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 6000 L photobioreactors in an industrial setting; one culture with direct addition of factory smoke, and one with fresh air 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*). The bioactivity was tested in two bacterial assays; growth inhibition assay, and anti-biofilm assay. Our 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 does 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.

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; 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 culprit is to boost production by injecting factory smoke with elevated CO_2 concentrations to the cultures[18]. Fossil carbon dioxide 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, i.e. 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 the 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 as 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.

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 the last 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 cultavtion 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 against 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 during the spring of 2014. Isolation was done under a microscope (Primo Vert, Zeiss) 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 (Supplementary Table 1) in photosynthesis to produce nutritious marine biomass to be applied as e.g. food end aquaculture feed, i.e. CCU. Cultivation was performed in parallel in two open 6000 L vertical column photobioreactors (**Feil! Fant ikke referansekilden.**). Both reactors were sylindrical with a diameter of 1.7 m and hight of 2.5 m, one built of steel and one of fiberglass (difference in material not affecting 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) and germicidal UV-lamps (Nordisk Vannteknik). Inorganic plant nutrients, i.e. Substral^{TM} (Scotts Company, Nordics A/S) was added to a concentration of 0.25 mL L^{-1} (Substral content found in supplementary Table 2). Silicate solution prepared from $Na_2O_3Si{\times}9H_20$ (Sigma-Aldrich) 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 addidtion of facorty 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 (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

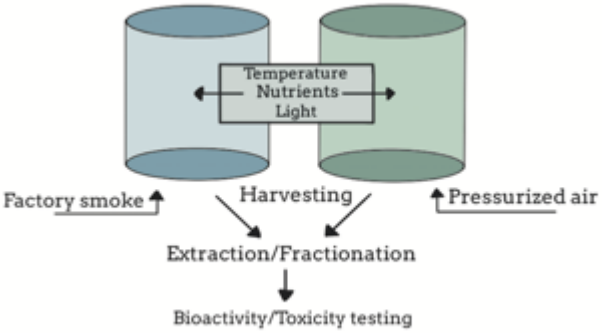

Fig. 1 Overview of cultivation experiment workflow.

using the method of Holm and Riemann [40] with ethanol (96%, Sigma Aldrich) as extractant and quantified using a Turner Designs fluorometer (TD 700). Temperature and pH were monitored throughout the cultivation period.

Cultures were harvested when 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 3000 L of both cultures were harvested using a continuous centrifuge (Algae Centrifuge, Sacramento, California, USA). After centrifugation the biomass was frozen and stored at -20 °C until further use. To continue cultivation, 3000 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 3000 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), 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) at 4 °C and subsequently centrifuged (4600 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). The organic extract was evaporated under reduced pressure (Laborata 4002, Heidolph). 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 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 and 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 and 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) 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), and 2000 cells were seeded into each well (4000 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 place 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 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), *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 company) 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 1:1000 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 600nm was measured in a VICTOR 1420 Multilabel Counter (Perkin Elmer).

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) 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 adding 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 washed 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 examined visually for the formation of biofilm that was 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 blank. Plates were incubated at 37 °C for 24 h. Enrichment media was removed by gently turning 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 between 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 toxic substances that can be deleterious to e.g. 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. 2 a). 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. 2 b) 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 our 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.

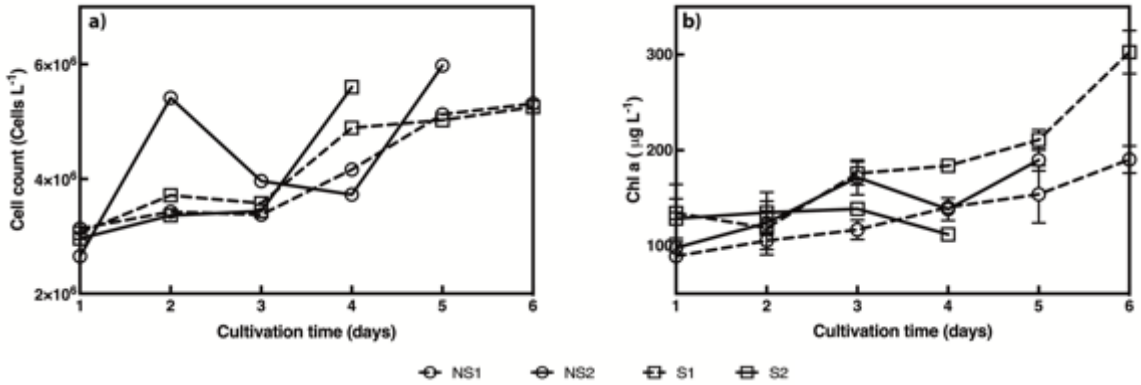

Fig. 2 Cell numbers in cells L^{-1} (a, left) and chlorophyll a (b, right) concentration (µg L^{-1}) vs. time for the four diatom cultures of *P. glacialis*; two cultures grown without factory smoke NS1 and NS2, and two cultures grown with factory smoke S1 and S2. Chl a represents values of three replicates.

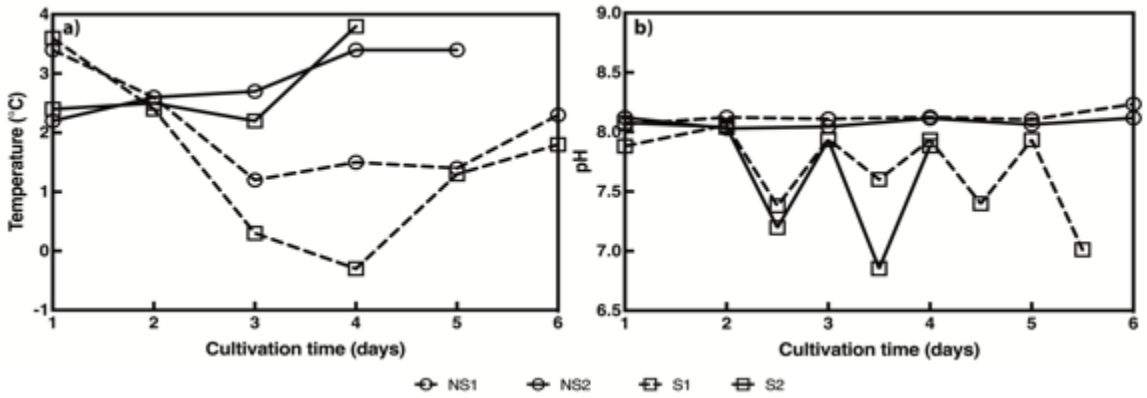

Fig. 3 Temperature (ºC) (a, left) and pH (b, right) measurements of samples NS1, NS2, S1 and S2 during cultivation of *P. glacialis*. Daily measurement was taken in cultures NS1 and NS2. pH in cultures S1 and S2 were measured twice each day, before and after addition of factory smoke.

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. 3 a). 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 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 100L 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 we measured were in the same range as the two aformentioned 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. 3 b) were made, once a day in cultures NS1 and NS2 and twice 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 CO2 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 in order 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; 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; human melanoma (a), human colon carcinoma (b) and normal lung fibroblasts (c). All the organic fractions, 64 in total, were tested at 100 μg mL^{-1}. Fig. 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 show a few differences between the treatments; Fraction 3 in NS2 and fraction 4 of S1 were not active against human colon carcinoma (Fig 4 b), and only fraction 5 of samples cultivated without smoke (NS1 and NS2) were active against human melanoma (Fig. 4 a) 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. 4 c). Our 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 have 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, and Lauritano et al.[48] showed that phosphocholines and chlorophyll derived molecules had anti-proliferate activity. Studies on *P. glacialis* are scarce, with less than a handful of publications on this species [35,49]. 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. Our 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 directly does not trigger any increase in toxicity against human cell lines.

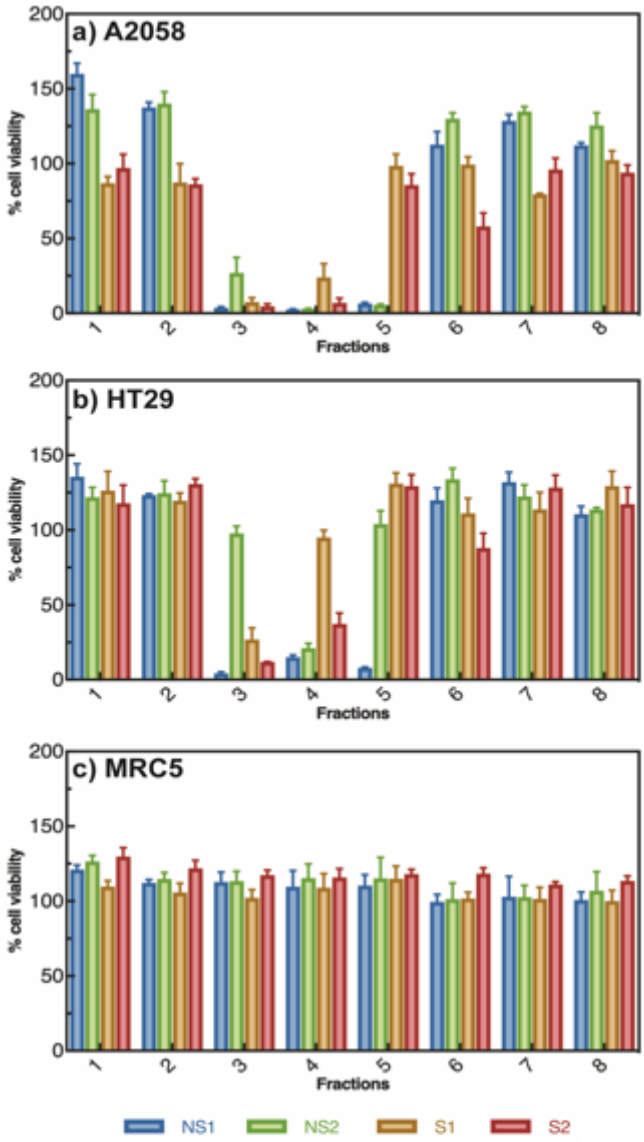

Fig. 4 Activity profiles of all fractions in samples NS1 (blue), NS2 (green), S1(yellow) and S2 (red) tested at 100 µg ml-1 against A2058 human melanoma (a), HT29 human colon carcinoma (b) and MRC5 normal lung fibroblasts (c). Fractions with cell viability lower than 50% is considered active. Data represents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

Sea urchin development assay

A sea urchin development assay was conducted to assess the impact of the extracts on larvae of *Paracentrotus lividus.* Our study revealed that the organic extracts of *P. glacialis* had toxic effects against *P. lividus* larvae. The toxicity assay was carried out in two stages; screening of flash fractions 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 very 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

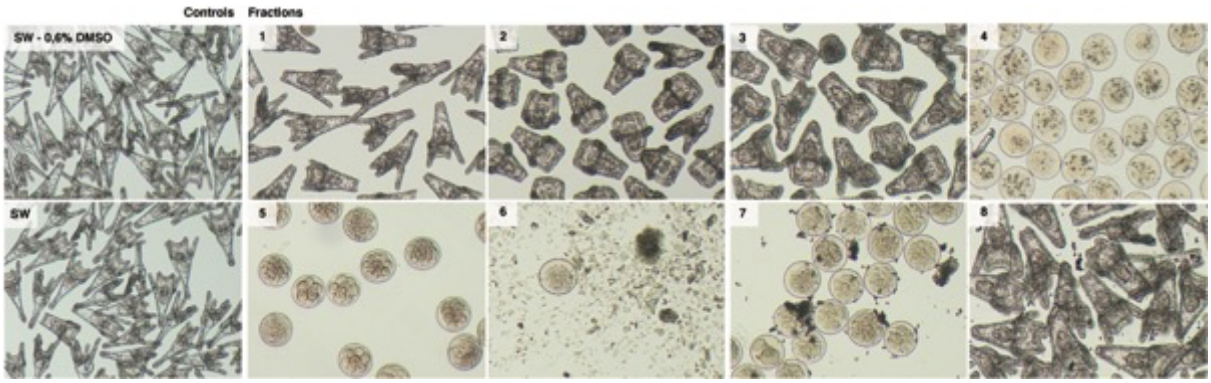

Fig. 5 Micrographs of sea urchin larvae (*P. lividus*) after being treated with Flash fractions 1-8 of NS1 at 100 µg mL^{-1} for 48h (4X magnification). Fractions 4-7 regarded as active fractions based on larvae survival. Controls are filtrated seawater (SW) and seawater with 0,6% DMSO.

fractions the same results applies to all four samples. There was little difference in fraction 1 and 8 compared to the two controls. These fractions were not regarded as active, and the larvae have been fully developed except for a few minor abnormalities. 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. Based on the results of the initial screening, a dose-response assay was performed with the concentrations 100, 75, 50, 25 and 5 µg

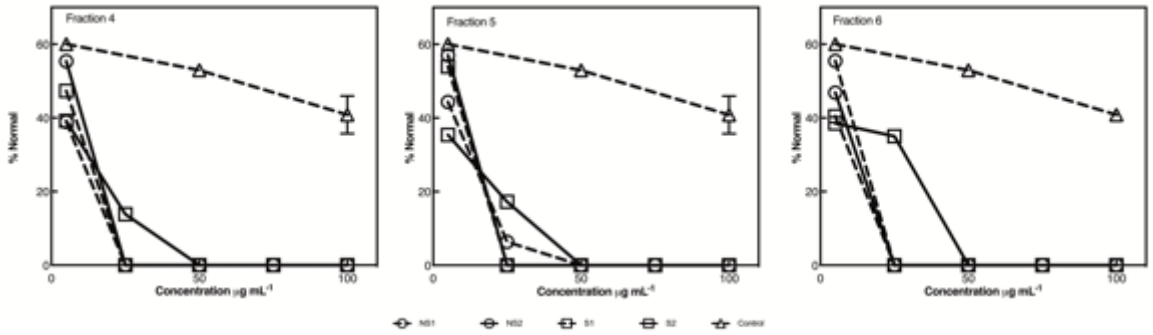

Fig. 6 Activity profiles from sea urchin toxicity assay of fraction 4, 5 and 6 in sample NS1, NS2, S1 and S2. Activity is measured in % normal developed larvae after 48 h of exposure to fractions at 100, 75, 50, 25 and 5 µg mL^{-1}. All malformed and dead larvae were regarded as not normal. Data represents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

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 normally developed sea urchin larvae for each of the treatment concentrations. As one can see 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 did neither increase or decrease 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 [32,50-53], and PUAs have been shown to be the one of the culprits [53,54]. A previous study by Gudimova et. al [55] 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 antiproliferative 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* has 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[29,56,57]. 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 the absorbance at 600 nm < 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, is seems as cultivation with factory smoke has 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. Our results show that mass cultivated *P. glacialis* have 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 if 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 [58,59], 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 600nm. As can be seen from the figure, 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, the 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 themselves, usually by pennate diatoms [60,61]. 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*. Our 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 e.g. plastic [62], and it is likely that the same applies to fiberglass and metal that were the materials of our bioreactors. Earlier experiments (not published) in the same bioreactors have shown 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

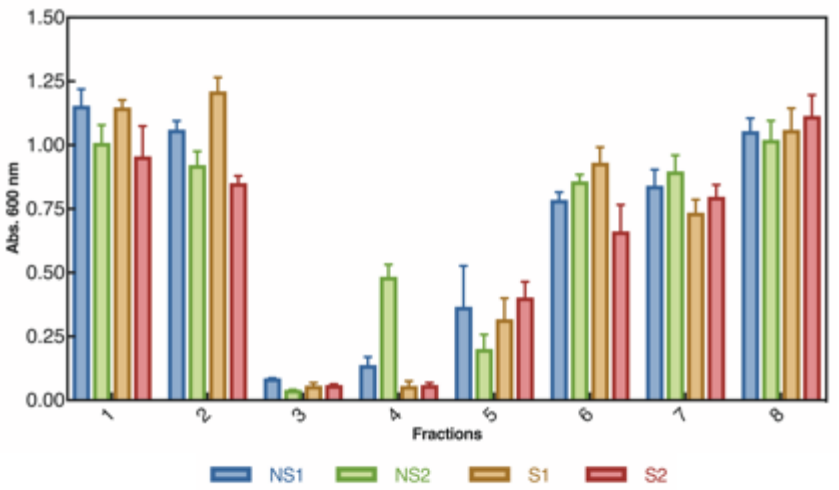

Fig. 7 Activity profile of Flash-fractions from NS1 (blue), NS2 (green), S1 (yellow) and S2 (red) showing inhibition of bacterial biofilm produced by S. epidermidis at 100 µg mL^{-1}. Fractions are characterized as active when absorbance at 600 nm <0.25. Data represents means of 3 replicates.

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.

Our 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 be useful in the search for new anti-fouling compounds.

Conclusions

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 mean of decreasing CO_2 emissions through carbon capture and utilization (CCU). 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 a safe production avoiding deleterious effects of harmful compounds and to comply to existing food/feed regulations.

Our results confirm the well-known fact that diatoms produce metabolites that may act toxic, but the toxicity does not increase when factory smoke are 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. Our research was based on the biological effects observed through a set of bioassays, while in the 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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