keibsiella pneumoniea* which, in planktonic state, has a MIC of 2mg mL^{-1}. When protected by the biofilm matrix, >50% survived a 4h treatment with 500mg mL^{-1} [19]. Research has shown that it is not the normal mechanisms of antibiotic resistance (e.g. efflux-pumps, mutations or modified enzymes) [20] that make the biofilm resistant. It is rather that the biofilm causes a slow penetration of the antibiotics, that an altered chemical environment inside the biofilm affects the effect if the antibiotics [21]. In addition,

the mutation frequency and horizontal gene transfer is significantly increased in biofilm forming bacteria, which could be one of the factors contributing to multi-resistant strains [18, 22, 23].

Diatoms and their predecessors co-evolved with bacteria. There is also evidence that many diatom genes were acquired from bacteria [24-26]. However, only a few groups are known to be regularly associated with diatoms such as *Alpha, Beta* and *gammaproteobacteria* and *bacteriodetes,* and these are represented by only a few taxa [24, 27, 28]. Some bacteria are shown to be commensialists that provide diatoms with much needed vitamins and trace elements in exchange for dissolved carbon and nitrogen compounds [26]. Diatom – bacteria interactions also include competitive interactions where bacteria sometimes are better at scavenging phosphate in environments where this is a limiting factor, thus inhibiting growth of diatoms [29]. This is further complicated by environmental factors that lead to beneficial interactions turning sour when e.g. nitrate is scarce while dissolved organic carbon is high [30].

When conditions are favorable, biofilms are present in virtually all environments [31]. Due to the opportunistic and persistent nature of biofilms, treatment is notoriously troublesome, causing harmful and chronic infections. Thus, finding new anti-biofilm agents is of great interest. Microbial biofilms in the marine realm usually consist of bacteria and benthic diatoms, e.g. species of *Navicula, Cylindrotheca* and *Nitzschia*, and a number of studies have therefore been conducted to find ways to combat diatom biofilm formation [32, 33]. In comparison, very little has been done to map the diatoms' own ability to fight bacterial biofilm. Previous bioactivity studies of the temperate marine diatom *Porosira glacialis* have revealed that the species harbor anti-biofilm activity against *S. epidermidis* [34, 35]. *S. epidermidis* is a common member of the human epithelial microflora skin and is usually harmless to humans, but it is also an important opportunistic pathogen [15]. Together with *S. aureus* it causes 2/3 of nosocomial infections in patients. *Staphylococcus epidermidis* is often used as test organism in anti-biofilm assays, as it easily forms biofilm and is a good indicator for possible anti-biofilm activity. The need for new treatments of bacterial biofilm combined with results from previous studies have motivated a further investigation of the anti-biofilm activity of *P. glacialis*. The purpose of the study was to investigate the anti-biofilm activity in organic extracts from the marine diatom *Porosira glacialis* using bioassay guided isolation with the aim of isolating and identifying the active compounds.

Materials and Methods

Mass cultivated diatoms

Cultivation was performed similar to [36], with some modifications. Mono algal - non-axenic mass culture of the cold-water diatom species *Porosira glacialis* was mass cultivated outdoors in a roofed and open 300 000 L fibreglass cylindrical open airlift photobioreactor. The cultivation took place during December 2019 at 5.1 – 5.9°C using solely artificial light provided by LED lamps. The diatoms were cultivated under a mean scalar irradiance of 36-50 µmol photons m^{-2} s^{-1} as measured at 0.7 m depth with a Li – 1500 light logger using a Li – 193SA spherical underwater quantum sensor (Li-Cor Biosciences, USA). The culture was maintained in exponential growth phase with cell numbers at 8-9 million cells L^{-1}. Nitrate was kept between 90-100 µM (Spectroquant® "Silicate (Silicic Acid) Test", Merk, Damstadt, Germany), while silicate varied from 18-59 µM (Spectroquant® "Nitrate test in seawater", Merk, Damstadt, Germany). Harvested biomass was centrifuged using a continuous centrifuge (Evodos 10, Evodos B.V., The Netherlands) and stored at -80°C.

Material preparation and separation

Extraction

Harvested biomass of *P. glacialis* was freeze-dried (Heto PowerDry PL9000, Thermo Fisher Scientific, Waltham, MA, USA) and ground into a fine powder. The dried biomass was extracted using MilliQ-H_2O overnight at 5°C, then centrifuged at 4600 rpm at 4°C for 30min. The pellet was resuspended in MilliQ-H_2O for 1h and centrifuged. The extracted pellet was freeze-dried and extracted for 4h with a 1:1 mixture of dichloromethane (Merck, Darmstadt, Germany) and methanol (MeOH, Merck) before being filtrated through a Whatman Ø 152 mm no. 3 filter. The organic extraction was repeated twice. The supernatant was dried in a rotavapor under reduced pressure and stored at -23°C until further use.

Flash fractionation

The extract was fractionated using a Biotage HPLC SP4 flash fractionation system (Biotage, Uppsala, Sweden). A subsample (1.5 g) of the organic extract was dissolved in 40 mL g^{-1} of hexane (Merck) and transferred to a separation funnel. A liquid:liquid partitioning was performed by adding 2x30 mL 90% MeOH (Merck). The lower phase (MeOH) was retrieved and mixed with 2 g of Dianon HP-20SS resin (Supelco, Bellefonte, PA, USA) and subsequently dried under reduced pressure. The columns were prepared by mixing 6.5 g of resin with MeOH

for 20 min, then washing with MilliQ-H_2O and transferring the material to a flash column (Biotage SNAP, Uppsala, Sweden). The column was equilibrated with 5% MeOH and the extract was loaded onto the column. Fractionation was done using two consecutive methods. First: a 5-100% MeOH gradient over 32 min with, second a MeOH:Acetone (Merck) gradient ending at 100% acetone over 18 min. Eight fractions were collected and dried under reduced pressure. The fractions were dissolved in 100 % DMSO (Merck) and stored at -20 °C until use. Plates with the desired concentration of fractions were prepared prior to bioactivity testing and diluted in dH_2O.

HPLC fractionation

Flash fractions with detected activity were re-fractionated into new 40 fractions using a preparative high-pressure liquid chromatography (prep-HPLC) system. The prep-HPLC consisted of a 600 HPLC pump, a 2996 photodiode array UV-detector, a 3100 - mass spectrometer, and a 2767 sample manager (Waters, Milford, MA, USA). The solvents used were A: MilliQ-H_2O + 0.1 % formic acid (FA) and B: Acetonitrile (ACN) + 0.1 % FA. Gradient 30-70% ACN over 40 min (1 fraction min^{-1}), flow 6 mL min^{-1}. Samples were injected into a Xterra Prep MS C_8 (10 μm, 10x250mm) column (Waters). Fractions were transferred to 96 well, deep well plates, freeze dried and stored at -20°C until further use. HPLC fractions were dissolved in DMSO (Merck) and diluted with dH_2O before bioactivity testing.

Bioactivity testing

Biofilm assay – inhibition of biofilm formation

The biofilm inhibition assay used *S. epidermidis* and was performed following Lauritano et al. (2016). In brief, triplicates of FLASH fractions from extracted diatom biomass were used to test biofilm formation the bacteria *Staphylococcus epidermidis* (ATCC – 35984). We added 50 µL fraction and 50 µL bacteria suspension to each well in microtiter plates (96-wells), using MilliQ water and enriched media (1:1) as blank, *Staphylococcus haemolyticus* as negative control, while *S. epidermidis* with MilliQ water was the positive control. Visual inspection of biofilm formation in the plates was backed by measuring absorbance at 450 nm using a VICTOR3 Multilabel Counter (Perkin Elmer). Biofilm coverage below 25 % were considered active [37].

Biofilm assay – eradication of established biofilm

The biofilm eradication assay was performed using *S. epidermidis* incubated overnight at 37°C and then diluted 1:100 in tryptic soy broth (TBS) with 1% glucose added. 50 µL Bacterial culture and 50 µl dH2O was then transferred to each well in a 96 well plate (Nunc). Blank consisted of TBS with 1% glucose mixed 1:1 with dH2O, *S. haemolyticus* was used as negative control, while positive control was *S. epidermidis.* The resulting plates were incubated for 24h at 37°C, then emptied and washed twice with phosphate-buffered saline (PBS). New TBS with 1% glucose was then added in addition to fractions (50 µL) in triplicates and incubated again for 24h at 37°C. Plates were then fixed, colored and measured following the same methodology as in the inhibition of biofilm formation assay [38].

Growth inhibition assay

Antibacterial testing was done following Ingebrigtsen et al (2016) [39]. In brief, we used the following strains (American Type Culture Collection Strain (ATCC) numbers in parentheses): *Enterococcus faecalis* (29212), *Staphylococcus aureus* (25923), *Streptococcus agalactiae* (*Streptococcus* Gr. B*,* ATCC 12386), *Escherichia coli* (25922) and *Pseudomonas aeruginosa* (27853). *E. faecalis* and *S. agalactiae* were enriched with Brain Heart Infusion (BHI, Sigma-Aldrich) and the other strains with Mueller – Hinton broth (MH, Becton Dickinson, Franklin, NJ, USA). Log phase cultures were diluted 1:1000 with their respective enrichment medium before being transferred (50 µL) to clear 96-well plates. Flash fractions were tested at a concentration of 50 µg/mL. Absorption at 450 nm values were measured using a 1420 Multilabel Counter VICTOR^3, values below 0.05 were regarded as "active". To allow for post – compensation, the plates were visually checked and measured prior to addition of bacteria as well. Blanks were 50 µL sterile water with 50 µL enrichment media, growth controls were 50 µL bacterial solution and 50 µL sterile water while Gentamicin (Aventis Pharma) was used as positive control.

Toxicity assay HepG2

Active HPLC fractions were screened for cytotoxicity using human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) and normal lung fibroblasts MRC5 (ATCC CCL-171). The HepG2 cells were suspended in MEM Earle's cell medium (Biochrom GmbH, Berlin, Germany) and MRC5 in Roswell Park Memorial Institute 1640 (RPMI, Sigma-Aldrich), both with 10% foetal bovine serum. 4000 (MRC5) and 2000 (HepG2) cells were seeded into the

wells of a 96-Well Nunc plate (Nunc, Roskilde, Denmark) and incubated at 37 °C in 5% CO_2 for 24h. The culture medium was then replaced with new VEKST with 10% FBS and gentamycin (10 µg mL^{-1}), the fractions or isolated compounds were added (25 and 50 µg mL^{-1}) and the plate was incubated for 72h at 37°C in 5% CO_2. All samples were tested in triplicates, negative control was growth medium and positive control was growth medium with 10% DMSO. After incubation, 10 µL CellTiter 96® AQueous One Solution Reagent (G3581, Promega, Madison, WI, USA) was added to each well and incubated for 1h. The plates were read using a DTX 880 plate reader (Beckman Coulter, CA, USA) at 495 nm.

Purification

The initial purification of compounds was performed using the same prep HPLC-MS system used for fractionation, using the same column (Xtrerra). The fraction collector was triggered by *m/z* of the selected compound, or by time (retention time) if trigger by *m/z* was not possible. Several gradients of A: MilliQ-H_2O + 0.1 % FA and B: Acetonitrile (ACN) + 0.1 % FA were used in the purification process to favour best possible separation and isolation of selected compounds. The second purification step was performed using a semi-preparative HPLC system coupled to a PDA and QDa-MS. The HPLC system was equipped with a Sample-manager FTN-R, Quaternary-solvent-manager (HPLC-pump), isocratic solvent manager, column-oven, QDa detector, a 2998 PDA detector and a fraction manager, all ACQUITY-Arc™ components, (Waters). For separation a Atlantis™ T3 3 µm, 3.0×150 mm C_{18} column (Waters) was used. Solvents used were, A: ddH2O + 0.1% FA and B: ACN + 0.1% FA. Gradients were adapted to achieve best possible separation and isolation of selected compounds.

HR-MS analysis

Active fractions and isolated compounds were analyzed using UHPLC-HR-MS for dereplication. The system used was a Acquity I-class UPLC (Waters, Milford, MA, USA) coupled to a PDA detector and a Vion IMS QToF (Waters). The chromatographic separation was performed using an Acquity C-18 UPLC column (1.7 m, 2.1 mm_100 mm) (Waters). Mobile phases consisted of A: ddH2O (Millipore, Burlington, MA, USA) + 1% FA and B: CAN (HiPerSolv, VWR, Radnor, PA, USA) + 1% FA. The gradient was run from 10% to 90% B in 12 min at a flow rate of 0.45 mL/min. Samples were run in ESI+ and ESI- ionization mode. The data was processed and analyzed using UNIFI 1.9.4 (Waters).

NMR

NMR spectra were acquired in d_6-DMSO on a Bruker Avance III HD spectrometer operating at 600 MHz for protons, equipped with an inverse TCI cryo probe enhanced for ^{1}H, ^{13}C and ^{2}H. $^{1}H/^{13}C$ chemical shifts were referenced to the residual solvent peak (d_1-DMSO: δ_H = 2.50 ppm, ^{13}C δ = 39.52 ppm).

Results

Large-scale cultivation

The cultivation of P. glacialis used in the present study was cultivated in large-scale in a 300,000 L fibreglass cylindrical open airlift photobioreactor. A visual control of the cells was performed daily using an inverted microscope to ensure that the culture remained healthy and that there was no contamination of other species of microalgae. The monoculture of *P. glacialis* remained healthy throughout the cultivation period. The harvest was performed using a semi-continuous centrifuge, removing app. 99 % of the water content resulting in 500 g wet weight of diatom biomass. Freeze drying and extraction yielded 41 g aqueous extract and 8,9 g organic extracts for flash fractionation and further isolation. Aqueous extract was not subject to in depth analysis in the present study, due to lack of bioactivity.

Bioactivity guided isolation

The organic extract of *P. glacialis* was fractionated into 8 fractions, which were all tested in an anti-biofilm assay for inhibition of biofilm formation by *S. epidermidis* at 50, 25, 20 and 5 µg mL^{-1}. All fractions were tested in 3 technical replicates. Out of the eight organic flash fractions tested, two fractions showed activity in a dose-response manner (Figure 1). Both fractions (F3 and F4) showed activity at 25 µg mL^{-1} as the fractions were regarded as active < 25% biofilm coverage.

Based on the activity, F3 and F4 were further fractionated using prep-HPLC into 40 fractions each. The HPLC fractions were tested in two anti-biofilm assays, both for inhibition of biofilm formation and eradication of established biofilm. Out of 80 tested fractions, 6 fractions showed

ability to inhibit biofilm formation (Figure 2), while 3 fractions removed established biofilm formed by *S. epidermidis* (Figure 3).

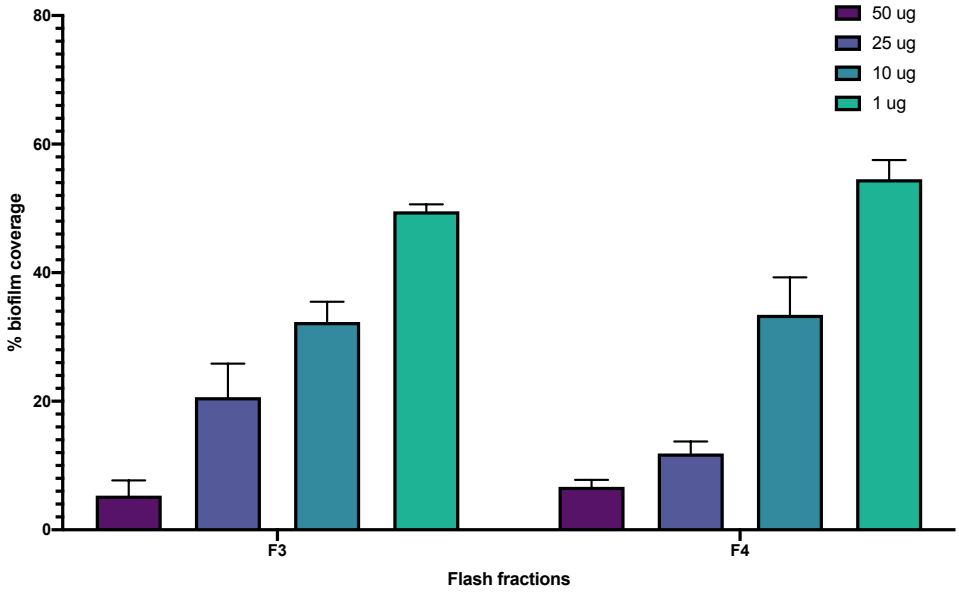

Figure 1: Dose-response assay on inhibition of biofilm formation by organic Flash fractions F3 and F4 from *P. glacialis*. Fractions were tested at concentrations: 50, 25, 10 and 1 µg mL^{-1}. Inhibition was measured in % biofilm coverage. Average of 3 replicates.

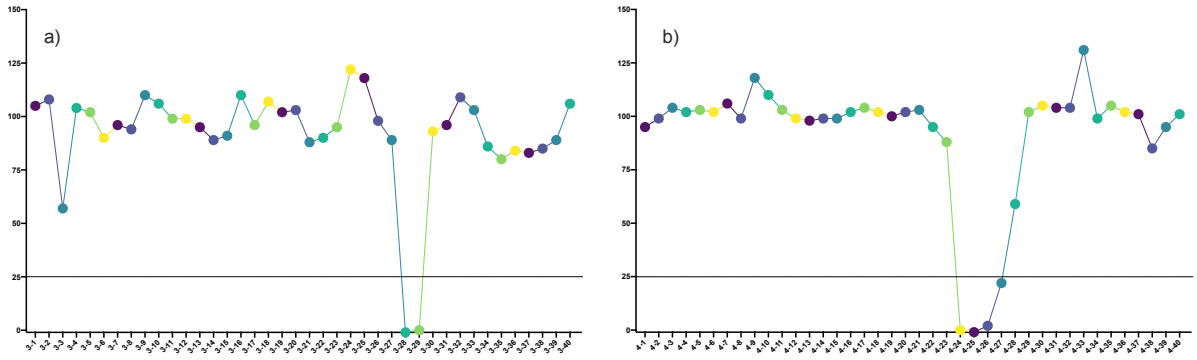

Figure 2: Inhibition of biofilm formation by re-fractionated active fraction F3 (a) and F4 (b) from organic extracts of *P. glacialis*. HPLC-Fractions were regarded as active below 25% biofilm coverage.

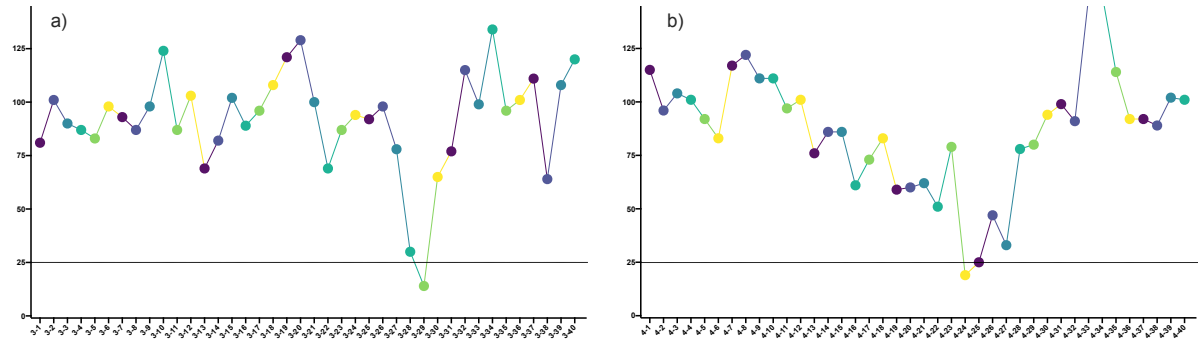

Figure 3: Inhibition of biofilm formation by re-fractionated active fraction F3 (a) and F4 (b) from organic extracts of *P. glacialis*. HPLC-Fractions were regarded as active below 25% biofilm coverage.

The HPLC fractions were also tested in an anti-bacterial growth inhibition against 5 bacterial strains: *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus agalactiae* (*Streptococcus* Gr. B), *Escherichia coli* and *Pseudomonas aeruginosa*. The results are presented in Figure 4, showing that 4 HPLC fractions inhibited growth of *S. aureus*, while 6 fractions inhibited the growth of *E. faecalis*. The active fractions and the surrounding, non-active fractions were tested for cytotoxicity against human hepatocellular carcinoma cell line HepG2. The results showed no toxicity against the human cell line (results in supplementary).

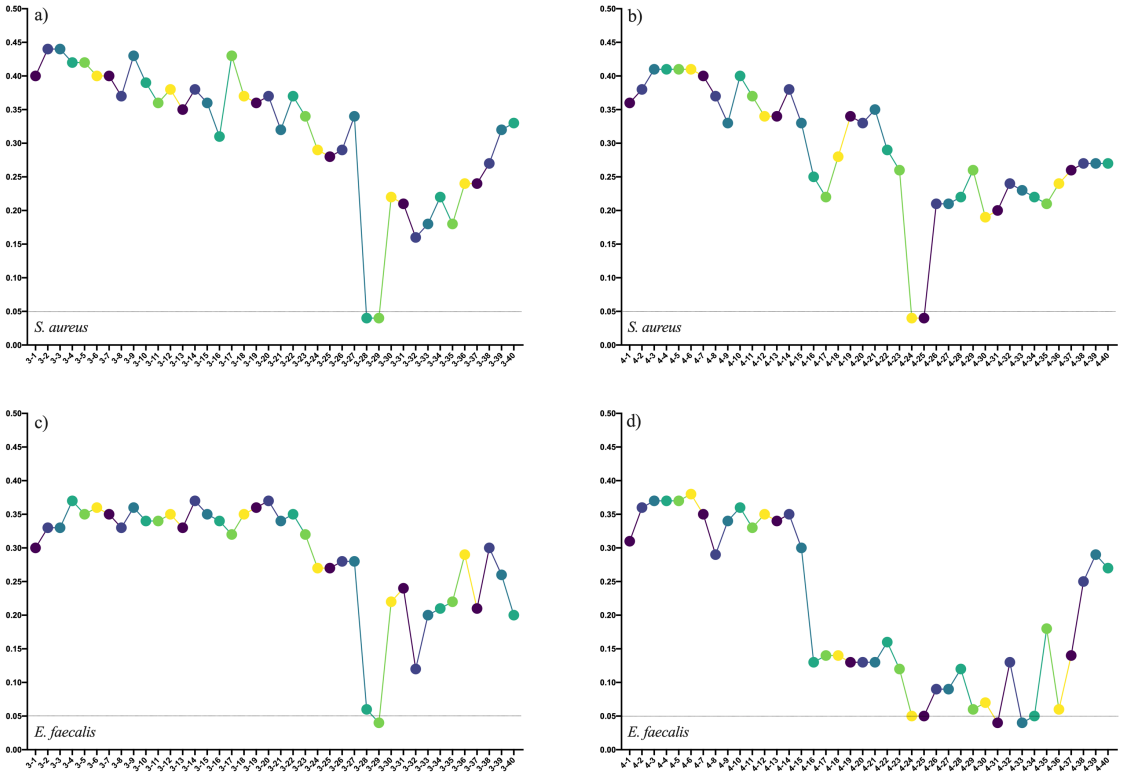

Figure 4: Activity profiles of HPLC fractions (re-fractionation of active fraction F3 and F4) of *P. glacialis* tested in a growth inhibition assay against *S. aureus* (a and b) and *E. faecalis* (c and d). Fractions were regarded as active at abs. <0.05 at 450nm.

Isolation and structure elucidation

The active and inactive HPLC fractions were analysed using HR-MS to find compounds that were unique for the active fractions, and these were purified using preparative HPLC. This resulted in six target compounds which were attempted isolated. The collected six fractions were dried under reduced pressure to about 10 % of collected volume and redissolved in MeOH. To remove impurities from the target compounds, they were purified a second time using a different prep-HPLC system. The second purification resulted in three compounds (mbc-346, mbc-347 and mbc-348) that were evaluated to be pure enough for structure elucidation using NMR. The remaining three compounds (mbc-343, 344 and 345) were included in the bioactivity testing. All compounds were freeze-dried. Structure elucidation by NRM resulted in the structures of mbc-346 and mbc-347 that could be determined based on the available NMR and MS data. Table 1 shows the freeze-dried yield of all six compounds isolated and tested.

Table 1: Compound yield of all 6 compounds isolated from *P. glacialis*

Compound ID	Weight (mg)	NMR structure
Mbc-343	0,2	No
Mbc-344	0,9	No
Mbc-345	1,5	No
Mbc-346	3,7	Yes
Mbc-347	0,1	Yes
Mbc-348	1,5	No

Compound mbc-346 was isolated as a light-yellow wax. Through 1D (^{1}H and ^{13}C, table 2, Figure S1 and S2) and 2D (HSQC, HMBC and COSY, Figure 5, S3 and S4) NMR experiments, mbc-346 was determined to be the previously known compound methyl 3-hydroxyoctadecanoate (Figure 5). Through HMBC and COSY, two subfractions of the molecule were unambiguously assigned (positions 1 – 7 and 17 – 19 in Figure 5). Signals for both hydrogen and carbon atoms 8 to 16 (all CH_2 and part of the aliphatic chain) overlapped and could not be distinguished from each other.

NMR data obtained for mbc-347 clearly indicates that this compound is or is closely related to pheophorbide A. However, only subfractions of the chlorophyll breakdown product could be

determined. Due to low sample amounts, only five clear peaks were visible in the carbon spectrum. Pheophorbide A and related compounds have a ratio of carbon to proton atoms of around 1:1, which complicated the structural elucidation proses. Furthermore, the sample was found to change over the course of the NMR experiments. The substructures that could be determined for mbc-347 using NMR data are highlighted in Figure 6.

Table 2: ^{1}H and ^{13}C NMR Assignments for mbc-346

	δ_C, type	δ_H, type (*J* in Hz)
1	51.1, CH_3	3.57, s
2	171.8, C	
3a	42.4, CH_2	2.39, dd (14.7, 4.7)
3b		2.28, dd (14.7, 8.3)
4	67.1, CH	3.80, m
5	36.9, CH_2	1.39 - 1.30, m*
6a	25.0, CH_2	1.39 - 1.30, m*
6b		1.24, m*
7	28.7, CH_2	1.28 - 1.18, m*
8	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
9	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
10	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
11	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
12	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
13	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
14	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
15	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
16	29.1 - 29.0, CH_2*	1.28 - 1.18, m*
17	31.3, CH_2	1.23, m*
18	22.1, CH_2	1.25, m*
19	14.0, CH_3	0.85, t (6.9)

*signals are overlapping

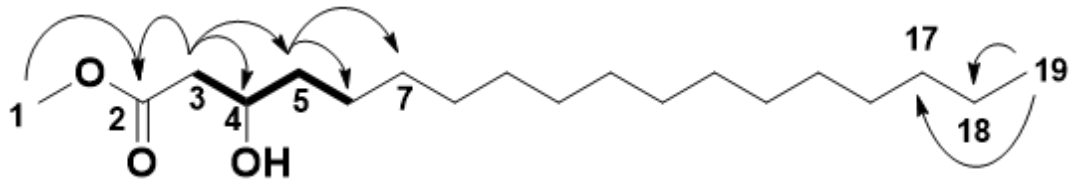

Figure 5: Selected HMBC (arrow) and COSY (bold) NMR correlations obtained for mbc-346

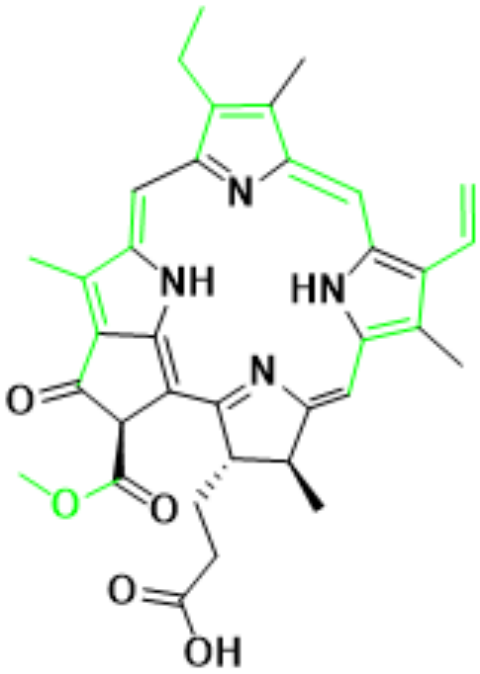

Figure 6: The structure of pheophorbide A. The subfractions that could be determined following NMR analysis of MBC-347 is highlighted in green.

Anti-biofilm activity of isolated compounds

All six isolated compounds were tested for anti-biofilm activity at two different concentrations 50 and 25 µg mL^{-1} (Figure 7). At 50 µg mL^{-1} activity was found for compound mbc-346, identified to be methyl 3-hydroxyoctadecanoate. Results showed that after 24h, there was 57 % biofilm coverage, compared to 120 % by non-active compounds. Compound mbc-347, pheophorbide a, showed some inhibition with 68 % coverage at 50 µg mL^{-1}. The compounds that could not be analysed by NMR were also tested, and compound mbc-348 (isolated by mass *m/z* 641) showed activity at both 25 and 50 µg mL^{-1} with 40 and 44 % coverage respectively. All compounds were also tested for eradication of established biofilm at 50 µg mL^{-1}, though no activity was found in this assay. A bacterial growth inhibition assay was performed, revealing no activity at 25 µg mL^{-1}. A viability assay towards normal lung fibroblasts (MRC5), revealed no cytotoxicity at 25 µg mL^{-1} for any of the tested compounds.

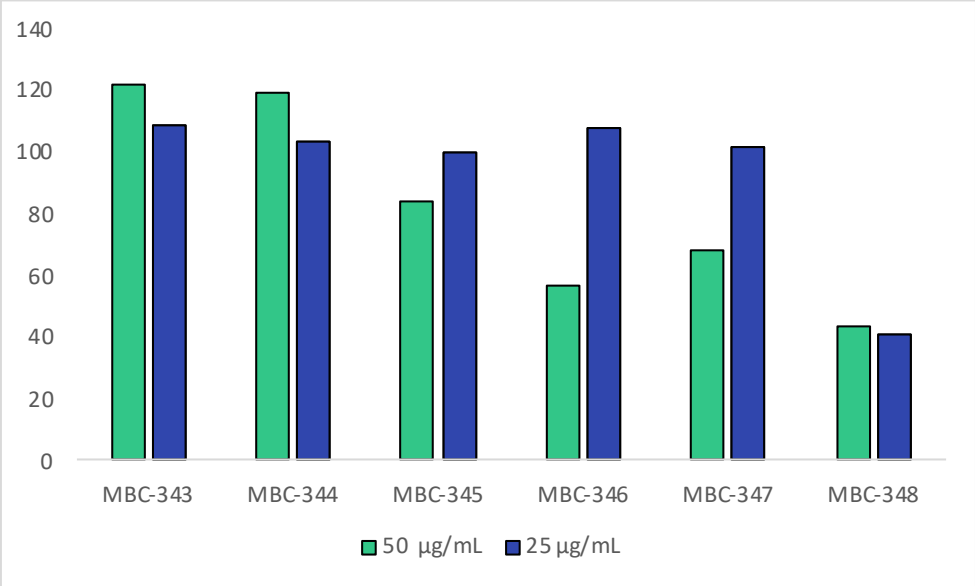

Figure 7: Bioactivity testing of isolated compounds for Inhibition of biofilm formation. Biofilm measured in % biofilm coverage. Compounds tested at 50 and 25 µg mL^{-1}.

Discussion

Bioassay guided isolation was used to identify compounds in the marine diatom *Porosira glacialis* with anti-biofilm activity towards *Staphylococcus epidermidis*. Continuous cultivation of *P. glacialis* in large scale over a period of > 4 months has showed very little visible formation of biofilm inside the photobioreactor, as well as on submerged equipment. This, in addition to previously found anti-biofilm activity in extracts of *P. glacialis* motivated an isolation of compounds with possible anti-biofilm activity [34, 35]. *S. epidermidis* was used as the test bacteria due its qualities as a biofilm forming bacteria, and as a good indication of anti-biofilm activity. Anti-biofilm activity was found in two organic Flash-fractions (F3 and F4) from the organic extract of the cultivated diatoms. In order to isolate individual compounds responsible for the observed bioactivity, the Flash fractions which were further fractionated using preparative HPLC into 40 fractions each. The bioactivity was recovered in six of these HPLC fraction, allowing us to compare active and inactive fractions to identify potential bioactive compounds, finally resulting in six compounds isolated.

Compound mbc-346 inhibited formation of biofilm by *S. epidermidis* at 50 µg mL^{-1} (57 % coverage, see Figure 7). The compound was confirmed to be methyl 3-hydroxyoctadecanoate by NMR analysis. Methyl 3-hydroxyoctadecanoate is a known hydroxy fatty acid methyl ester previously isolated from the essential oil fraction of the plant species *Minuartia recurva* and

Acokanthera schimperi [40, 41] and from the bacteria *Thiobacillus thioxidans* [42]. There are no previous reports on the compound being isolated from diatoms, or microalgae in general. Another hydroxy fatty acid methyl ester, Urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3β) isolated from the plant *Barleira courtallica* have shown antimicrobial, anti-inflammatory and cytotoxic activity [43]. Apart from this there are little reports connecting hydroxy fatty acid methyl esters to bioactivity, and none to anti-biofilm activity, but there are previous studies reporting antibacterial activity in free fatty acids isolated from diatoms, e.g. eicosapentaenoic acid (EPA) [44]. This could suggest that the anti-biofilm activity could possibly be linked to fatty acids in *P. glacialis*.

Compound mbc-347 identified was as a pheophorbide-like compound and showed some degree of inhibition of biofilm formation at 50 μg mL^{-1} compared to the non-active compounds (68 % biofilm coverage). Pheophorbide a has previously shown anti-inflammatory [45] activity and anti-viral activity (hepatitis C virus) [46]. Pheophorbide-like compounds have also showed some cytotoxic effect [47]. Compound mbc-348 showed activity at both 25 and 50 μg mL^{-1}, although the biofilm coverage was 40 % at each concentration showing that the activity was not dose-dependent. Compound mbc-348 was not of high enough purity for NMR, thus a structural elucidation is not available. Data form the UPLC-HR-MS analysis of this compound were very hard to interpret and we were unable to calculate a reliable elemental composition, but the mass used as fraction trigger for this compound (*m/z* 641) on the preparative HPLC, suggested that it could also be a chlorophyll derivate.

The bioactivity testing of HPLC fractions revealed fractions which removed established biofilm as well as having antibacterial activity towards *Staphylococcus aureus* and *Enterococcus faecalis.* These activities were not detected in the isolated compounds, suggesting that either the test concentration (50 μg mL^{-1}) was too low or that the activity was caused by synergies between several compounds. Another option is certainly that there could be more bioactive compounds yet to be isolated form the *P. glacialis* extract.

Conclusion

The absence of biofilms in large scale cultures of *P. glacialis* is a valuable asset, and in this study, we have been able to at least partially ascribe this activity to two isolated and characterized compounds; methyl 3-hydroxyoctadecanoate (mbc-346) and a pheophorbide-like structure (mbc-347). The bioactivity of compound mbc-346 is interesting as it is the first report

on the isolation of this compound from a diatom. It is also the first evidence of anti-biofilm activity of this group of compounds. Further investigation of the anti-bacterial and anti-biofilm activity of fatty acids from *P. glacialis* could be of interest. Such activity, if shown to be the result of degradation of primary metabolites present in most, if not all, microalgae species used in microalgae mass cultivation, would aid in explaining the low level of biofilm in bioreactor systems using similar cultivation conditions. In turn, this could inspire efforts to optimize towards cultivation conditions where the microalgae produce higher levels of the compounds leading to less biofilm formation, thus saving money on costly cleaning. Furthermore, the isolated compound, methyl 3-hydroxyoctadecanoate, could be of commercial interest in itself as a possible detergent or treatment of biofilm, biologically produced from a renewable and sustainable source.

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SUPPORTING INFORMATION

Compounds isolated from the marine diatom Porosira glacialis inhibits formation of biofilm by *Staphylococcus epidermidis*

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Figure S1. ^{1}H NMR spectrum of mbc-346

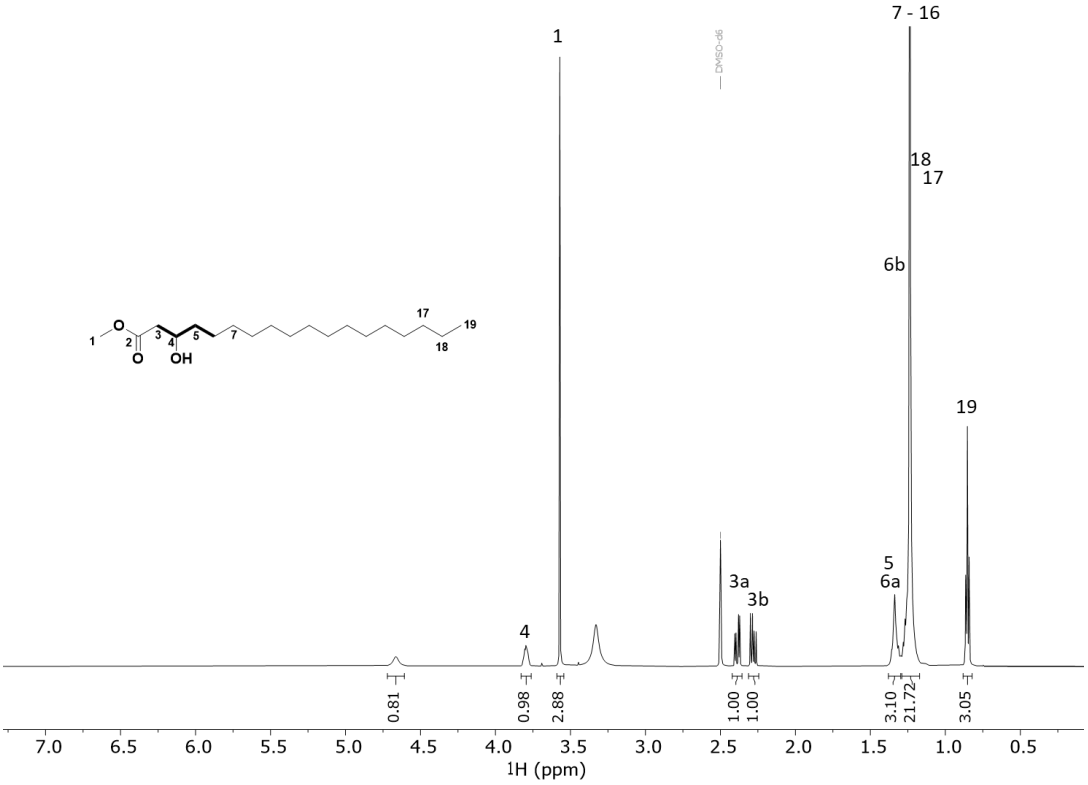

Figure S2. ^{13}C NMR spectrum of mbc-346

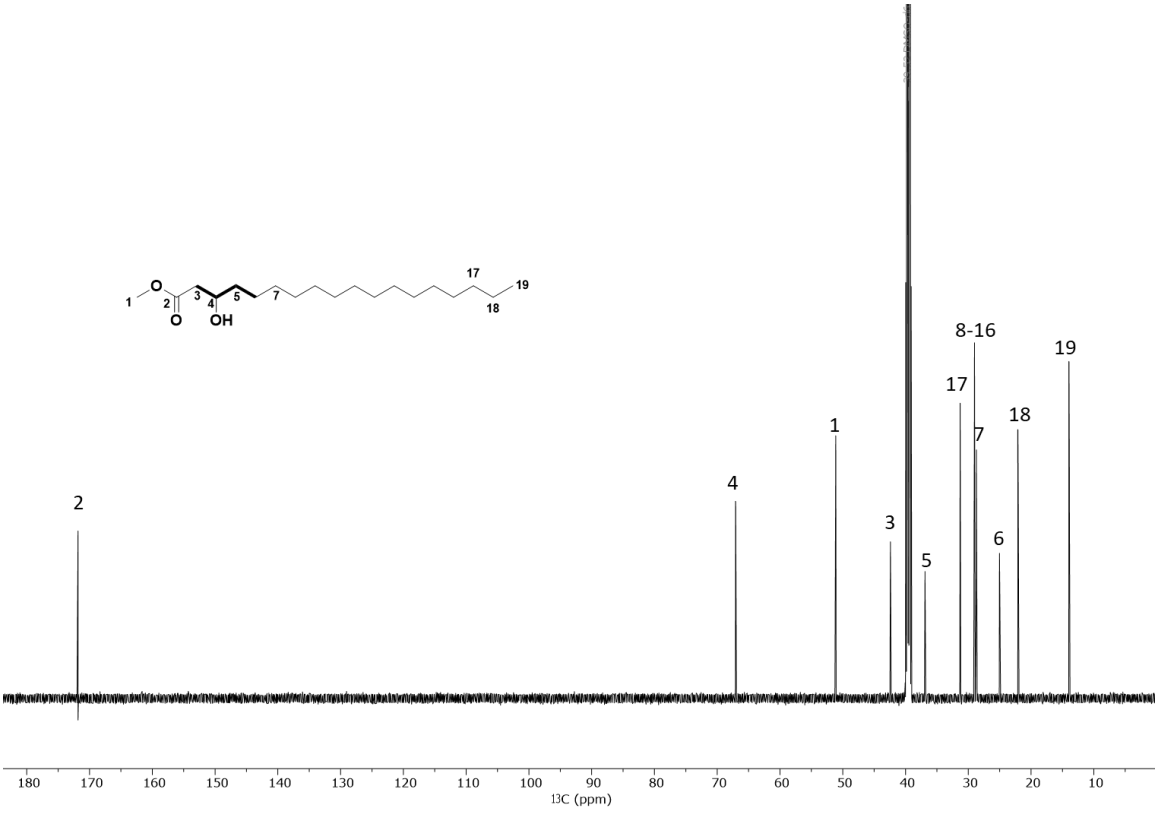

Figure S3. HSQC + HMBC spectrum of mbc-346

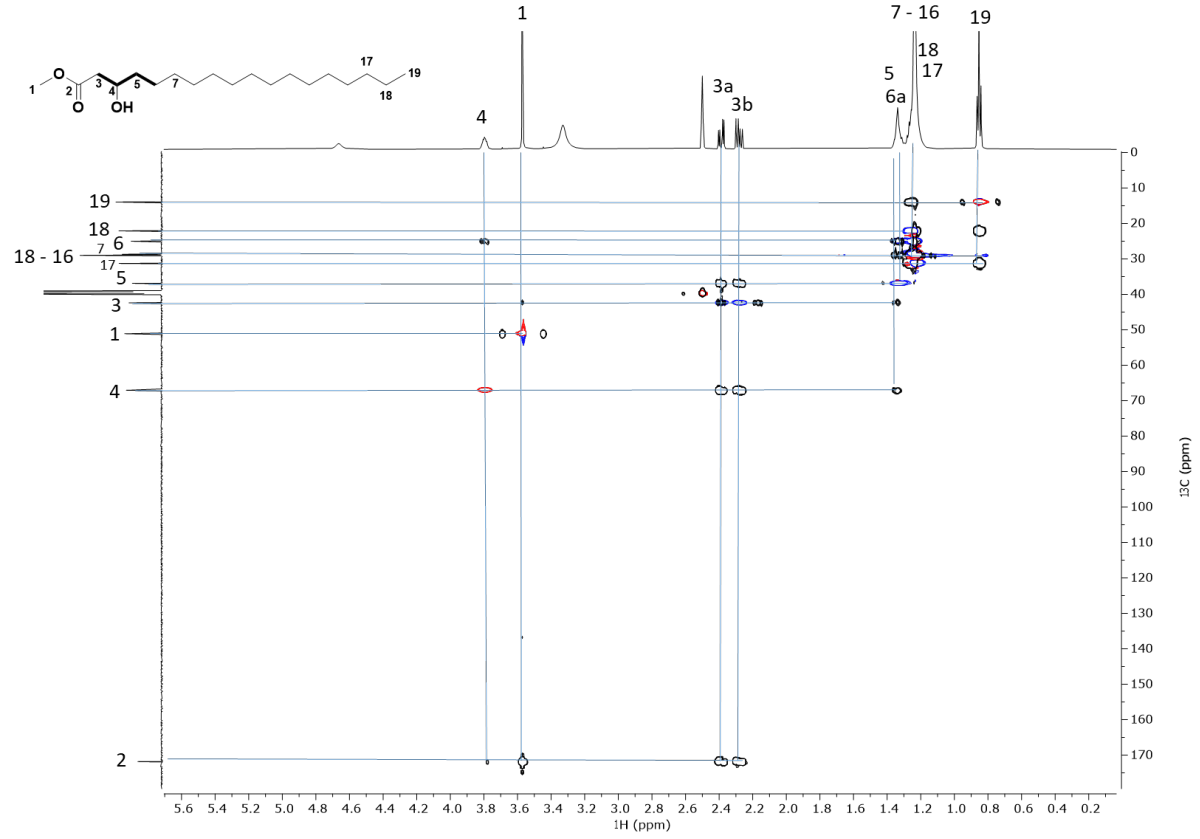

Figure S4. COSY spectrum of mbc-346