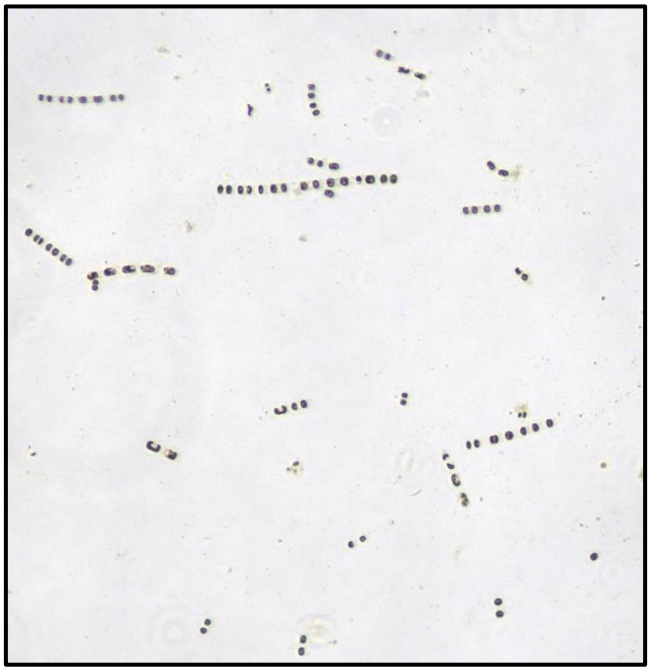
UNIVERSITAT ROVIRA i VIRGILI

GÖTEBORGS UNIVERSITET

EFFECTS OF COPEPODAMIDES ON DIATOMS

Marc Benet Caballero

FINAL BACHELOR THESIS IN BIOTECHNOLOGY



Chain formation of the diatom species *Skeletonema marinoi*

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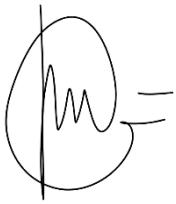
Jo, **Marc Benet Caballero**, amb DNI 48138440S, sóc coneixedor de la guia de prevenció del plagi a la URV Prevenció, detecció i tractament del plagi en la docència: guia per a estudiants (aprovada el juliol 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) i afirmo que aquest TFG no constitueixen cap de les conductes considerades com a plagi per la URV.



Göteborg, 7 de juny del 2023.

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1. About Signals of the Sea (University of Gothenburg)

Gothenburg University, one of the largest universities in the Nordic countries, is located on the west coast of Sweden, in the city of Gothenburg. It was founded in 1891 and it has more than 37.000 students and 7000 employees.

It is a major power and one of the only ones in Europe in the field of marine sciences, where it has several groups working on topics such as marine biology, oceanography, marine chemistry, marine ecology, effects of climate change on the marine environment, evolutionary biology, polar research, aquaculture, among many others.

The University of Gothenburg is also involved in many national and international projects and has two marine stations located along the west coast of Sweden, and owns the R/V Skagerak, a new modern research vessel for research and education (figure 1).

Figure 1. *R/V Skagerak.*

The stay has been made in the Signals in the Sea group, which targets the individual signalling compounds and translate their function in the pelagic ecosystem. Their research focuses on the isolation of signalling molecules produced by pelagic organisms, and their subsequent chemical study and characterization, transmission, and effects caused on other organisms living in their environment. In doing so, they intend to study the different relationships that are established between different marine organisms, such as zooplankton or phytoplankton.

Currently, Signals of the Sea is focusing its research on a new group of recently discovered molecules, called copepodamides. These polar molecules are synthesized by copepods (a group of crustaceans). In marine environments they can trigger different defence responses in other organisms, such as the synthesis of secondary metabolites like toxins, changes in behaviour or changes in metabolism.

2. Abstract

Many pelagic organisms living in the oceans are not able to perceive information from their environment in any other way than by responding to chemical signalling traces. Therefore, many species have developed mechanisms to analyse their surroundings in order to avoid predators or dangerous situations. In the following study, copepodamides were extracted from *Calanus finmarchicu*s, a species of Copepoda, a crustacean belonging to the zooplankton group. Copepodamides are polar molecules containing a fatty acyl group derived from ω-3, which act as chemical signalling molecules that trigger diverse survival responses in many species of phytoplankton.

Cultures of the diatom *Skeletonema marinoi* resting stages (non-hatched individuals) were grown in different concentrated media inoculated with the extracted copepodamides (0nM, 1nM and 5 nM). After 4 and 7 days, the groups of *Skeletonema marinoi* cultures were analysed and compared. Results show that cell growth and division appeared to be increased in copepodamide-free cultures. Regarding the formation aggregation of cells in chains, they were significantly shorter in groups treated with copepodamides. The levels of chlorophyll relative fluorescence seem to be higher in copepodamide-treated cultures in relation the number of cells after 4 and 7 days.

These results suggest that copepodamides synthesized by *Calanus finmarchicu*s trigger diverse survival mechanisms on *Skeletonema marinoi* that might help them escape from their predators.

Key words: pelagic organisms, bioprospecting, copepodamides, phytoplankton, zooplankton, chemical signalling, *Calanus finmarchicu*s, *Skeletonema marinoi*, resting stages, cell growth, chain forming-species, chlorophyll.

3. Introduction

3.1 Plankton

In the depths of the ocean, where red and yellow light radiation no longer reaches, a world of green and blue extends (Wimpenny et al. 1966). This underwater world is inhabited by one of the first life forms to appear on the Earth, tiny creatures which can generate their own energy from sunlight and nutrients present in the environment. These are called phytoplankton, a group of organisms comprising a large number of species, including cyanobacteria and single-celled protists (such as dinoflagellates, diatoms and coccolithophores). The phytoplankton ais characterised by being able to perform photosynthesis and obtain the necessary energy for its vital functions. This is possible thanks to specialised structures such as chloroplasts (present in protists) or photosynthetic membranes (present in cyanobacteria) (Sardet et al. 2015).

Phytoplankton plays an essential role for life on the planet. They are able to fix atmospheric carbon and transform it into organic matter, while releasing oxygen at the same time. Phytoplankton-rich areas are also called "forests of the ocean", as they generate around 70% of the global oxygen production (Nelson et al.), and about the 50% of the oxygen we breath (Abida et al. 2013). Phytoplankton is additionally a major source of food for many other organisms, from large animals to zooplankton, that feed by ingestion of processed organic matter.

Thus, between the two types of plankton, a close relationship is established in which zooplankton survives on the basis of phytoplankton. This second group includes a large number of species as well, examples of which are some crustaceans, single-celled animals, sponges, jellyfish, different worms, certain molluscs… Zooplankton can live in deep-ocean areas below the sunlit zone (below 200 meters), where photosynthesis is no longer possible. They can move to areas closer to the surface to feed themselves on phytoplankton using ocean currents or displacement mechanisms (Wimpenny et al. 1966).

As mentioned previously, plankton gathers a wide range of species (see Table 1) from really big organisms, such as jellyfish, to femtoplankton, like viruses (Abida et al. 2013). Plankton can also be classified in two different groups: those organisms referred as holoplankton, which are strictly planktonic, ore those called meroplankton, which belong to the planktonic community only during a specific phase of their life cycle (Bowler et al. 2009).

***Table 1**. Size of classes of plankton, together with representatives of taxa present in each. Colouring as it follows: Bacteria, Protozoa, Chromista, Plantae, Fungi and Animalia. Modified source from (Abida et al. 2013).*

Plankton breakdown by size	Examples of diversity by taxonomy
Megaplankton over 20 mm	Scyphozoa (large jellyfish), ctenophora, tunicata (salp, pyrosoma), mollusca (cuttlefish), heterokontophyta (kelp)
Macroplankton from 2 mm to 20 mm	Scyphozoa (jellyfish), ctenophora, tunicata (salp, pyrosoma, doliolida), mollusca (small squid), chaetognatha, crustacea (krill)
Mesoplankton from 0.2 mm to 2 mm	Arthropoda (copepods, cladocera, ostracoda), scyphozoa (small jellyfish), chaetognatha, crustacea (krill), mollusca (pelagic "snails", "slugs", "butterflies"), amoebozoa (amoeba)
Microplankton from 20 µm to 200 µm	Heterokontophyta (diatoms, oomycetes), chlorophyta (green algae), rhodophyta (red algae), dinoflagellata, ciliata (euplotes, astomes), apicomplexa (cyclospora, selenidium), haptophyta (coccolithophores, prymnesium), cryptophyta, foraminifera, radiolaria, euglenozoa, amoebozoa
Nanoplankton from 2 µm to 20 µm	Cyanobacteria, blastocladiomycota, ascomyta, basidimycota, smaller heterokontophyta, chlorophyta, rhodophyta, dinoflagellata, ciliata, apicomplexa, haptophyta, cryptophyta, foraminifera, radiolaria, euglenozoa
Picoplankton from 0.2 µm to 2 µm	Cyanobacteria, proteobacteria, verrucomicrobia, nitrospirae (nitrosopumilus), crenarchaeota, smallest heterokontophyta (pelagophyceae, chrysophytes, eustogmatophyuceae)
Femtoplankton	Viruses

3.2 The relevance of plankton and ecosystem function: an emerging field

Bioactive molecules produced by planktonic organisms might seem something that we do not need to pay attention to, but actually, understanding the chemical ecological responses tiggered by these molecules can be crucial to help and preserve the biodiversity and the ecosystem functionality. In a marine environment, many key processes such us food selectivity and location, chemical defence, mate finding, and many other, rely on a complex ned of interactions via chemical ecology that is established in both inter- and -intra-specific specie levels (Ianora et al. 2011).

Nowadays, in a time of ecological and environmental crisis, when the word population is rapidly increasing, aging for a longer time, and demanding bigger amounts of sources, it is essential to focus our attention towards the sea to find possible solutions for a sustainable future. A study carried on by (Abida et al. 2013) strongly believes that disruptive solutions could be found in bioprospecting the oceans, focusing on the arsenals of molecules, enzymes

and genes that have been not yet explored. It is believed that the marine microbiology combined with an increasing awareness of environmental consciousness could be the key for the solution to many actual problems (Bowler et al. 2009).

Bioprospecting products related to marine organisms can be of great value in the market. And there are many that are already used in the field of biocatalysts. In figure 2, different steps of the biocatalytic process development are shown. Marine enzymes offer enzymatic properties that other organisms do not have. Marine enzymes can function under special conditions of pressure, salinity, temperature, or solar exposure. Therefore, these qualities, such as halo-stability or thermo-stability, are extremely interesting for industrial applications (Rodrigues et al. 2022).

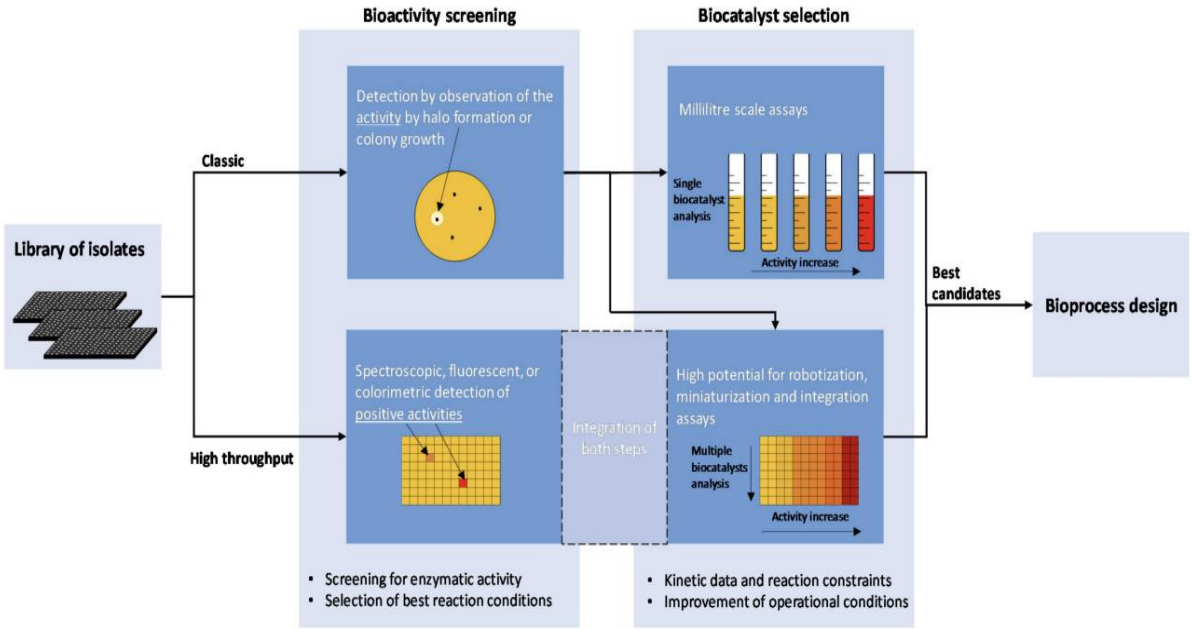


Figure 2. *Different steps of the biocatalytic screening and selection for a biocatalytic process development. Source from (Rodrigues et al. 2022).*

To give some examples, amylases from the halophilic species *Pseudoalteromonas undina*, are being used to make possible the conversion of sugars for bioethanol production from marine microalgae biomass in a high salinity environment. Using land-based enzymes would imply an extra desalination step (Shafiei et al. 2011).

Marine enzymes that can operate in different pH ranges are also widely used, and which operate in the detergent industry, for example. Marine habitats offer also a wealth of thermophilic

biocatalysts with thermostable (80-108 ºC) and barophilic properties. But the focus can also be put on the biotechnological exploitation of cold-adapted enzymes of marine planktonic species living in the Arctic and Antarctic areas. These can be used in the detergent industry, food preservation at low temperatures or bioremediation processes due to their low energy consumption and their low temperature operation where many other enzymes cannot work (Rodrigues et al. 2022).

For all the above reasons, it is important to focus research attention on the wide range of bioresources that ocean bioprospecting can offer. The study carried out by (Abida et al. 2013) concludes that planktonic biodiversity could provide new insights into how to deal with several today's global problems. Using phytoplankton as a resource for biofuels could lessen the impact that fossil fuels have on the planet's carbon cycle. It could also be used as a source of nutraceutical and pharmaceutical components that could help treat a variety of diseases. In addition, the search for new bioactive molecules that different organisms use to communicate or protect themselves could lead to the discovery of pharmaceutical and antibiotic compounds that could help fighting multi-resistant microbes in several areas, such as the food industry, healthcare and medicine, the detergent industry, cream and cosmetics production, or agriculture.

3.3 What are copepods?

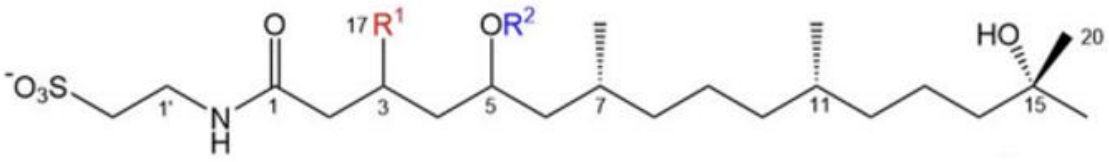
This project focuses on some specific types of plankton. One of them are copepods, specifically the species *Calanus finmarchicus*, commonly used in research. Copepods are a group of crustaceans within the zooplankton group. They are, in fact, the most abundant animals in the world, and are described as active, free-swimming, segmented, shell-forming organisms. Most copepods migrate during the night to more superficial areas to feed, mostly on diatoms and dinoflagellates, and dive to deeper areas during the day to evade being seen by predators (Sardet et al. 2015). *Calanus finmarchicu*s can be found all over the world except the Antarctic Ocean. They are very common in the northern hemisphere at depths of up to 4000 metres and serve as a source of protein for many multicellular organisms such as pelagic fish, whales or even other zooplankton species (Wimpenny et al. 1966).

But they also act as predators. As studied by Selander et al. (2019), copepods cause a unique fear effect on their phytoplankton prey through a group of molecules called copepodamides. Phytoplankton have no other way of analysing their environment and sensing predatory attacks

than by chemical signalling traces. In the water, copepods move around in a cloud of copepod amides that are chemically detectable by prey. This triggers different defence mechanisms depending on the type of prey; secretion of secondary metabolites such as toxins, change of swimming trajectories and speed, or separation of chains and aggregations formed by different individuals to avoid being found all at once (Selander et al. 2015). Or in some cases, certain planktonic species that have not hatched remain in a dormant state (cysts) until the signal is no longer detectable (Regnefors et al. 2004).

3.4 Copepodamides and their structure

Copepodamides were first described by Selander et al. (2015) from extracts of the copepod species *Centropages typicus* and *Calanus finmarchicus*. They represent the first isolated molecules that serve as chemical mediators between phytoplankton and zooplankton. Copepodamides (Figure 2) are characterised by containing a fatty acyl group derived from ω-3, such as docosahecaenoic acid (DHA), eicosapenaneoic acid (EPA) or stearidonic acid (SDA). Copepodamides are polar lipids that were named from A-F according to their molecular weight. Copepodamides A and D were found to contain DHA, B and E contained EPA and in the case of copepodamides C and G, SDA was present. Furthermore, two scaffolds were found to be the skeleton of the other copepodamides in lack of their fatty acyl groups (DHA, EPA and SDA) and named with the letters G (part of copepodamides A-C) and H (part of copepodamides D-F).



Copepodamide	MW (Da)	R^1	R^2
A	761.5	$-CH_3$	Docosahexaenoic acid (DHA)
B	735.5	$-CH_3$	Eicosapentaenoic acid (EPA)
C	709.5	$-CH_3$	Stearidonic acid (SDA)
D	759.5	$=CH_2$	Docosahexaenoic acid (DHA)
E	733.5	$=CH_2$	Eicosapentaenoic acid (EPA)
F	707.5	$=CH_2$	Stearidonic acid (SDA)
G	451.3	$-CH_3$	-H
H	449.3	$=CH_2$	-H

Figure 3*. Chemical structure of copepodamides A to H, indicating their radicals (R^1 and R^2). MW referring to molecular weight expressed in Daltons. Figure from (Selander et al. 2015).*

3.5 Resting stages - *Skeletonema marinoi*

A second focus will be set on the survival mechanisms of diatom from the species *Skeletonema marinoi*, a unicellular photosynthetic organism common in Scandinavian waters. *Skeletonema marinoi* has the ability to remain in abiotic marine sediments in a dormancy/resting stage when conditions are not favourable, i.e. when there are insufficient nutrients or when the environment becomes anoxic (Stenow et al. 2020). Therefore, these organisms are able to remain as cysts for more than a century (Johansson et al. 2019) and do not hatch until conditions become favourable, i.e. there are sufficient nutrients, good oxygen availability, and light (Stenow et al. 2020).

Furthermore, after sequencing the genome of *Skeletonema marinoi*, it was possible to classify this diatom as a chain-forming species, in which many unicellular individuals attach together to form aggregates, which is believed to have advantages, for their vital functions, photosynthetic needs, ability to emerge and sink and as protection against predators (Johansson et al. 2019). Previous studies suggest that diatoms form and dissolve aggregates to escape from predators, as these can only detect, capture and feed on preys up to a certain size (Hansen et al. 1993).

However, it has been observed that the length of the chains formed by the diatom *Skeletonema marinoi* varies when getting close to grazing predators (such as copepods), where the chains appear shorter, as opposed to when they get close to non-grazing predators (Bergkvist et al. 2012).

4. Objective and hypothesis

The main objective is to perform the extraction of copepodamides from the zooplankton species *Calanus finmarchicus* and use them as inoculum to test for effects of predator chemical cues on the hatching, growth and chain length of resting stage/cyst cells of the diatom *Skeletonema marinoi*.

It is hypothesized that the presence of copepodamides in *Skeletonema marinoi* cultures will reduce the number of hatched cells and decrease the cell division rate compared with the copepodamide-free cultures. In addition, a shortening of the chains formed by several individuals are expected to be observed in cultures with copepodamides.

5. Materials and methodologies

5.1 Copepodamide extraction

Extraction of copepodamides fromfreeze-dried *Calanus finmarchicus* was carried out following the protocol designed by "Signals of the Sea" and based on the experimental design on the BUME method described by Löfgren et al. (2012), as follows. The whole extraction process was carried out for 7 different batches of copepods.

5.1.1 Purification of non-polar molecules

Different batches of approximately 45 g of copepods *C. finmarchicus* were measured and kept in 0.4 L of methanol 99.9%. Ultrasonication was performed during 20 minutes in order to release the various molecules, including copepodamides for extraction, and stored during 24 hours at -20 °C. Next, the methanol was drained using a cellulose filter and methanol was added a second time to the remining copepods.

Ultrasonication and filtration were repeated a second time, and the copepod extract in methanol was kept.

5.1.2 Rotational evaporator

The extract was placed in a round-bottom flask and placed in the rotational evaporator, where the methanol was evaporated, condensed, and discarded, until the extract reached a near-solid state.

Rotational evaporator was used among all the steps to evaporate the medium in which the copepodamides were kept and redissolved afterwards.

5.1.3 Liquid-liquid lipid extraction

After the extract was dried, it was dissolved in 100 mL of a methanol : 1% NH_4 in H_2O (95:5) solution. The new copepod extract was mixed with a heptane : methanol (98:2) buffer in a liquid-separating funnel in a 1:1 proportion. Two different phases appeared: a lower polar-

phase containing the copepodamides in methanol, and a higher non-polar-phase containing unwanted lipid in heptane.

Methanol containing the copepodamides was kept and mixed again with the heptane : methanol (98:2) buffer in the liquid-separating funnel in a 1:1 proportion. The previous separation was repeated in order to obtain a higher purified product, and the methanolic phase was kept. Following, step 5.1.2 (rotational evaporator) was repeated to dry the copepod extract for the next step.

5.1.4 Solid phase extraction (SPE)

The dried extract was dissolved in 100 mL of a methanol : MQ H_2O (1:4) solution. For solid phase extraction, a vacuum chamber was used, and 60 mL SPE columns were activated twice by adding 30 mL of 99.9% methanol. After, a volume of 15-20 mL of the extract was added and passed through each SPE column.

Once the extract was filtered, each column was washed in four steps using four solutions of methanol in MQ H_2O with increasing methanol concentrations (20%, 50%, 70% and 100%). The filtrates corresponding to the 70% and 100% solutions were saved. Only the 100% methanol solution was used onwards, and the 70% methanol solution was kept as a "back-up".

The whole step was repeated for the remaining copepod extract, activating the SPE columns twice with 99.9% methanol, loading them with the extract, and performing the 4 washings. Following, step 5.1.2 (rotational evaporator) was repeated to dry the copepod extract for the next step.

5.1.5 Fractionation

The dried extract was dissolved in approximately 9 mL of 99.9% methanol and transferred in 1.5 mL HPLC vials. Fractions were separated using reversed phase HPLC using a Hitachi Elite LaChrom HPLC coupled to a Hitachi LaChrom L-2455 diode array UV detector and a Waters Fraction Collector III. Two buffers were used to separate the different components of the extract: buffer A containing methanol : acetonitrile : MQ H_2O with 0.2% formic acid (v/v) (45:40:15), and buffer B containing isopropanol 100% with 0.2% formic acid (v/v). The different samples were separated based on their light absorption under a wavelength of 461

nm. Sample injections were set to 70 µL and the software was set to 19 rounds for each vial. Time between the injections was 36 minutes.

After 25 hours, different fractions were obtained for further analyse.

5.1.6 Mass spectrometry

The fractions were analysed using mass spectrometry. Agilent Technologies 1290 Infinity were utilized, performing direct infusion and using the detector 6470 Triple Quand LC/MS. The equipment was set to a gas temperature of 260 °C, gas flow 5 L/min, nebulizer 20 psi, sheath gas temperature at 200 °C and sheath gas flow at 6 L/min. Capillary was settled to 3500 V and nozzle voltage to 500 V. Fractions were all placed in a 96-well plate. Two buffers were used for the analysis: buffer A containing methanol : acetonitrile : MQ H_2O with 0.2% formic acid (v/v) (35:30:30), and buffer B containing isopropanol 100% with 0.2% formic acid (v/v).

QQQ quantification software was used to determine the amount of copepodamides found in every fraction. Those containing a significant amount of them were pooled together. Following, step 5.1.2 (rotational evaporator) was repeated to dry the copepod extract for the next step.

5.1.7 Finalization

The dried extract containing the copepodamides was dissolved in approximately 3 mL of methanol 99.9% and topped with nitrogen.

5.2 Resting stages experiment preparation

Copepodamide effects were tested on hatching, growth and behaviour of the species *Skeletonema marinoi* (strain R05). It was cultivated in S/2 + Si medium (recipe in annexes) during 7 days, at 16 °C under and average of 50 fphotons/m^2 on a plankton wheel (see figure 4) at a speed of 2.5 rpm.

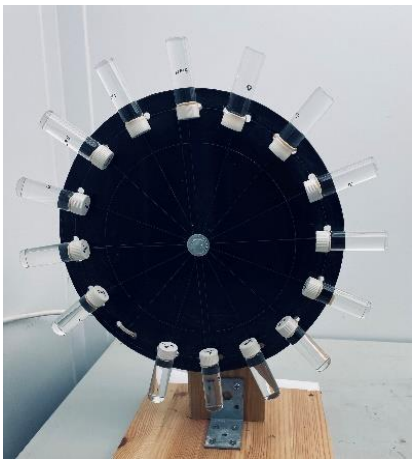


Figure 4*. Plankton wheel (2.5 rpm).*

Skeletonema marinoi was manipulated in the dark until right before the vials were ready to be set on the plankton wheel in orther to minimize chlorophyll activation, cell hatching and cell growth and division until the starting point of the experiment.

The culture was distributed in 8 mL vials, which were divided into 3 groups with different treatments: control group (methanol added), 1 nM group (copepodamides added for a final concentration of 1 nM) and 5 nM group (copepodamides added for a final concentration of 5 nM). In addition, an extra group was separated at the beginning of the experiment to mesure the initial parameters.

In order to add the copepodamides, solutions were prepared using the stock solution obtained after the extraction, with a concentration of 28 nM, and then added to the vials before the culture with *Skeletonema marinoi*, so the mehtanol could evaporate and cause a minimal impact on the growth. To minimize oxidation of the copepodamides, methanol evaporation was performed under nitrogen.

Growth analysis were performed at three points of the experiment: at day 1, when the vials were prepared (Starting Point, "SP"), at day 4 and at day 7. The group of vials analyzed on day 7 were coated a second time with their respective treatments on day 4. This was done by inoculating methanol or copepodamides (1 nM or 5 nM) into new vial caps. The inoculum was left to evaporate and the old caps were replaced with new caps loaded with the new dose of copepodamides. The replicates used for day 4 analysis were discarded afterwards. The analyses performed to the different groups are described next:

a) **Chlorophyll relative flourescence**. Samples from the cultures were dsitributed in 48-well plates and chlorophyll relative flourescence was mesured using a Thermo Scientific Varioskan Flash equipment.

b) **Cell growth and division**. Cells were counted under an inverted microscope Zeiss Vert.A1, using Gridded Sedgewick Rafters of 1 mm^2.

c) **Chain length**. Mesured by counting the number of cells forming the first 50 chains encountered. Performed under an inverted microscope Zeiss Vert.A1, using Gridded Sedgewick Rafters of 1 mm^2.

A diagram of the experiment group distribution and preparation is showed below (figure 5).

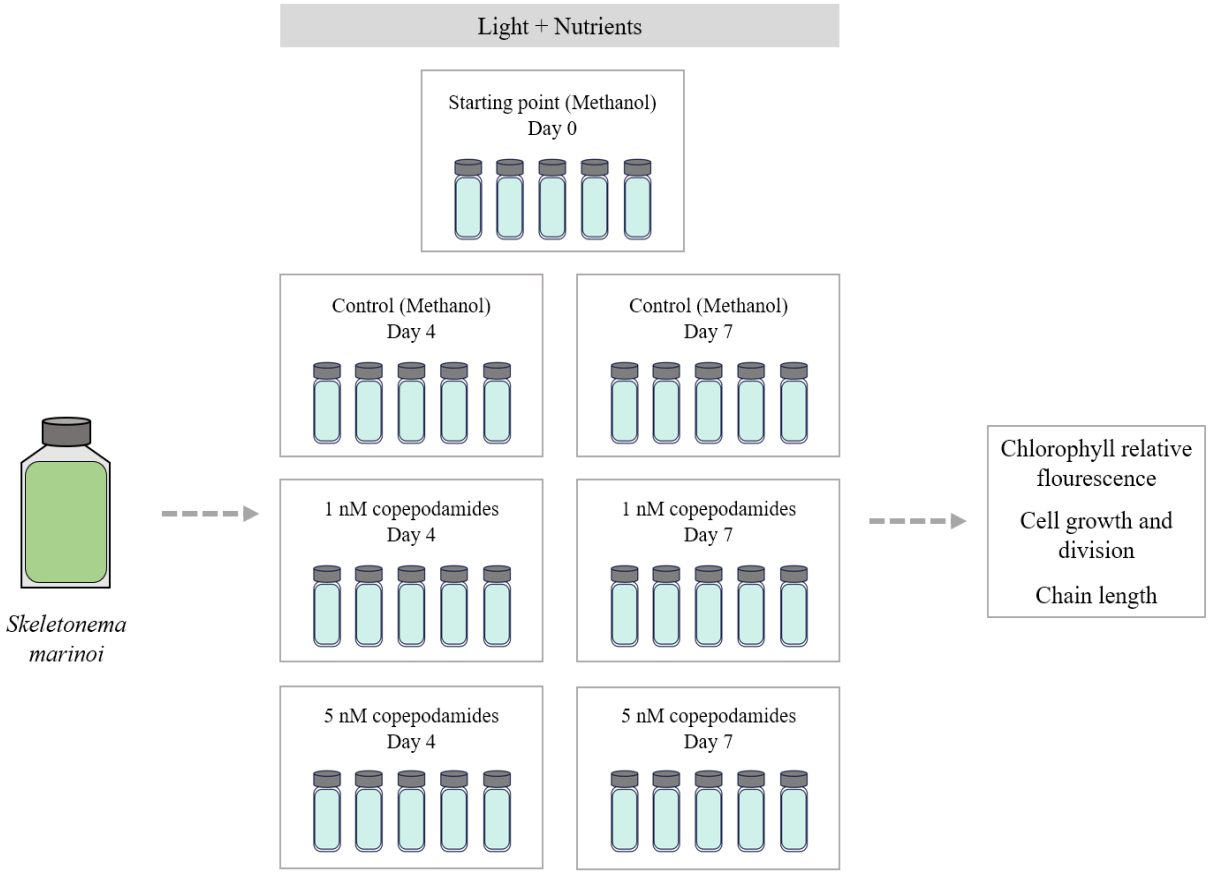


***Figure 5**. Diagram of the resting stages experiment using* Skeletonema marinoi*, showing the treatments applied in each group of vials and the day on which the analyses were performed.*

5.3 Statistical analyses

Statistical analyses were performed using RStudio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.)

6. Results and discussion

The extraction of copepodamides was successful, with a final solution of a concentration of 28 nM, which was used for the resting stages experiment with *Skeletonema marinoi.* Results regarding this experiment are presented hereunder:

6.1 Chlorophyll relative fluorescence, cell growth and division

Both the results obtained for chlorophyll relative fluorescence and those obtained from the cell counting reflect that *Skeletonema marinoi* hatches after day 4. Results show that there are no significant differences between the levels of chlorophyll mesured at the starting day and day 4. Something similar is observed in cell counting, although the number of cells is slightly higher on day 4, no significant growth is observed. This also indicates that the resting cells have not yet hatched on day 4. The appearance of the cells on the inverted microscope is also very similar in the culture analysed on the starting day and the ones analysed on day 4. All these observations apply to all treatments: control, 1 nM and 5 nM (See figure 9).

However, on day 7 both the levels of chlorophyll relative fluorescence and the number of cells in the culture were much higher compared to the starting day and day 4, indicating that the resting cells had hatched after the analysis on day 4. Incrase precentatges can be accessed in (Table 1) and graphics can be seen at annexes (Figure 10 and 11).

***Table 2**. Increment of chlorophyll relative fluorescence and cell number increase in percentages (%) comparing samples from day 4 and day 7.*

Chlorophyll relative fluorescence and cell number increase		
Treatment	Increase in chlorophyll (%)	Increase in cell number (%)
0 nM	2127	363
1 nM	907	269
5 nM	1111	332

The different treatments seemed to not have an effect on the chlorophyll relative flourescence at first. Howerver, this parameter is not totally linearly proportional to the number of cells in the culture (see figure 7). Furthermore, as an aside, the resting cells of *Skeletonema marinoi* contain chlorophyll, which is rapidly activated after being exposed to light.

Regarding the effect of the treatments, differences in cell number between them can only be observed after day 7. The number of cells appeared to be a decreased in the group of cultures growing with the presence of copepodamides compared to the control, where no copepodamides were added. Concretely, the cell count was a 20 % less in the vials with a concentration of 1 nM copepodamides in realtion with the control, while it appeared to be a 37 % decreassed in the vials with a concentration of 5 nM. A p-value could not be calculated because only one of the cultures from the contol group from day 7 hatched.

Nevertheless, a significant p-value of 0.0057 was obtained when comparing the cell count values from the cultures treated with 1 nM and 5 nM copepodamides. A 21 % decrease in the number of cells was calculated between these two groups (Figure 6).

No difference in chlorophyll relative fluorescence levels was found between cultures treated with 1 nM and 5 nM copepodamides (p-value was 0.1365). The control that hatched had a similar value to the trated groups as well. (Figure 6).

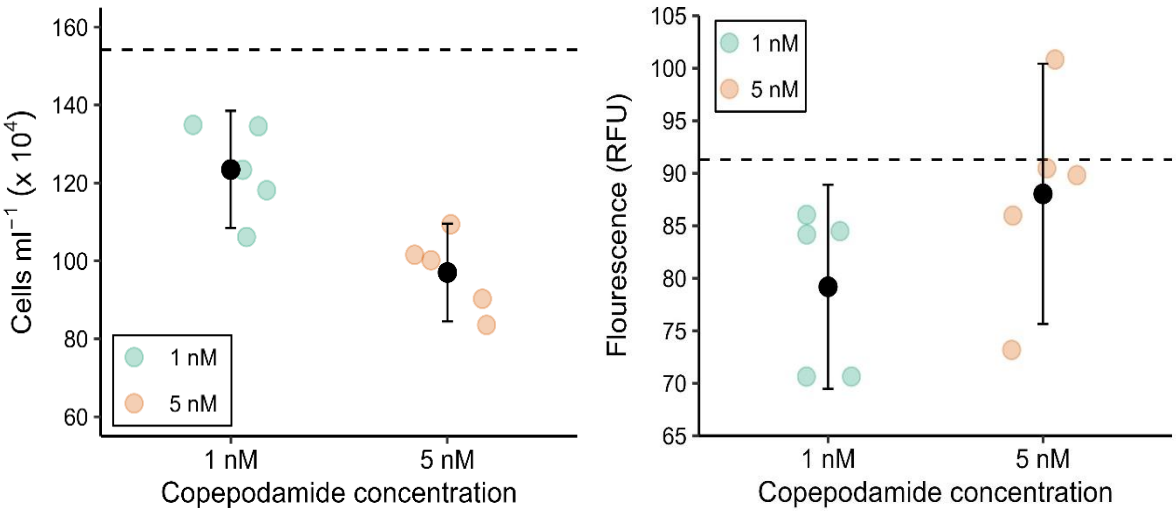


Figure 6. *Graphs showing the number of cells of Skeletonema marinoi per mL of culture (left) and chlorophyll relative florescence (right) in RFU (Relative Fluorescence Unites) of the vials corresponding to the control group (represented by a dotted line), and the copepodamide treatments at concentrations of 1 nM (green dots) and 5 nM (red dots). Data from day 7. The black dots show the mean of each group, and the bars indicate the confidence intervals.*

These results suggest that cell division and growth are inhibited when copepodamides are present in their environment, meaning that *Skeletonema marinoi* senses when there are predators approtching. In response, cells save energy and do not divide as much as cells living in a safe environment.

In the results comparing the linearity between the number of cells in the culture and the chlorophyll relative flourescence, it can be seen that the groups treated with different concentrations of copepodamides differ slightly from the regression. The graphical representation (Figure 7) suggests that the group treated with 5 nM copepodamides could be synthesizing similar levels of chlorophyll as the group treated with 1 nM and the control, although the number of cells is smaller. In the graphs presented above (figures 4 and 5) it can be seen that there is a significant difference in cell number (p-value = 0.0057) between treatments, but no difference in relative chlorophyll levels (p-value = 0.1365). This leads us to think that when *Skeletonema marinoi* is in a dangerous situation where it is attacked by copepods, it could increase the expression of chlorophyll and chloroplasts in order to generate more energy in case it has to separate from the chains or has to move further to escape.

Similar results can be observed in the values obtained on day 4 (Figure 6). These suggest that cultures treated with 5 nM copepodamides could express higher relative chlorophyll levels per cell compared to the levels of the control group or the group treated with 1 nM. This could indicate that upon the preception of a danger in the environment, *Skeletonema marinoi* could already increase its chlorophyll production in the early stages of hatching or after getting activated by the light during the first days.

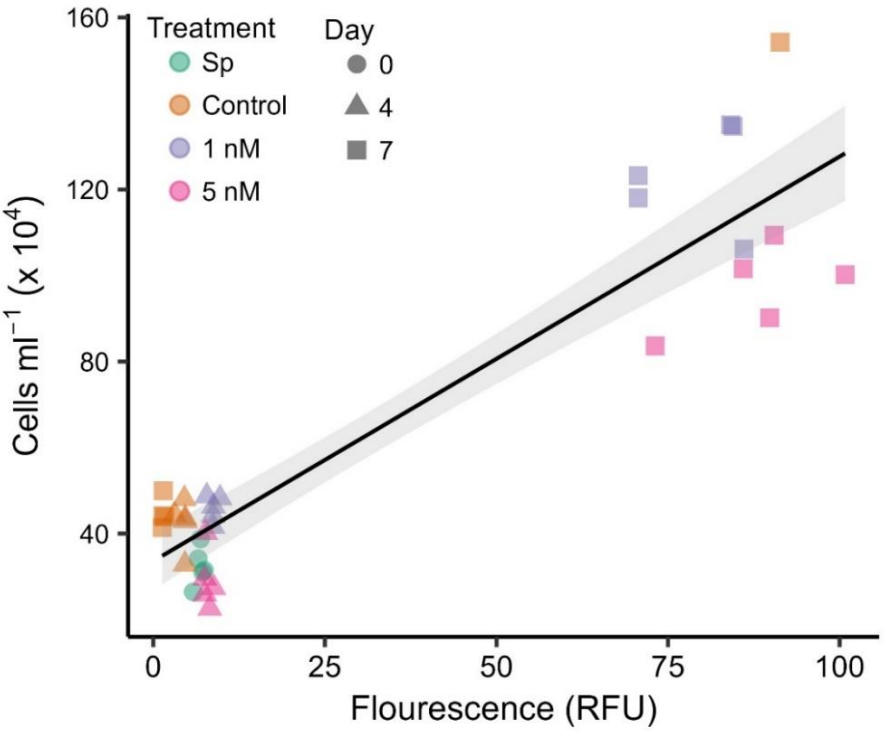


Figure 7*. Graph showing the number of cells of Skeletonema marinoi per mL of culture, and chlorophyll relative florescence in RFU (Relative Fluorescence Unites) of the vials corresponding to the Starting point analysis (Sp), the control group, and the copepodamide treatments at concentrations of 1 nM and 5 nM. The different colours represent the type of treatment applied to the culture, and the shape represents the day of analysis. A solid line represents the regression relation between the number of cells and Fluorescence (RFU). The shades around the line are the confidence intervals.* R^2 *= 85%*

6.2 Chain length

Chain lengths differences in the cultures were only observed after day 7. On day 4, hardly any chains had formed. Only a few were present in all cultures regardless of the treatment applied. No significant differences were found between the copepodamide-treated groups and the control group on day 4 (these results can be found in the annexes).

Results from the cell counting on day 7 showed that the control group appeared to have longer chains compared to the groups treated with copepodamides. In the control culture, the chains exceeded 7 cells per chain on average, where there could be found chains of up to 16-18 cells. In cultures treated with 1 nM copepodamides, chains were formed with an average of 3 cells, representing a 59% decrease compared to the control group. In cultures treated with a

concentration of 5 nM, the chain average was approximately 2 cells, representing a 73% decrease compared to the control.

Because only one of the control group cultures had hatched on day 7, a p-value could not be calculated. However, significant differences were found (p-value of 0.000056) between the groups treated with 1 nM and 5 nM copepodamides. See (figure 8).

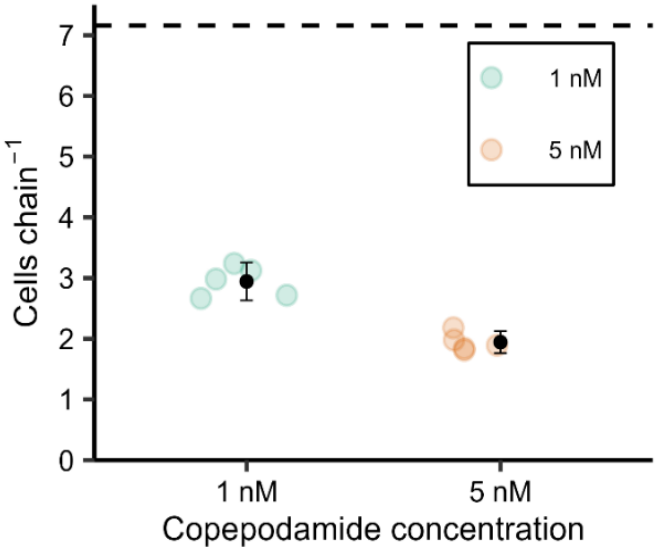


***Figure 8**. Graph showing the average number of cells per chain of the vials corresponding to the control group (represented by a dotted line), and the copepodamide treatments at concentrations of 1 nM (green dots) and 5 nM (red dots). Data from day 7. The black dots show the mean of each group, and the bars indicate the confidence intervals.*

The appearance of the cultures on day 4 was practically the same as the resting stage culture analysed at the starting point (day 0), suggesting that the cells did not hatch until after day 4. Under the inverted microscope, the resting stage cells looked more rounded in shape and organised into aggregates. Almost no chains are visible (Figure 9).

On day 7, however, longer chains were visible, and no clustering of cells was observed. Specifically, in the control group, very long and well-organized chains were found. The cells appeared to be more squared and elongated, and well distributed throughout the culture fluid. (Figure 9).

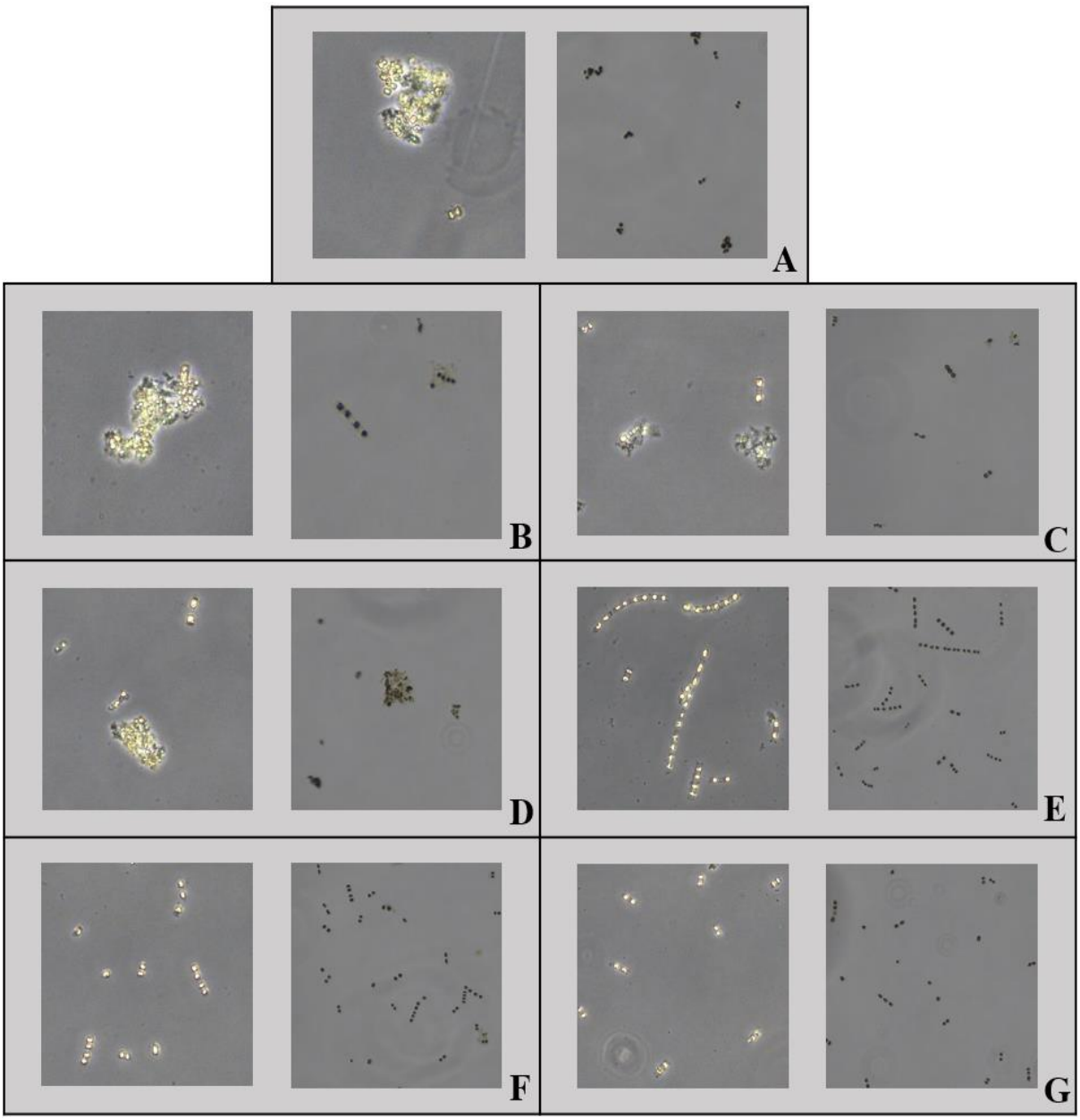


***Figure 9**. Pictures taken under the optical microscope at a magnification of 40x (left picture) and 10x (right picture). A) starting day, B) Control on day 4, C) 1 nM copepodamides on day 4, D) 5 nM copepodamides on day 4, E) Control on day 7, F) 1 nM copepodamides on day 7, G) 5 nM copepodamides on day 7.*

The results obtained indicate that, at the level of group behaviour, *Skeletonema marinoi* breaks up the chains it forms in safe environments in order to form smaller units, or even separate into individual units to achieve lower chances of being found all together by predators. With this strategy the survival probabilities increase when a danger approaches. This experiment showed that copepods (*Calanus finmarchicus)* mean a real danger to *Skeletonema marinoi*, and that copepodamides represent an aid to diatoms in detecting dangers and acting in response.

7. Conclusion and future perspectives

The abundance of pelagic species in our oceans is very important to maintain a healthy environment on the planet Earth, as they help to preserve the balance between oxygen production and carbon dioxide consumption.

This project reflects on a small scale the balance between producers (first level of the trophic chain) and their consumers (second level of the trophic chain). The experiments carried out show the effects of copepodamides produced by copepods (consumers) on the growth, development, and behaviour of diatoms (producers). In addition, an example of communication between different species through chemical signals in water is observed. Diatoms, in this case, are able to analyse their environment by sensing chemical signals produced by copepods.

The results of the experiment reveals that *Skeletonema marinoi* can generate various survival responses in the presence of copepodamides emitted by *Calanus finmarchicus*. It has been shown that the growth and cell division of diatoms is diminished in the presence of a risk of being hunted. With higher concentrations of copepodamides, the lower their reproduction, and this corresponds to the level of stress caused by predation.

The group behaviour of *Skeletonema marinoi* is also affected by the presence of copepods. In safe environments, *Skeletonema marinoi* forms chain-like groupings of about 7 cells per cluster on average. However, copepodamides induce a chain-splitting response in diatoms to reduce the chances of being encountered by predators. Therefore, chains of less than 3 cells on average can be seen in copepodamide cultures.

The linearization of cell number with respect to chlorophyll relative fluorescence has also shown changes in chlorophyll production (and probably also in photosynthesis rate) caused by the presence of copepodamides in the culture. It has been observed that relative chlorophyll levels per cell number are higher in cultures with copepodamides. This suggests that the survival response generated by *Skeletonema marinoi* focuses on increasing chlorophyll levels to increase energy production.

Regarding the hatching of resting stages, no effect of copepodamides has been seen. All the responses observed were post-hatching. However, the experiment should be repeated, because only one of the cultures of the control group on day 7 hatched. The results would be more reliable if we had several replicates of the control group.

Copepodamides may have some applications in the field of biotechnology. As tested in this project, the presence of copepodamides affects phytoplankton by putting on pressure to change their metabolic rates, and a clear example is an increased chlorophyll synthesis when species such as *Skeletonema marinoi* are grown in a medium with copepodamides.

At an industrial biotechnology level, copepodamides could be used to increase the yield of various species. For example, algae farms are booming currently in Sweden, allowing the production of multiple products which are useful for a variety of applications. One of their applications is operating in photobioreactors, which are basically fermenters used for the elimination of waste products such as CO2 from factories. If copepodamides have the same effect on algae as on *Skeletonema marinoi*, the rate of photosynthesis could be increased, hence improving the efficiency of photobioreactors.

Another specific example where signalling molecules can be used is to grazing pressure on other organisms to produce metabolites of interest, such as carotenoids (produced industrially by species such as *Dunaliella salina*), which can be useful as antioxidants, for example. They are presumed to have positive health effects such as the prevention of cardiovascular disorders, certain ageing-related diseases, such as Alzheimer's, or certain types of cancer. They can be also used as additives in food, in animals feed (aquaculture), cosmetics, among others.

But the overall challenge for one of the biggest fields in marine research is to use algae as biofuel. Therefore, molecules that can help increase the rate of biomass combined with proper nutrient and light management could be key to accelerate this branch of research.

In summary and in a broader context, this experiment has shown how there are non-physical communication signals between different marine species, which on a large scale could be useful for bioprospecting. The fact that signalling molecules such as copepodamides produce a response in other organisms could be used as a tool to initiate the synthesis of secondary metabolites, changes in photosynthesis wage, or changes in behaviour and metabolic rates in other species. Which could help solving current problems, such as global warming, or useful for industrial purposes (pharmaceutical, cosmetic, food, agricultural, etc.). That is why understanding the established interactions between the extensive biodiversity living in the oceans could be key to a better and sustainable future.

8. Self-evaluation

During the internship I learned a lot of things that I had not learned before in my studies. I think I will be able to apply them many times in the future and they have given me an idea of how the real world of research works. Not only have I learned many techniques that I could use in the lab, but also concepts that I think are more important, such as working in a team, learning how the dynamics in a lab work (helping each other, sharing information, having meetings to catch up, etc.), being able to think critically and evaluate results, learning to work with statistics, among others.

I really enjoyed the dynamics in the lab, every week we would meet about 6 or 7 people of all kinds: professors, PhD students, master students from other countries, and me. All these people were from the same group or had projects in common. Every Wednesday they would meet to discuss what they were working on and express doubts or problems in their research to discuss as a group and find a solution. They were very productive sessions and I really enjoyed seeing how everyone expressed their ideas, it seems impossible how it can help to see things from another point of view.

I think I have made very positive use of the tasks assigned to me. Regarding the extraction of copepodamides, I had to learn a lot of techniques and use equipment that was difficult to programme. At least, after being supervised by my tutor, I went through the different steps of the extraction several more times independently, so I managed to master all the techniques and equipment used. I also elaborated my own protocols and gave my opinion on how to improve the procedures.

Regarding the *Skeletonema marinoi* part of the project, I think this is the part where I have seen the most reflection of what the real research is like. As I mentioned before, in the group meetings I was guided and helped to design the experiment. After running the experiment for the first time and seeing no results, I talked to several members of the Signals of the Sea group and by modifying different parameters and running it again, we succeeded. I found the whole procedure very exciting. I felt very independent doing it on my own and actually getting results that could be useful for the whole group. I think all of this taken together, I think, is "the spirit" of research that many of us studying science careers are looking for.

I learned how to think a little bit more critically, tried to look at things form other perspectives and also how to organise events in time in order to succeed with the combination of writing a

project, working at a lab, keeping up with a lab notebook and managing to get enough time to perform all the experiments.

In conclusion, I would like to say that the opportunity to do an internship in a real laboratory has helped me to have a vision of how I could see myself in the future. I enjoyed being part of a group that works as a team where you help each other. I do not rule out going into something similar in the next few years.

9. References

Abida, H., Ruchaud, S., Rios, L., Humeau, A., Probert, I., De Vargas, C., Bach, S., & Bowler, C. (2013). Bioprospecting marine plankton. Marine drugs, 11(11), 4594–4611. https://doi.org/10.3390/md11114594

Bergkvist, J., Thor, P., Jakobsen, H. H., Wängberg, S. Å., & Selander, E. (2012). Grazer-induced chain length plasticity reduces grazing risk in a marine diatom. Limnology and Oceanography, 57(1), 318-324.

Bowler, C., Karl, D.M., Colwell, R.R. (2009). Microbial oceanography in a sea of opportunity. Nature. 459, 180–184.

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. pp 26-60. In Smith W.L. and Chanley M.H (Eds.) Culture of Marine Invertebrate Animals. Plenum Press, New York, USA.

Guillard, R.R.L. and Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. 8: 229-239.

Hessen, D. O., & Van Donk, E. (1993). Morphological changes in Scenedesmus induced by substances released from Daphnia. Archiv fur Hydrobiologie, 127, 129-129.

Ianora, A., Bentley, M. G., Caldwell, G. S., Casotti, R., Cembella, A. D., Engström-Öst, J., Halsband, C., Sonnenschein, E., Legrand, C., Llewellyn, C. A., Paldavičienë, A., Pilkaityte, R., Pohnert, G., Razinkovas, A., Romano, G., Tillmann, U., & Vaiciute, D. (2011). The relevance of marine chemical ecology to plankton and ecosystem function: an emerging field. Marine drugs, 9(9), 1625–1648. https://doi.org/10.3390/md9091625

Johansson, O. N., Töpel, M., Pinder, M. I., Kourtchenko, O., Blomberg, A., Godhe, A., & Clarke, A. K. (2019). *Skeletonema marinoi* as a new genetic model for marine chain-forming diatoms. Scientific reports, 9(1), 5391.

Löfgren, L., Ståhlman, M., Forsberg, G. B., Saarinen, S., Nilsson, R., & Hansson, G. I. (2012). The BUME method: A novel automated chloroform-free 96-well total lipid extraction method for blood plasma [S]. Journal of lipid research, 53(8), 1690-1700.

Nelson, D., B.S. Education, M.S. Aeronautics, Master Teacher K-12. Save the Plankton, Breathe Freely. Reviewed for National Geographic. https://www.nationalgeographic.org/activity/save-the-plankton-breathe-freely/

Rengefors, K., S. Gustafsson & A. Stahl-Delbanco (2004) Factors regulating the recruitment of cyanobacterial and eukaryotic phytoplankton from littoral and profundal sediments. Aquatic Microbial Ecology, 36, 213-226.

Rodrigues, C. J., & de Carvalho, C. C. (2022). Marine Bioprospecting, Biocatalysis and Process Development. Microorganisms, 10(10), 1965.

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Sardet, C., Rafael D. Rosengarten, Theodore Ohman, Rosengarten, Theodore, Sardet, Dana, and Ohman, Mark. Plankton : Wonders of the Drifting World. 2015. Print.

Selander, E., Berglund, E. C., Engström, P., Berggren, F., Eklund, J., Harðardóttir, S., ... & Andersson, M. X. (2019). Copepods drive large-scale trait-mediated effects in marine plankton. Science Advances, 5(2), eaat5096.

Selander, E., Kubanek, J., Hamberg, M., Andersson, M. X., Cervin, G., & Pavia, H. (2015). Predator lipids induce paralytic shellfish toxins in bloom-forming algae. Proceedings of the National Academy of Sciences, 112(20), 6395-6400.

Shafiei, M., Ziaee, A. A., & Amoozegar, M. A. (2011). Purification and characterization of an organic-solvent-tolerant halophilic α-amylase from the moderately halophilic Nesterenkonia sp. strain F. Journal of Industrial Microbiology and Biotechnology, 38(2), 275-281.

Stenow, R., Olofsson, M., Robertson, E. K., Kourtchenko, O., Whitehouse, M. J., Ploug, H., & Godhe, A. (2020). Resting stages of *Skeletonema marinoi* assimilate nitrogen from the ambient environment under dark, anoxic conditions. Journal of phycology, 56(3), 699-708.

Wimpenny, Ronald Stenning. The Plankton of the Sea. London: Faber and Faber, 1966. Print.

10. Annexes

Next, graphics of all the data are shown, including data from day 4, which wasn't relevant regarding the number cells, chlorophyll relative fluorescence and number of cells per chain.

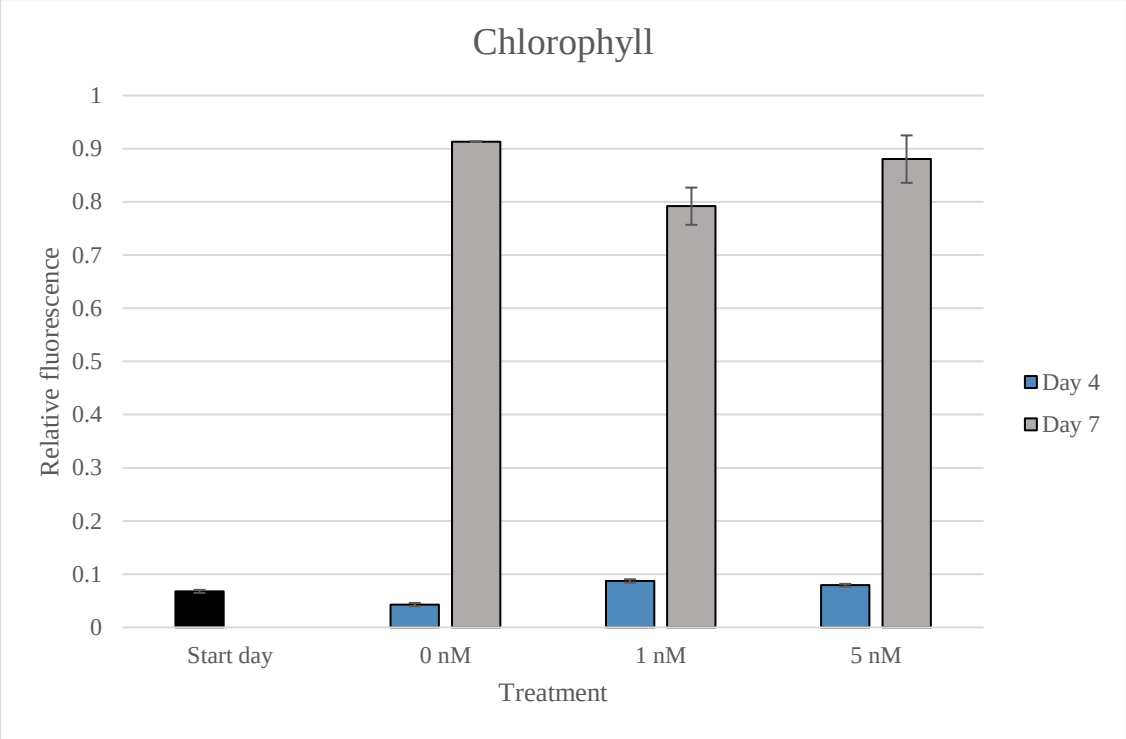


***Figure 10**. Graph showing chlorophyll relative florescence in RFU (Relative Fluorescence Unites) of the vials corresponding to the start day, the control group (0 nM), and the copepodamide treatments at concentrations of 1 nM and 5 nM. Cell counts are shown in different times: Start day, day 4 and day 7. Black lines are error bars. * Grup control on day 7 has only one value.*

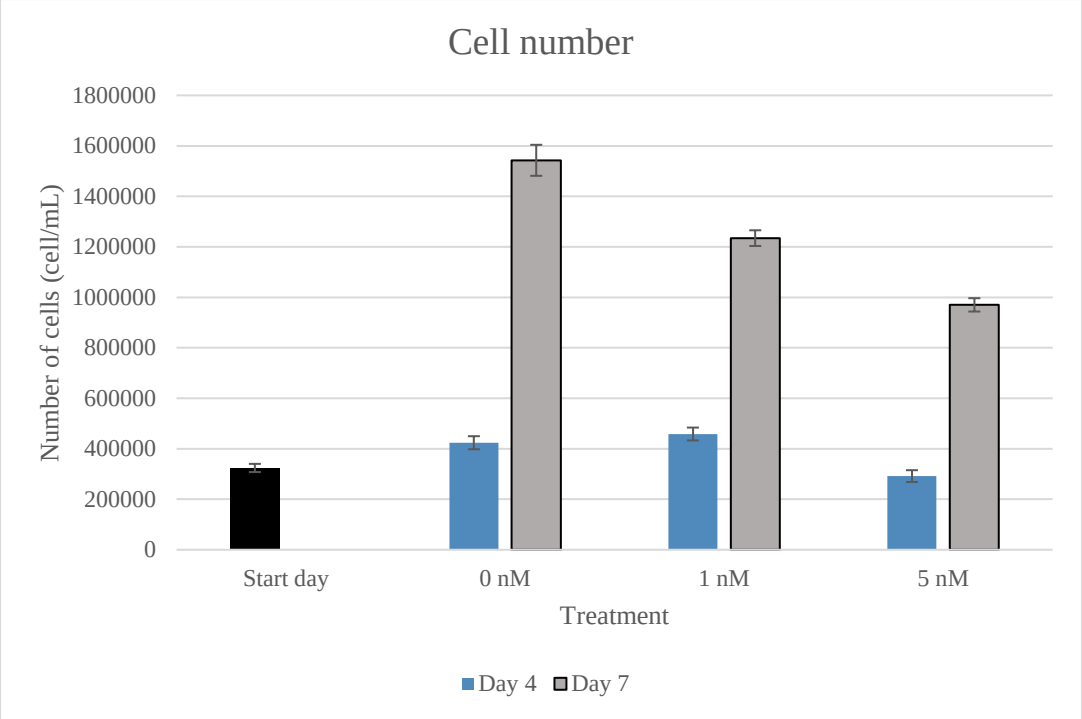


Figure 11. *Graph showing the number of cells per mL of the vials corresponding to the start day, the control group (0 nM), and the copepodamide treatments at concentrations of 1 nM and 5 nM. Cell counts are shown in different times: Start day, day 4 and day 7. Black lines are error bars. * Grup control on day 7 has only one value.*

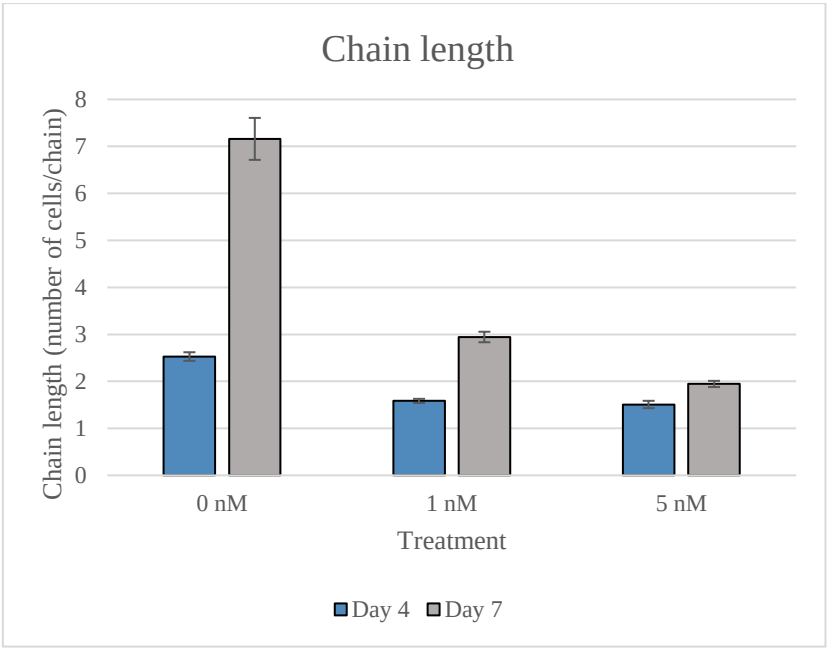


Figure 12. *Graph showing the average number of cells per chain of the vials corresponding to the control group (0 nM), and the copepodamide treatments at concentrations of 1 nM and 5 nM. Data from day 7. Black lines are error bars. * Grup control on day 7 has only one value.*

f/2 Medium

(Guillard and Ryther 1962) and (Guillard 1975)

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half. To prepare, begin with 950 mL of filtered natural seawater and add the following components. The trace element and vitamin solutions are provided below. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
NaH_2PO_4 H_2O	5 g/L dH_2O	1 mL	3.62×10^{-5} M
Na_2SiO_3 $9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

f/2 Trace Metal Solution

To prepare, begin with 950 mL of dH_2O, add the components and bring final volume to 1 liter with dH_2O. Autoclave. Note that the original medium (Guillard and Ryther 1962) used ferric sequestrene; we have substituted $Na_2EDTA \cdot 2H_2O$ and $FeCl_3 \cdot 6\ H_2O$.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3$ $6H_2O$	---	3.15 g	1.17×10^{-5} M
Na_2EDTA $2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93×10^{-8} M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
$ZnSO_4$ $7H_2O$	22.0 g/L dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2$ $6H_2O$	10.0 g/L dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10×10^{-7} M

f/2 Vitamin Solution

First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add 1 mL of the primary stocks and bring final volume to 1 liter with dH_2O. Filter sterilize. Store in refrigerator or freezer.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
Thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
Biotin (vit. H)	1.0 g/L dH_2O	1 mL	2.05×10^{-9} M
Cyanocobalamin (vit. B12)	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M