

Allelopathic metabolites of Rhodophyta: Influence on microalgal growth and cellular function

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2 List of abbreviations

Abbreviation	Explanation
ATP	Adenosine triphosphate
BPNS	Belgian part of the North Sea
CCM	Carbon-concentrating mechanisms
DHA	Docosaheptaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EPA	Eicosapentaenoic acid
FW	Fresh weight (wet weight)
G3P	glyceraldehyde-3-phosphate
GC-MS	Gas chromatography-mass spectrometry
GtC	Gigaton carbon
HAB	Harmful algae bloom
LC-MS	Liquid chromatography-mass spectrometry
MAA	Mycosporine-like amino acid
NADPH	Nicotinamide adenine dinucleotide phosphate
PBP	Phycobiliprotein
PC	Particle count
PE	Phycoerythrin
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEP-CK	Phosphoenolpyruvate carboxykinase
PES	Polyethersulfone
PET	Polyethyleentereftalaat
PGA	3-phosphoglycerate
PS	Photosystem
RuBP	Ribulose-1,5-bisphosphate
S	Solvent control
SP	Sulfated polysaccharides
UV	Ultra violet
VHOC	Volatile halogenated organic compounds

3 Abstract

This thesis investigates the allelopathic effects of three red macroalgae—*Acrochaetium secundatum*, *Gracilaria gracilis*, and *Palmaria palmata*—on the growth of two microalgae species: *Tisochrysis lutea* (Haptophyta) and *Phaeodactylum tricornutum* (Bacillariophyta). For each macroalga, three types of extracts were prepared: a polar extract, a nonpolar extract from the algal surface, and a nonpolar extract from the entire algal tissue. These extracts were tested in concentration series to assess their effects on microalgal growth.

In addition to growth inhibition assays, the chemical composition of the extracts was analyzed to check for potential allelochemicals. A full spectrophotometric profile was obtained for the polar extracts, with quantification of phycoerythrin and phycocyanin. The fatty acid composition of the nonpolar extracts was determined using GC-MS. The most abundant fatty acid identified, eicosapentaenoic acid (EPA), was subsequently tested separately on the microalgae to evaluate its specific effect. Finally, the potential role of oxidative stress was examined using a DPPH radical scavenging assay.

The results show that the extracts cause species- and extract-specific growth inhibition. *T. lutea* was more sensitive than *P. tricornutum*. The strongest inhibitory effect was observed with the total nonpolar extract of *P. palmata*. However, EPA alone did not fully account for the observed inhibition. Polar extracts, particularly those of *A. secundatum*, also showed significant inhibition, potentially due to the phycobiliproteins. However, these pigments may also exert indirect effects through light absorption.

This study aims to identify active components and elucidate their mechanisms of action, with implications for both ecological understanding and biotechnological applications.

4 Nederlandstalig abstract

Deze thesis onderzoekt de allelopathische effecten van drie roodwieren—*Acrochaetium secundatum*, *Gracilaria gracilis* en *Palmaria palmata*—op de groei van twee microalgen: *Tisochrysis lutea* (Haptophyta) en *Phaeodactylum tricornutum* (Bacillariophyta). Van elk wier werden drie types extracten bereid: een polair extract, een apolair extract van het zeewieroppervlak, en een apolair extract van het gehele wier. De extracten werden getest in concentratiereeksen om hun effect op microalgengroei te evalueren.

Naast groeiremming werd ook de chemische samenstelling van de extracten geanalyseerd om potentiële allelochemicaliën te karakteriseren. Er werd een volledig spectrofotometrisch profiel genomen van het polaire extract, waarbij fycoërythrine en fycocyanine werden gekwantificeerd. Verder werd de vetzuursamenstelling in de apolaire extracten bepaald met GC-MS. Het meest dominante vetzuur in de extracten, eicosapentaeenzuur (EPA), werd afzonderlijk getest op de microalgen om het specifieke effect ervan te evalueren. Tot slot werd ook de mogelijke rol van oxidatieve stress onderzocht via een DPPH-radicaalassay.

De resultaten tonen dat de roodwierextracten soort- en extract-specifieke groeiremming veroorzaken. *T. lutea* bleek over het algemeen gevoeliger dan *P. tricornutum*. De sterkste inhibitie werd waargenomen bij het apolaire totaalextract van *P. palmata*. Uit de extra test met EPA, bleek EPA op zich de remming niet volledig te verklaren. Polaire extracten, vooral van *A. secundatum*, vertoonden ook duidelijke remming, vermoedelijk door fycobiliproteïnen zoals fycoërythrine en fycocyanine. Deze pigmenten kunnen echter ook indirecte effecten veroorzaken via lichtabsorptie.

Deze studie benadrukt het belang van gecombineerde chemische en biologische analyses om actieve componenten en hun werking te identificeren, met oog op zowel ecologische als biotechnologische toepassingen.

5 Introduction

5.1 Algae

“The lungs of the planet”, a well-deserved nickname for algae as they (together with cyanobacteria) are responsible for up to 80% of the earth’s oxygen (Valavanidis, 2023). This is due to the fact that, like plants, algae rely on photosynthesis to convert carbon dioxide (CO₂) into biomass and thereby release oxygen (O₂). By using carbon dioxide to produce biomass, photosynthetic organisms are significant contributors to carbon sequestration, playing a crucial role in climate change mitigation. This is no different for algae; as primary producers, algae absorb CO₂ from seawater and the atmosphere, helping to regulate global carbon cycles (Wang et al., 2024). The ocean absorbs 41% of anthropogenic carbon emissions, with algae playing a vast role in this process by capturing and storing carbon through photosynthesis, ultimately contributing to environmental sustainability (Gu & Yin, 2022; Wang et al., 2024).

For comparison, the terrestrial carbon sink stores over 2000 GtC (Gigaton carbon) in soil and vegetation, sequestering around 3 GtC per year, while the oceanic sink holds 38,000 GtC, absorbing 2 GtC annually through the solubility and biological pumps (Farrelly et al., 2013). While terrestrial sinks provide short- to medium-term storage, they are vulnerable to deforestation and fires, whereas the ocean stores carbon for thousands of years, it plays a more stable and long-term role in climate change mitigation (Farrelly et al., 2013).

What separates the algae from terrestrial plants, in addition to living under water, is that algae do not have a vascular system, transporting nutrients through their body based on diffusion (L. Pereira, 2021). This distinction is made to avoid confusion with seagrasses, a relatively small group of angiosperms (flowering plants) containing 72 species divided over four families (Unsworth & Cullen-Unsworth, 2017). They are the only flowering plants that can live fully submerged in saline conditions, with most of them having a fully oceanic life cycle (Unsworth & Cullen-Unsworth, 2017).

Furthermore, algae are a very diverse group of organisms of different evolutionary origin and can be either single- or multicellular. Typically, a distinction is made between microscopic algae and the so called seaweeds, often referred to as micro- and macroalgae, respectively (L. Pereira, 2021). Although the term microalgae usually describes eukaryotic unicellular organisms, sometimes the prokaryotic cyanobacteria (or blue-green algae) are also included (Torres-Tiji et al., 2020). This is because both are included under the term phytoplankton, and together they are responsible for the majority of all primary production (*i.e.*, photosynthesis) in the ocean (Daniel C. O. Thornton, 2012). The link between cyanobacteria and algae even extends back to the evolutionary transition from prokaryotic to eukaryotic life forms (Martin et al., 2015).

Cyanobacteria are the first known organism to ever perform photosynthesis and are the reason that ca. 2500 million years ago the atmosphere slowly began containing oxygen, making life as we know possible (Drews, 2011). Their importance does not stop there: according to the endosymbiosis theory, chloroplasts—the cell organelles where photosynthesis occurs—find their origin in cyanobacteria (Archibald, 2015). This theory entails that two completely different types of cells merge on an intracellular level. It was first described by Russian scientist Constantin Mereschkowsky in 1905, but only became publicly accepted after Lynn Margulis' take on the theory in 1970 (Archibald, 2015; Gray, 2012; Martin et al., 2015).

It entails that the chloroplasts originate from a photosynthetic cyanobacterium that is taken up by a non-photosynthetic cell. The same theory applies for the mitochondria, which can be found in almost all eukaryotic organisms and play a very important role in the energy household of the cell, but in this case the origin is an aerobic bacterium. It is very difficult to pinpoint exactly how this endosymbiosis occurred and what types of cells were involved, therefore multiple theories about the host cell and the process of uptake exist. Most theories are based on phagocytosis and agree that cyanobacteria and aerobic bacteria are the origin of chloroplast and mitochondria, respectively (Martin et al., 2015).

The most prominent evidence supporting the theory is that both mitochondria and chloroplasts contain their own DNA, which closely resembles bacterial genomes, including its circular structure (Gray, 2012; Martin et al., 2015). Furthermore, the presence of double membranes in mitochondria and chloroplasts supports the hypothesis that these organelles originated through an endosymbiotic event (Berry, 2003). It is proposed that the host cell began to engulf the prokaryotic cell—initially to increase the surface area of interaction—which eventually led to its complete internalization, retaining both its original membrane and a membrane derived from the host (Berry, 2003). In some algae, the chloroplasts have three or four membranes, which indicates the repeated uptake of photosynthetic cells by non-photosynthetic cells (Archibald, 2015; Berry, 2003). This is often referred to as secondary endosymbiosis.

Although photosynthesis finds its evolutionarily origin in prokaryotic cyanobacteria, the earliest known eukaryotic organism capable of photosynthesis is *Bangiomorpha pubescens*, a fossilized red alga dating to over 800 million years ago (Drews, 2011; Gibson et al., 2018).

Similar to the debate surrounding the origin of chloroplasts, the classification of algae has been a complex and evolving issue, subject to numerous revisions over time. Historically, most algae were grouped under Protista, along with other organisms that did not clearly fit into the plant or animal kingdoms, such as amoebae and, briefly, even bacteria (Heimann & Huerlimann, 2015). Despite numerous efforts to develop more coherent classification systems, none have proven universally satisfactory. As a result, multiple classification schemes continue to be used concurrently (Heimann & Huerlimann, 2015).

5.2 Macroalgae

Seaweeds have been used for over 2500 years, albeit very differently depending on the region. For example, in Ireland, the species Irish moss (*Chondrus crispus*) was seen as food for the poor, while in China seaweed was traditionally seen as a delicacy (Ferdouse et al., 2018). Currently, the most commonly known uses of seaweed are nori, kombu and wakame, all of which used in the food industry (Ferdouse et al., 2018). In 2015, global seaweed production reached 30.4 million metric tonnes, more than doubling in 10 years compared to the 14.7 million tonnes produced in 2005 (Ferdouse et al., 2018). Most is used as a food source, however the cosmetic and industrial seaweed industry are also growing markets. Seaweed extracts already contribute 40% of the global hydrocolloid market (Ferdouse et al., 2018). The continued expansion of the seaweed market is therefore expected, if not certain (Ferdouse et al., 2018). This makes seaweeds an interesting field of study.

5.2.1 Morphology

Although algae do not have root systems and vascular systems like plants, there is still differentiation between tissues. Their body is called the thallus and can take many shapes and sizes, from thin filaments to wide sheets (L. Pereira & Gaspar, 2020). The thalli are typically made up of three morphologically distinct parts, visualized in Figure 1; the blade, the stipe and the holdfast. The blade makes up the largest part of the thallus. The stipe is a stem-like structure found at the base. For fixation purposes, some algae (mainly the larger species like kelp), have a specialized structure called the holdfast, with which they can hold on to the substrate (L. Pereira, 2021; Pereira & Gaspar, 2020). Another specialized structure found in some seaweeds are gas-filled compartments called pneumatocysts, floats, bladder or gas vesicles (Dromgoole, 1981). These pneumatocysts provide the algae with positive buoyancy (J. A. Raven, 1996). Algae that do not have these specialized structures instead accumulate gas (O_2) in their tissue to maintain positive buoyancy (Dromgoole, 1982).

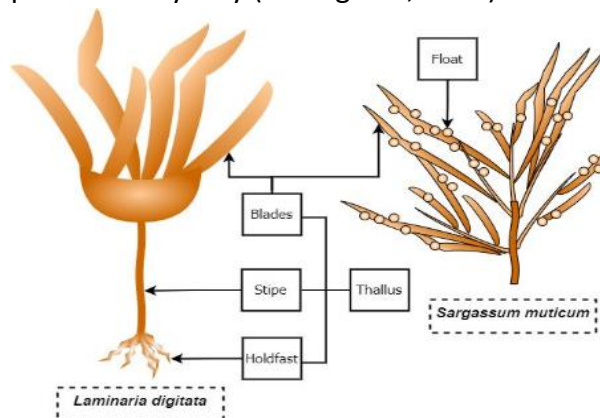


Figure 1. Morphology of macroalgae (Y. Zhao et al., 2022).

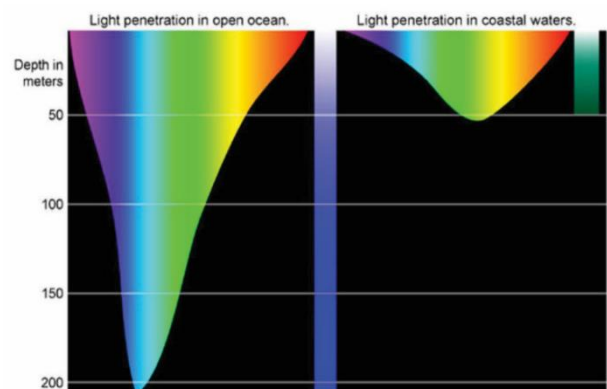


Figure 2. Light penetration in function of depth: comparison between the open ocean (left) and coastal waters (right) (NOAA, n.d.).

5.2.2 Ecology

Almost all macroalgae need marine conditions, light and a fixation point (L. Pereira, 2021). For this reason, they are predominantly found in coastal areas and more precisely in rocky shores, as they can better attach to solid substrates compared to sand (L. Pereira, 2021). Seaweeds typically grow at depths shallower than 100 meters; however, occurrences have been reported at depths of up to 295 meters in the Bahamas (Suzanne Fredericq, 2003).

Seaweeds are typically classified into three groups—red, green, and brown algae—corresponding to the divisions *Rhodophyta*, *Chlorophyta*, and *Phaeophyceae*, respectively (Cikoš et al., 2022). However, these groups do not make up monophyletic clades (Bykova et al., 2020). In biological cladistics, monophyly refers to a taxonomic grouping that constitutes a clade—meaning it includes its most recent common ancestor (or ancestral population) and all of its descendants, while excluding any unrelated organisms. Since red, green, and brown algae do not share a single common ancestor exclusive to them, they are polyphyletic, meaning they originate from multiple evolutionary lineages rather than a single one. Hence, these groups simply refer to the color of the algae, which is a result of the dominant pigments they contain and use to capture light in order to do photosynthesis (L. Pereira, 2021).

The light quality algae can use is dependent on their pigments, which is why they are of such importance. While the specific role and function of these pigments in photosynthesis will be addressed in Section 5.2.3, their broader ecological significance will be discussed here. In short, they are the driving factor behind seaweed distribution in the ocean. In coastal regions, where most algae live, the wavelengths corresponding with blue and red light are absorbed by the water column in the first meters, while green to yellow light penetrates furthest as seen in Figure 2 (NOAA, n.d.). Chlorophylls primarily capture light in the blue (400 – 500 nm) and red (600 – 700 nm) regions of the spectrum, while carotenoids mainly absorb blue light (Nobel, 2020). As these are the main photosynthetic pigments in most algae, it is beneficial for algae to try to get as close to the surface as possible, since those wavelengths do not penetrate deep into the water column (Guidi et al., 2017; NOAA, n.d.; Nobel, 2020). However, phycobiliproteins (also known as phycobilins), the accessory light-harvesting pigments present in red algae, absorb light in the 520 – 670 nm range, corresponding to the green to yellow region of the visible spectrum. (Nobel, 2020). Consequently, red algae can usually also be found at greater depths of the coastline (L. Pereira, 2021).

5.2.3 Photosynthetic pigments

5.2.3.1 Photosynthesis

Photosynthetic pigments are arranged within light-harvesting complexes, where a central chlorophyll *a* molecule—commonly referred to as the trap chlorophyll or reaction center—is surrounded by accessory pigments (Nobel, 2020). Contrary to this reaction center, there are many different types of accessory pigments (depending on the seaweed species). The main groups are discussed in more detail in Section 5.2.3.2 and an overview of the common accessory pigments per seaweed species is provided in Table 1. The function of these pigments is to capture photons (light particles or light energy) and pass this on to the reaction center, where the chemical reactions of photosynthesis start (Nobel, 2020). This process is visually represented in Figure 3. In more detail, when an accessory pigment captures a photon of light that matches the energy difference between the pigment ground state and excited state, the electron absorbs the photon's energy and transitions to a higher energy level (Nobel, 2020). This energy can be transferred between pigment molecules via resonance energy transfer, ultimately reaching the reaction center. Here, the specialized trap chlorophyll will act as an oxidant, splitting water molecules (H_2O) to provide a source of electrons (Nobel, 2020). This reaction produces oxygen as a byproduct and makes it of such great importance to all aerobic organisms including humans. The electrons derived from splitting water molecules will be passed on through a series of molecules, which is hence called the electron transfer chain. Ultimately, this leads to the generation of ATP (adenosine triphosphate) and NADPH (Nicotinamide adenine dinucleotide phosphate), which serve as energy carriers for the next stage of photosynthesis: the fixation of inorganic carbon (Gómez & Huovinen, 2012; Nobel, 2020).

For the fixation of inorganic carbon, algae face a unique challenge compared to terrestrial plants: The limited availability of CO_2 in seawater. Dissolved CO_2 constitutes only about 0.5% of the total inorganic carbon pool (Gómez & Huovinen, 2012). Atmospheric CO_2 dissolves in the water and

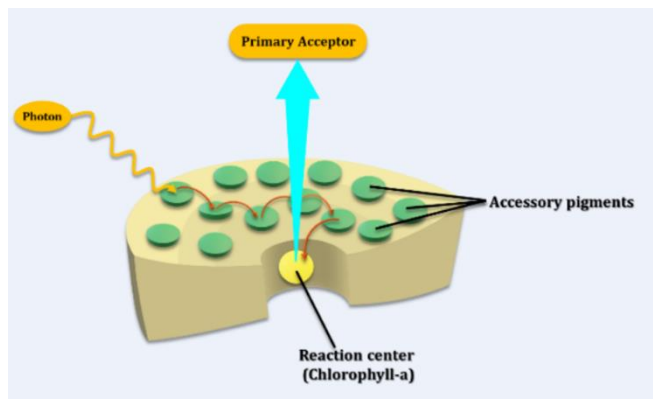


Figure 3. Light harvesting complex (ALLEN Career Institute, n.d.).

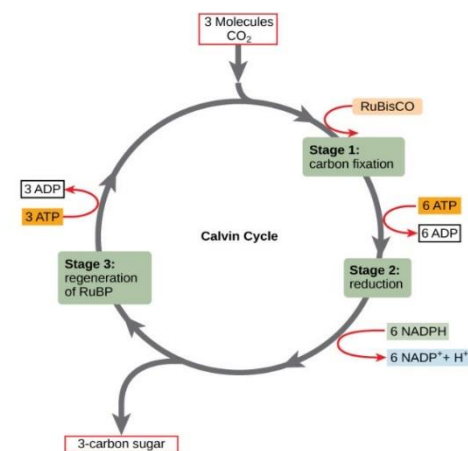


Figure 4. Schematic illustration of the Calvin-Benson cycle (Molnar et al., 2015).

forms an equilibrium with bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), creating a buffer system. In marine conditions this equilibrium strongly leans towards HCO_3^- (Gómez & Huovinen, 2012). Furthermore, simple diffusion of CO_2 inside the algal cells is limited by the diffusion boundary layer around the algal surface, which can result in carbon limitation (Maberly, 1990). For comparison, the diffusion rate at the thalli surface of seaweed is about 10,000 times lower than that on the surface of terrestrial plants (Maberly, 1990). Due to the strong preference for CO_2 of RuBisCO, the key enzyme in carbon fixation, seaweeds must find a way to maintain a sufficient supply (Gómez & Huovinen, 2012; J. Raven, 1997).

Therefore, many seaweeds have evolved carbon-concentrating mechanisms (CCMs) that actively transport CO_2 and HCO_3^- into the cell (Gómez & Huovinen, 2012; J. Raven, 1997). These mechanisms primarily involve the active uptake of HCO_3^- via specialized anion exchange transporters, facilitating its accumulation within the cytoplasm (Giordano et al., 2005; J. Raven, 1997). Once internalized, HCO_3^- is converted to CO_2 through the action of carbonic anhydrase (CA), an enzyme that catalyzes the reversible interconversion between inorganic carbon species (Giordano et al., 2005). This process ensures a sustained CO_2 supply near RuBisCO. Some seaweed species also localize CA within the periplasmic space or chloroplast stroma, thereby establishing localized microenvironments with elevated CO_2 concentrations (Giordano et al., 2005). By overcoming diffusion barriers and minimizing photorespiration, these CCMs allow seaweeds to maintain efficient carbon fixation despite the low availability of dissolved CO_2 in seawater (Giordano et al., 2005; J. Raven, 1997).

Once CO_2 is available, it is incorporated into organic molecules through the Calvin–Benson cycle, the primary biochemical pathway for photosynthetic carbon fixation, visualized in Figure 4. This cycle begins with RuBisCO fixing CO_2 onto ribulose-1,5-bisphosphate (RuBP), forming two molecules of 3-phosphoglycerate (PGA), referred to as the carbon fixation stage (Gómez & Huovinen, 2012; Molnar et al., 2015; J. Raven, 1997). The second stage is the reduction, where these molecules are converted into glyceraldehyde-3-phosphate (G3P) using ATP and NADPH from the light-dependent reactions discussed above. Lastly, in the regeneration stage, some of the G3P molecules are used to make RuBP, this also requires ATP and allows the cycle to continue (Gómez & Huovinen, 2012; Molnar et al., 2015; J. Raven, 1997). However, RuBisCO is not entirely specific to CO_2 ; it can also bind O_2 , leading to a competing process called photorespiration (Gómez & Huovinen, 2012; J. Raven, 1997). In photorespiration, O_2 is consumed and CO_2 is produced, virtually reverting the entire carbon uptake process discussed above. RuBP is oxidized to glycolate and PGA in the C2 oxidative photosynthetic carbon cycle (Gómez & Huovinen, 2012). Since photorespiration is energetically costly, especially when CO_2 levels are low, seaweeds have developed strategies to minimize its impact (J. Raven, 1997).

To enhance carbon efficiency, seaweeds utilize additional light-independent carbon fixation pathways, sometimes called β -carboxylation (Gómez & Huovinen, 2012). Some sources refer to this as light-independent fixation and classify the Calvin cycle as part of light-dependent fixation. Unlike the Calvin cycle, which is indirectly dependent on light through ATP and NADPH, these alternative pathways do not rely on products of light-dependent reactions and function even when photosynthesis is not actively occurring. This mechanism is somewhat analogous to C_4 and CAM plant metabolism and provides an alternative means of fixing carbon, especially in conditions where CO_2 availability is restricted (Gómez & Huovinen, 2012; J. Raven, 1997).

The β -carboxylation occurs through the irreversible carboxylation of phosphoenolpyruvate (PEP) by the enzyme phosphoenolpyruvate carboxylase (PEPC), which fixes CO_2 in the presence of bicarbonate (HCO_3^-) to form oxaloacetate and inorganic phosphate (Pi) (O'Leary et al., 2011). This reaction is facilitated by magnesium ions (Mg^{2+}) as cofactors and is independent of light-driven energy carriers (O'Leary et al., 2011). Oxaloacetate can then be converted into malate or aspartate, which serve as intermediates in various metabolic pathways, including carbon storage, nitrogen assimilation, and respiration (O'Leary et al., 2011). Another enzyme, phosphoenolpyruvate carboxykinase (PEP-CK), helps in recycling carbon by converting oxaloacetate back into PEP while preserving energy. These processes contribute to enhanced carbon-use efficiency by providing an alternative carbon fixation pathway that minimizes losses due to photorespiration (Gómez & Huovinen, 2012; O'Leary et al., 2011). This can contribute up to 48% of total carbon fixation in some species, especially in actively growing parts of the thallus (Gómez & Huovinen, 2012). This combination of light-dependent and light-independent processes allows seaweeds to efficiently capture and convert inorganic carbon into organic compounds, ensuring their survival in dynamic marine environments (Gómez & Huovinen, 2012).

5.2.3.2 Accessory pigments

Photosynthetic pigments are specialized molecules that absorb light energy and play a crucial role in the process of photosynthesis, when a pigment absorbs light and passes its excitation towards the reaction center, it is called an accessory pigment (Nobel, 2020). These pigments belong to different molecular classes, primarily including tetrapyrroles (such as chlorophylls and phycobilins) and isoprenoids (such as carotenoids). Their ability to absorb light is determined by their conjugated double-bond systems, which allow electrons to become excited upon photon absorption (Nobel, 2020).

Cyclic tetrapyrroles are arguably the most important molecular structure regarding the energy household of all living organisms. It makes up both the heme (part of the haemoglobins) in our blood, as well as the most abundant light capturing pigments; chlorophylls (Van Dooren et al., 2012). Cyclic tetrapyrroles are a class of organic molecules composed of four pyrrole rings arranged in a cyclic, planar structure (Figure 5). They frequently contain a metal ion at their core,

which is central to their role in biological redox reactions. Chlorophylls, for instance, are tetrapyrrole-based pigments with a magnesium ion at their center, enabling them to efficiently absorb light energy (Van Dooren et al., 2012). The evolution of tetrapyrroles played a critical role in the emergence of photosynthetic and respiratory pathways in early life forms. The biosynthesis of tetrapyrroles, such as chlorophyll and heme, is a highly conserved process across different domains of life. Chlorophylls first evolved in bacteria and were later transferred to eukaryotes via endosymbiosis (explained in Section 5.1), leading to the development of plastids and the emergence of oxygenic photosynthesis (Van Dooren et al., 2012).

Phycobilins, found in cyanobacteria and red algae, are open-chain tetrapyrrole pigments (Nobel, 2020). Unlike chlorophylls, they lack a central metal ion but still maintain an extended conjugated system, allowing them to absorb wavelengths of light that penetrate deeper into aquatic environments, particularly in the green and yellow parts of the spectrum (Nobel, 2020). These pigments are covalently bound to apoproteins via thioether bonds, forming functional phycobiliproteins, which facilitate their role in energy transfer (Glazer, 1989; Ji et al., 2023; Nobel, 2020). The phycobiliproteins are typically arranged in assemblies of 300 to 800, called phycobilisomes. This is illustrated in Figure 6. Phycobilisomes function as highly efficient light-harvesting complexes, transferring absorbed energy towards chlorophyll in the photosystem (Glazer, 1989; Ji et al., 2023). These complexes are composed of phycobiliproteins and linker proteins, which stabilize the structure and influence energy transfer efficiency (Gantt, 1975; Ji et al., 2023).

Phycobilins are derived from the degradation of tetrapyrrole macrocycles such as heme or chlorophylls, with biliverdin acting as an intermediate in their biosynthesis (Taniguchi & Lindsey,

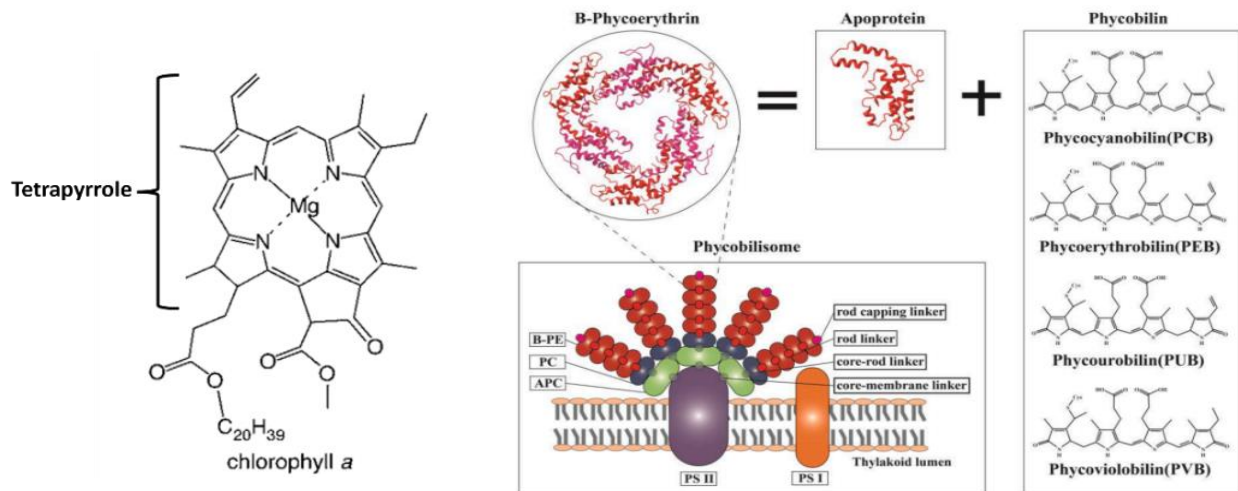


Figure 5. Tetrapyrrole structure of chlorophyll *a*. (Van Dooren et al., 2012)

Figure 6. Visualization of how phycobilins combine with apoproteins to make up phycobiliproteins, that in turn are structured in phycobilisomes. B-PE, B-phycoerythrin; PC, phycocyanin; APC, allophycocyanin; PS I, photosystem I; PS II, photosystem II (Ji et al., 2023).

2023). Biliverdin is further reduced to form phycoerythrobilin, which can subsequently be converted into phycocyanobilin (Taniguchi & Lindsey, 2023). Phycoerythrobilin is the bilin of phycoerythrin, while phycocyanobilin is that of phycocyanin. These two phycobiliproteins are the most abundant in red algae (Cikoš et al., 2022; Nobel, 2020). The absorption spectra of phycobilins vary depending on their conjugation system and interaction with protein scaffolds, enabling them to function as highly efficient light-harvesting pigments (Taniguchi & Lindsey, 2023).

Carotenoids, such as the ones depicted in Figure 7, are isoprenoid molecules composed of long hydrocarbon chains with alternating single and double bonds (Nobel, 2020). This conjugated system allows them to absorb blue and green light efficiently, playing a crucial role in photosynthetic light harvesting (Nobel, 2020). Many more carotenoids than those illustrated in Figure 7 exist within algae, enabling them to optimize light absorption under different aquatic environments. As accessory pigments, carotenoids transfer excitation energy to the trap chlorophyll in both photosystem I (PSI) and photosystem II (PSII) (Nobel, 2020; Takaichi, 2011). Additionally, carotenoids serve as essential photoprotective agents by dissipating excess light energy in response to high-light conditions (Takaichi, 2011).

Furthermore, some algae possess water-soluble carotenoid-protein complexes, such as peridinin-chlorophyll-protein and orange carotenoid protein, which enhance light capture and photoprotection (Takaichi, 2011). The structural diversity and functional specialization of carotenoids highlight their critical role in algal photosynthesis and adaptation to varying light environments (Nobel, 2020; Takaichi, 2011).

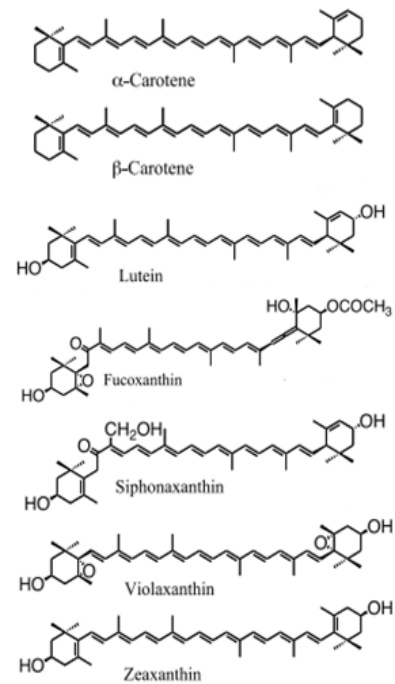


Figure 7. Structures of some common carotenoids in seaweeds (Takaichi, 2011).

The specific pigment composition of seaweeds, which varies among algal groups, determines their characteristic coloration; a summary of the pigments associated with each group is provided in Table 1. Fucoxanthin dominates in the Phaeophyceae and is the pigment to which brown algae owe their color (Wehr, 2015). In Rhodophyta phycobiliproteins, such as R-phycoerythrin, are dominant and result in a red color (Bryant, 1982). Green algae thank their color to the dominance of chlorophylls (Cikoš et al., 2022). Many environmental stressors are known to have an effect on the composition of these pigments in algae, the most famous ones being temperature (Cikoš et al., 2022), light (Korbee et al., 2005) and nutrient availability (Figuerola et al., 2010). This is one of

many reasons why anthropogenic activities leading to climate change, pollution and eutrophication can vastly affect algae.

Table 1. The main pigments from three different macroalgal groups (brown (Phaeophyceae), green (Chlorophyta) and red (Rhodophyta) (Cikoš et al., 2022).

Macroalgae group	Main pigments	References
Phaeophyceae (brown algae)	fucoxanthin	(G. S. Matos et al., 2021)
	β -carotene	(Aryee et al., 2018)
	chlorophyll <i>a</i>	(Biris-Dorhoi et al., 2020)
	chlorophyll <i>c</i>	
Rhodophyta (red algae)	phycobiliproteins	
	α -carotene	
	β -carotene	
	zeaxanthin	
	lutein	
	chlorophyll <i>a</i>	
	chlorophyll <i>d</i>	
	β -carotene	
Chlorophyta (green algae)	xanthophylls (lutein, violaxanthin, antheraxanthin, zeaxanthin, neoxanthin)	
	siphonaxanthin	
	siphonein	
	chlorophyll <i>a</i>	
	chlorophyll <i>b</i>	

The pigments are not only important in photosynthesis, but have other properties such as anti-oxidant, anti-inflammatory, anticancer, antidiabetic and anti-obesity properties when exposed to human cells (Cornish & Garbary, 2010; Priyanka et al., 2022), which have potential for, or are currently being utilized in, pharmaceutical and/or cosmeceutical applications. In addition to their pigment-related properties, algae produce a wide range of other bioactive compounds with diverse and potentially beneficial biological activities. (Okechukwu et al., 2024). This makes them well suited for bioprospecting.

5.3 Bioprospecting

Bioprospecting, also known as biodiscovery, involves the exploration of organisms and their natural compounds to identify bioactive substances with potential applications in product development. (Oyemitan, 2017; Tamayo, 2014). Natural products form the basis of approximately half of all clinically approved drugs, a proportion that increases further when synthetic drugs inspired by natural compounds are included. This is illustrated in greater detail in Figure 8.

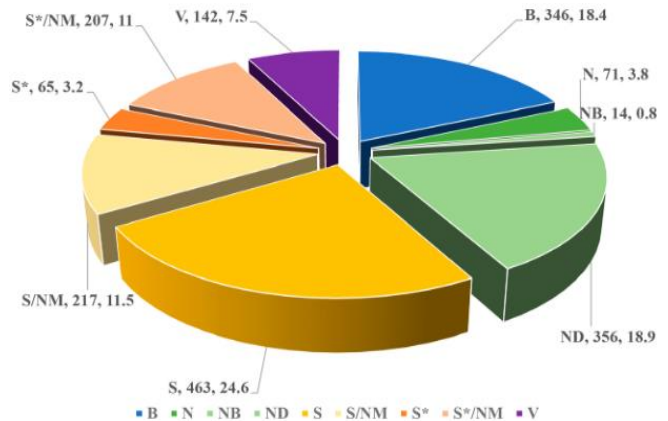


Figure 8. Overview of the sources of approved drugs between 1981 and 2019. B: biological macromolecule, N: unaltered natural product, NB: botanical drug, ND: Natural product derivative, V: Vaccine, S: Synthetic drug, S*: Synthetic drug (NP, pharmacophore) and NM: Mimic of natural product (Newman & Cragg, 2020).

The marine environment represents a vast and largely untapped reservoir of bioactive compounds, with marine organisms offering significant potential for biodiscovery (Okechukwu et al., 2024; Sigwart et al., 2021). Evolution in the ocean predates terrestrial life, resulting in extensive evolutionary diversity and, consequently, a broader spectrum of bioactive compounds (Sigwart et al., 2021). The extent of marine biodiversity is highlighted by the fact that 90% of marine species remain undescribed as of 2020, further enhancing the likelihood of discovering novel compounds (Sigwart et al., 2021). Furthermore, the success rate of drug development from marine-derived natural products is estimated to be two to four times higher than that from terrestrial sources, reinforcing the promise of marine biodiscovery (Sigwart et al., 2021).

5.3.1 Bioprospecting in red algae

A wide array of marine organisms serve as a source of bioactive compounds, including bacteria, fungi, algae, seagrasses, invertebrates, and vertebrates (Okechukwu et al., 2024). In this thesis, we focus on red algae (Rhodophyta) for several reasons. Firstly, they are well represented in the Belgian part of the North Sea (BPNS) (VLIZ, n.d.). Secondly, Rhodophyta constitute the most diverse group of seaweeds in terms of species richness (Bykova et al., 2020). They are the oldest evolutionary lineage of autotrophic (photosynthesis performing) eukaryotes and have a very complex life history, further highlighting their diversity (Gibson et al., 2018; Richard, 1980). Moreover, they are the most prolific producers of bioactive compounds; Leal et al. (2013) reported that 53% of all macroalgal bioactive compounds discovered between 1965 and 2012 originated from Rhodophyta. Regarding allelopathy (defined in Section 5.4), red algae account for the largest proportion of the 123 taxa in which this phenomenon has been described, representing 37% of all cases (Budzałek et al., 2021). In comparison, green and brown algae contribute 33% and 30%, respectively. However, most research in this field has primarily focused

on green algae (Budzałek et al., 2021). While some bioactive compounds derived from red algae are widely exploited, many remain underused (Carpena et al., 2023). Hence, red algae are a rich source of diverse bioactive compounds, which can be broadly categorized into carbohydrates, proteins, lipids, secondary metabolites, and essential micronutrients such as vitamins and minerals (Carpena et al., 2023). In the following sections, each of these groups will be explored in more detail, highlighting their chemical characteristics, biological activities, and potential applications in biodiscovery.

5.3.2 Carbohydrates

Carbohydrates represent a diverse group of compounds unified by a common elemental composition: carbon, hydrogen, and oxygen (Soult, 2025). Their structure typically features carbon chains with one or more aldehyde or ketone groups and multiple hydroxyl groups. Most are composed of repeating monosaccharide units, with glucose being the most prominent example. As energy-rich molecules, carbohydrates serve as primary energy sources in the human diet and function in energy storage across plants and animals. While once thought to serve only this metabolic role, research has since highlighted their broader functions in cellular communication and molecular recognition, influencing processes such as fertilization, immune response, and cell growth (Jéquier, 1994; Osborn, 2003; Rioux & Turgeon, 2015).

Rioux & Turgeon (2015) offer a comprehensive overview of carbohydrates in seaweeds, focusing on polysaccharides. In red algae, these are primarily structural components of the cell wall, with some also functioning in energy storage and osmoregulation. Notably, the sulfated polysaccharides agar and carrageenan—widely used in the food industry—are key red algal cell wall constituents (Rioux & Turgeon, 2015). In addition to these, red algae synthesize several storage carbohydrates, including the low molecular weight compounds floridoside, isofloridoside, and digeneaside, and the high molecular weight floridean starch (Rioux & Turgeon, 2015). The following sections examine the main groups of carbohydrates in more detail.

5.3.2.1 Sulfated polysaccharides

A well-known group of carbohydrates derived from red algae are the sulfated polysaccharides (SP). However, SPs are not exclusive to Rhodophyta; well-known examples from other algal groups include ulvans from Chlorophyta and fucans from Phaeophyceae (Patel, 2012). The most described SPs from red algae are the sulfated galactans carrageenan and agar (Patel, 2012; Rioux & Turgeon, 2015). Galactans are polysaccharides made from the monosaccharide galactan. In the case of agar and carrageenan, some of the functional groups on the sugar-chain are substituted by sulfate groups, hence the name sulfated galactans. Typically, agar has many anhydrogalactose rings, giving it its gelatine-like properties, whereas carrageenan contains significantly more sulfate groups and less ring structures (Rioux & Turgeon, 2015). However, their exact composition (the

amount of sulfate groups and anhydrogalactose rings) and therefore their physiochemical properties vary among different seaweed species (Rioux & Turgeon, 2015).

The antioxidant activity of SPs derived from Rhodophyta has been demonstrated through various assays, including DPPH and hydroxyl radical scavenging tests (L. S. Costa et al., 2010; Souza et al., 2020; Yang et al., 2011). Beyond their antioxidant properties, SPs exhibit diverse bioactivities. For instance, Luo et al. (2015) reported antitumor effects of λ -carrageenan in mice, while Pratoomthai et al. (2018) observed a reduction in melanin synthesis in melanoma cells treated with Rhodophyta-derived SP *in vitro*. The immunostimulatory potential of these polysaccharides has also been highlighted: Khongthong et al. (2021) demonstrated that SP from *Gracilaria fisheri* enhanced macrophage activation, phagocytic activity, and the expression of pro-inflammatory mediators *in vitro*. Similarly, Wongprasert et al. (2014) confirmed *in vivo* immunostimulatory effects in *Penaeus monodon* (shrimp), noting increased hemocyte counts and elevated antioxidant enzyme activities. Additionally, shrimp fed with SPs from *G. fisheri* exhibited significantly reduced mortality and viral load following infection with white spot syndrome virus (WSSV). Other antiviral activities have also been documented. For example, M. Kim et al. (2012) showed that SPs from *Gyrodinium impudicum* could inhibit influenza A virus entry *in vitro*, while Bouhlal et al. (2011) demonstrated that SPs from Rhodophyta species effectively interfered with the adsorption and replication of HSV-1 and HIV-1. Additional pharmacological activities of Rhodophyta-derived SPs, such as antibacterial, antithrombotic, and gastrointestinal-stimulating effects, have been reported, although these are generally less potent or less extensively studied compared to conventional pharmaceuticals (Arokiarajan et al., 2022; Patel, 2012).

Furthermore, some non-pharmaceutical applications are discussed in literature. For example, agar extracted from *Gracilaria* and *Gelidium* species is widely utilized as a culture medium in microbiology (*e.g.*, in Petri dishes) and as the food-grade hydrocolloid known as E406 (Alvarenga et al., 2011; Martin Glicksman, 1983). Additionally, D. Kim & Kang (2020) demonstrated the antifouling potential of λ -carrageenan when applied as a surface coating, significantly inhibiting diatom attachment on nylon surfaces—an effect comparable to commercial antifouling agents, though challenges remain for large-scale application.

5.3.2.2 Floridoside, isofloridoside and digeneaside

The main function of these low molecular weight carbohydrates is the storage of photosynthetic reserves, as well as osmoregulation of the cell (Rioux & Turgeon, 2015). Furthermore, they also play a role in regulating the production of cell wall polysaccharides and floridean starch (Goulard, Diouris, et al., 2001; Goulard, Le Corre, et al., 2001). Although (iso)floridoside and digeneaside function as energy storage compounds in red algae, they are not currently utilized in food products or industrial applications. (Martinez-Garcia & van der Maarel, 2016; Rioux & Turgeon,

2015). However, they have potential qualities suitable for therapeutic implementation and industrial applications.

P. Torres et al. (2019) reviewed the bioactivity potential of Rhodophyta, particularly members of the order *Gracilariales*, and identified three key medical applications of floridoside and digeneaside: anti-inflammatory, osteogenic, and antioxidant activities. M. J. Kim et al. (2013) reported floridoside's anti-inflammatory effects in microglial cells. Ryu et al. (2015) demonstrated its promotion of osteogenic differentiation in mouse mesenchymal stem cells through upregulation of bone-related markers and transcription factors. Antioxidant activity was reported by Y. X. Li et al. (2010), who found that floridoside and D-isofloridoside enhanced cellular defenses and inhibited enzymes involved in inflammation and tissue degradation.

Floridoside also shows industrial potential, similar to that of sulfated polysaccharides, but less explored due to more difficult and less optimized extraction processes (Hellio et al., 2004; Martinez-Garcia & van der Maarel, 2016). Hellio et al. (2004) demonstrated antifouling activity against *Balanus amphitrite* larvae, with low toxicity to crustacean nauplii. Furthermore, *Acanthophora spicifera* extracts containing floridoside and digeneaside, significantly improving *Ostrea edulis* larval survival following *Vibrio coralliilyticus* exposure, highlighting their potential for vibriosis control in aquaculture (Guillén et al., 2024).

5.3.2.3 Floridean starch

Floridean starch is the main photosynthetic reserve storage carbohydrate in red algae and is found in small, usually spherical, granules (up to 3.4 μm) in the cytoplasm, in contrast to starch in plants and Chlorophyta, where it is stored in the chloroplasts (Rioux & Turgeon, 2015; Yu et al., 2002). Its average chain length (18 glucose units) is short compared to higher plants, has a similar structure to amylopectin with a high branching frequency, and completely lacks any amylose structures (Yu et al., 2002).

In their chapter, Rioux & Turgeon (2015) briefly discuss floridean starch, but found no pharmaceutical applications. However, a potential application in food is indicated by Yu et al. (2002), who isolated floridean starch isolated from three red algae species.

Its physicochemical properties—low gelatinization and pasting temperatures, low viscosity, high clarity, and resistance to retrogradation—are attributed to its short chains, lack of amylose and low lipid and protein content. These characteristics show potential for food applications such as frozen food products where clarity and thermal stability are essential and instant noodles (Yu et al., 2002).

5.3.3 Proteins

Similar to carbohydrates, protein are a large group of bioactive molecules produced by algae and varies a lot between red, green and brown algae (Belghit et al., 2017; Carpena et al., 2023).

Rhodophyta have considerably higher amounts of protein compared to green and especially brown seaweeds (Øverland et al., 2019). This high protein content is gaining them some attention as potential sustainable feed ingredients for livestock and aquaculture. However, inconsistent results from past studies are linked to variability in macroalgal composition, digestibility issues, and differing experimental setups (Øverland et al., 2019). Many species lack sufficient digestible protein for use as primary protein sources, though protein levels can be improved through extraction. Hence, biorefinery approaches and further studies are needed to optimize macroalgae use in animal feed (Øverland et al., 2019). Besides a food source, protein from Rhodophyta can hold potential other applications. The most prominent protein group within red seaweeds are the phycobiliproteins as they can make up to 50% of total protein content (Cian et al., 2015). Therefore, they will be discussed in more detail below.

5.3.3.1 Phycobiliproteins

The literature review on phycobiliproteins (PBP) by Ji et al. (2023) divides the implementation of PBP into four major uses: food, cosmetic, fluorescence and medical. The most tested PBP is C-phycocyanin produced by cyanobacterium *Arthrospira Platensis* (Ji et al., 2023). However, in this thesis the emphasis will be on Rhodophyta derived PBP.

In the food industry, PBPs, especially phycoerythrin (PE), are gaining interest as natural alternatives to synthetic colorants due to their antioxidant activity and health benefits (Campos Assumpção de Amarante et al., 2020). PE was successfully applied in pancakes and yogurts, retaining color for up to two months despite some heat sensitivity (Campos Assumpção de Amarante et al., 2020; T. Pereira et al., 2020). While not yet approved as a food colorant in Europe, PE is already used in several Asian countries, where it is extracted from *Porphyra* sp.

In cosmetic applications, Rhodophyta derived PBPs are used as colorants in cosmetic powders, eye-shadow, eyeliner and lipstick (Arad et al., 1996; Balboa et al., 2015; Borowitzka, 2013). Companies are also commercializing creams with (allegedly) antioxidant and anti-aging properties based on or at least containing phycoerythrin and phycocyanin (Ji et al., 2023). Further ongoing developments aim to expand their use in natural and sustainable cosmetics (Ji et al., 2023).

Furthermore, PBPs are widely used as fluorescent markers in biological research, including fluorescence-activated cell sorting (FACS) and flow cytometry (Ji et al., 2023; Taniguchi & Lindsey, 2023). PE-conjugated antibodies have been used to evaluate T cell activation and lymphocyte phenotypes in ovarian cancer patients, and to label reticulocytes and leukocytes for micronuclei detection (Aldona et al., 2012; Zeng et al., 2020). Additionally, phycocyanin has been applied as a selective fluorescent sensor for mercury detection in water samples (Girmatsion et al., 2021).

Lastly, PBP show potential for clinical use due to their antioxidant, anti-inflammatory, antitumor, and antiviral properties (Ji et al., 2023; W. Li et al., 2019; Okechukwu et al., 2024). Antioxidant activity has been demonstrated in various PBP sources, including *Pyropia yezoensis*, where PE-derived peptides reduced oxidative stress (Bermejo et al., 2008; Ji et al., 2023; Kavitha et al., 2019;

E. Y. Kim et al., 2018). Anti-inflammatory effects were reported for C-phycocyanin from cyanobacteria and for PE-containing water extracts from *P. palmata* (Ji et al., 2023; Lee et al., 2017). Antitumor activity was observed in R-PE-rich extracts from *Portieria hornemannii*, showing hepatoprotective and anticancer effects *in vitro* (liver cells) and *in vivo* (rats) (Senthilkumar et al., 2018). Antiviral activity has been studied mainly in cyanobacterial PBP and in *Griffithsia sp.*, which produces the lectin griffithsin with demonstrated activity against HIV and coronaviruses, including *in vivo* protection against SARS-CoV by strongly binding its receptor binding domain (Okechukwu et al., 2024; O'Keefe et al., 2010; Petit et al., 2021; Sigwart et al., 2021).

5.3.4 Lipids

The total lipid content in red seaweeds is relatively low; Rodrigues et al. (2015) reports 0.6%, 0.9%, 2.2% for *Gracilariaria gracilis*, *Osmundea pinnatifida* and *Grateloupia turuturu*, respectively. El Maghraby & Fakhry (2015) reported 1.56% total lipid concentration in the red algae *Jania rubens*. Both of these studies tested species of all three seaweed groups and indicated red seaweeds to contain the least amount of total lipids (El Maghraby & Fakhry, 2015; Rodrigues et al., 2015). However, compared to Chlorophyta species the red seaweeds have a very high PUFA/SFA ratio, where SFA are saturated fatty acids (H. Pereira et al., 2012). These fatty acids are especially important for the human diet, as the body has a limited capacity to synthesize them (H. Pereira et al., 2012). This makes them eligible for low-caloric food applications (Cotas, Leandro, Pacheco, et al., 2020).

Despite their relatively low total lipid content, Rhodophyta are rich in polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are highly valued for nutritional and therapeutic applications (Cotas, Leandro, Pacheco, et al., 2020; H. Pereira et al., 2012). EPA and DHA are associated with numerous health benefits, including reduced plasma triglyceride levels, lower blood pressure, improved endothelial function, increased high-density lipoprotein over low-density lipoprotein ratio of cholesterol, and anti-atherosclerotic and antithrombotic effects (Robinson & Stone, 2006). These fatty acids also contribute to anti-inflammatory responses, particularly in conditions such as rheumatoid arthritis, and offer protection against cardiovascular disease (Calder, 2010, 2021). Crude lipid extracts from Rhodophyta species such as *Palmaria palmata*, *Porphyra dioica*, and *Grateloupia turuturu* demonstrate potent COX-2 inhibition and antioxidant activity, confirming their anti-inflammatory and protective potential (E. da Costa et al., 2021; Lopes et al., 2020). Additionally, EPA and DHA are linked to neurological and developmental benefits, including fetal support, reduced allergy incidence, and mitigation of early cognitive decline (Calder, 2021; Swanson et al., 2012).

5.3.5 Essential micronutrients

Both vitamins and (inorganic) minerals are considered essential micronutrients, with vitamins extracted from red seaweeds already being used for increasing the nutritional value of foods (Carpena et al., 2023). Škrovánková (2011) suggests that marine macroalgae serve as an excellent source of both water-soluble (B1, B2, B12, C) and fat-soluble (β -carotene, vitamin E) vitamins, with several of these compounds contributing not only to essential physiological functions but also to broader health benefits. For instance, vitamin C is associated with reduced blood pressure and cancer risk, β -carotene supports cardiovascular health, and vitamin E has been linked to lower mortality from cerebrovascular diseases and certain cancers (Škrovánková, 2011). Despite their low lipid content, seaweeds contain appreciable levels of fat-soluble vitamins, particularly vitamin E (Škrovánková, 2011). The high vitamin B12 content in certain red algae, particularly *Porphyra* species (commonly used to produce nori), is especially significant for individuals adhering to strict vegetarian or vegan diets, which are typically deficient in this essential nutrient (Škrovánková, 2011). Functional foods and nutraceuticals incorporating seaweed-derived vitamins offer promising strategies to address deficiencies, especially among populations with increased nutritional demands or impaired vitamin absorption (Škrovánková, 2011). These applications highlight the potential of seaweed as a sustainable and health-promoting ingredient in the food industry.

Moreover, edible seaweeds contain significant concentrations of essential minerals such as sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu), phosphorus (P), iodine (I) and selenium (Se), often exceeding the levels found in terrestrial plants (Rupérez, 2002; Wen et al., 2006). This elevated mineral content is attributed to their marine environment (Rupérez, 2002).

5.3.6 Secondary metabolites

In addition to primary metabolites, red algae also produce a diverse array of secondary metabolites that play crucial roles in their defense and adaptation to environmental stressors (Carpena et al., 2023). These compounds, synthesized through secondary metabolic pathways, are often linked to various biological activities (Carpena et al., 2023). Notably, phenolic compounds (mainly polyphenols), terpenoids (mainly terpenes), and alkaloids have been frequently identified as the dominant classes of secondary metabolites in red seaweeds (Aziz et al., 2021; Carpena et al., 2023).

5.3.6.1 Terpenoids

Terpenes represent the largest class of secondary metabolites, composed of repeating five-carbon isoprene (C_5H_8) units assembled in numerous structural configurations (Paduch et al., 2007; Perveen, 2018). They are mainly known in plants where they take on the role as attractant and/or repellent (Paduch et al., 2007). Terpenes can be both polar and nonpolar depending on the functional groups (Paduch et al., 2007).

While terpenes are simple hydrocarbons, terpenoids are their modified counterparts, distinguished by functional groups and structural changes such as oxidized or repositioned methyl groups (Perveen, 2018). Based on the number of carbon atoms, terpenoids are categorized into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, and triterpenes. Many of these compounds exhibit significant biological activity and are widely applied in pharmaceuticals (Perveen, 2018).

Terpenoids are a diverse group of compounds with applications in medicine, nutrition, cosmetics, and the production of hormones and vitamins (Paduch et al., 2007; Perveen, 2018). They include anticancer agents like taxol, antimalarial compounds, and are widely used for their aromatic properties in flavorings and fragrances (Perveen, 2018).

The recent review paper on terpenes derived from red algae by Peixoto et al. (2025) indicates that the broad biotechnological relevance of terpenes has attracted global scientific interest due to their wide range of potential applications. Terpenes derived from red algae have demonstrated notable anti-inflammatory, antioxidant, anticancer and tumor (melanoma, lung, breast, prostate and colon), and acetylcholinesterase inhibitory (possible Alzheimer therapy) properties (Campos et al., 2012; Del Prado-Audelo et al., 2021; Farooqi et al., 2012; Knott et al., 2019; Machado et al., 2015; Minhas et al., 2024; Peixoto et al., 2025; Shapumba et al., 2017).

Among red algal genera, *Laurencia* stands out for its rich repertoire of bioactive terpenes (Peixoto et al., 2025). The sesquiterpene elatol, one of the most prominent secondary metabolites found in *Laurencia* species, has demonstrated strong antitumor properties and notable biocidal activity, suggesting potential applications in antifouling and antimicrobial formulations (Campos et al., 2012; da Gama et al., 2008). Additionally, elatol has shown promise as an antiproliferative agent in the treatment of leishmaniasis (a parasite transmitted disease), with its effect specific towards protozoa (Dos Santos et al., 2010).

5.3.6.2 Phenols

Phenolic compounds, known for their inherently strong antioxidant properties, play a protective role in plants and have gained increasing attention for their health benefits in the human diet (Rodríguez et al., 2023). While traditionally sourced from terrestrial plants, marine organisms such as seaweeds, seagrasses, and sponges are also rich in polyphenols (Rodríguez et al., 2023). The concentration and bioavailability of these compounds depend on various factors, including source, environmental stability, and extraction methods (Rodríguez et al., 2023).

Macroalgae contain many different types of phenols, all of which with a wide variety of bioactivity and applications. In their review paper, Rodríguez et al. (2023) combined all these in a schematic overview which is adopted in this thesis and presented in Figure 9. The main groups in red algae are flavonoids, bromophenols and phenolic terpenoids (Rodríguez et al., 2023).

Polyphenols have garnered significant interest for their wide-ranging applications across various fields (Verma et al., 2024). In environmental and industrial contexts, they are utilized for the treatment of wastewater and industrial effluents, as well as in anticorrosion strategies (Verma et al., 2024). In material science, polyphenols are employed in the production of dyes, packaging materials, and textiles, and are increasingly integrated into chemosensing, biosensing, and general sensor technologies (Verma et al., 2024). Their incorporation into hydrogels and nanocomplexes has also expanded their use in advanced material formulations (Verma et al., 2024). In the context of public health, polyphenols have been explored for their potential in controlling COVID-19, as well as for their broader roles in dietary supplementation, health management, and disease prevention (Verma et al., 2024). Furthermore, they exhibit strong antimicrobial, disinfectant, and pest control properties, alongside well-established antioxidant activity (Verma et al., 2024). In the food and pharmaceutical industries, polyphenols serve as natural food additives and are instrumental in the development of biomaterials and therapeutic agents (Verma et al., 2024).

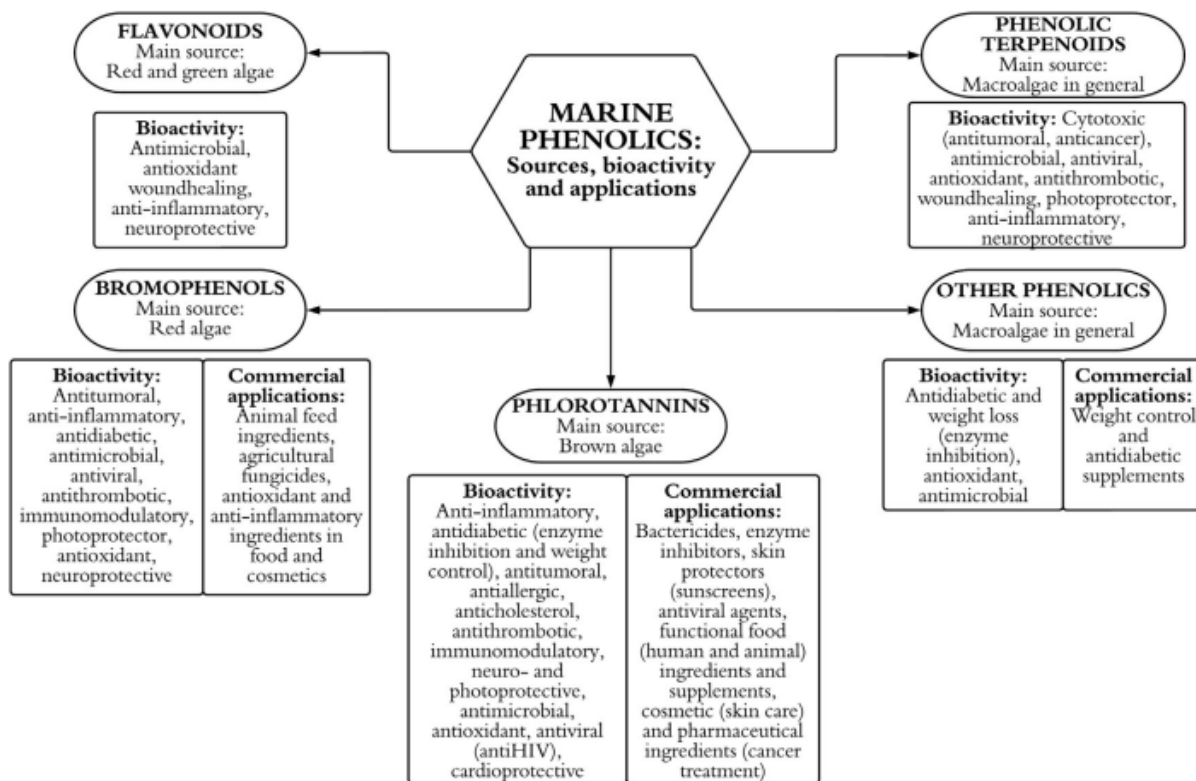


Figure 9. Overview of phenolic compound groups, their bioactivity and commercial application (Rodríguez et al., 2023).

5.3.6.3 Alkaloids

Alkaloids are a diverse and widespread class of natural products found across nearly all marine and terrestrial life (Fattorusso & Tagliatela-Scafati, 2008). Their complex structures and biological activities make them valuable for research and as natural compounds in drug development (Fattorusso & Tagliatela-Scafati, 2008). They do not have a specific structural component that defines them and their classification remains ambiguous, but they are usually defined by their ecological function as chemical defense against predation (Fattorusso & Tagliatela-Scafati, 2008).

Research on seaweed-derived alkaloids remains limited compared to terpenes and phenols (Carpena et al., 2023). Given the well-established anti-inflammatory properties of plant-derived alkaloids and the promising findings from the limited studies on seaweed-derived alkaloids, these marine compounds may represent valuable bioactive candidates for bioprospecting initiatives (Souza et al., 2020). Red algae from the *Gracilaria* genus have been identified as particularly rich sources of these nitrogen-containing compounds (de Almeida et al., 2011). In the paper of de Almeida et al. (2011), they are also discussed regarding their antioxidant activity.

5.4 Allelopathy

Allelopathy was first defined by Hans Molisch in 1937 as “the biochemical interaction, both stimulatory and inhibitory, between primary producers and/or microorganisms” (Gross, 2003; Semmouri et al., 2024). However, it is often more narrowly defined as the beneficial or harmful effects of one plant or microorganism on another, mediated through the release of chemical compounds into the environment (Dakshini & Inderjit, 1994; Macías et al., 2008). These compounds are known as allelochemicals. The use of the term “plant” rather than “primary producer” in traditional definitions has contributed to the misconception that allelopathy is primarily a terrestrial phenomenon. In reality, allelopathy also plays a significant role in aquatic ecosystems, particularly in shaping the distribution, abundance, and community dynamics of microalgae (Macías et al., 2008). Moreover, both laboratory and field tests indicate that allelopathy can be found in every aquatic habitat and that all primary producers can produce allelochemicals (Gross, 2003).

Another note is that these definitions include positive effects as allelopathy. For example, some algae have been found to be auto stimulatory: they release chemicals that promote growth of other individuals of their species, giving successors a competitive advantage over other algae (Dakshini & Inderjit, 1994). However, most studies about allelopathy cover the negative effects, such as growth inhibition (Gross, 2003). Therefore, allelopathy is mainly regarded to provide an advantage for the organism excreting the allelochemical by deterring competing organisms (Semmouri et al., 2024). In this thesis, the main focus will be on this growth inhibitory effect of allelochemicals.

5.4.1 Ecological importance of allelopathy

Allelopathy is well studied in plants, so its ecological relevance is easiest explained using terrestrial examples. It has important ecological implications as it plays a key role in shaping where plant species grow, how they interact, and how biodiversity is maintained (Hierro & Callaway, 2021). Allelopathic effects influence plant distributions across ecosystems such as grasslands, shrublands, and forests worldwide. Although it can be difficult to prove allelopathy as the sole cause of species suppression, a growing body of evidence shows that many plants produce chemicals that affect the growth of neighboring species (Hierro & Callaway, 2021). Allelopathy also contributes to species coexistence by promoting complex competition patterns, such as intransitive competition, where no single species dominates (Hierro & Callaway, 2021). These interactions can be shaped by coevolution, especially when native and invasive species interact, leading to the development of new chemical defenses or sensitivities. In some cases, this can even give invasive species an advantage, helping them spread more easily (Hierro & Callaway, 2021).

Recent research suggests that plants may also recognize and respond to the chemical signals of others, adding a new layer of complexity to plant interactions. Overall, allelopathy is more than just a chemical curiosity—it is a powerful ecological force that influences plant communities, species diversity, and the dynamics of ecosystems (Hierro & Callaway, 2021). Interactions from macroalgae with their environment are well described, but these are often the interactions with animals, and therefore more anti-predatory than true allelopathic interactions. These include the influence of excreted chemicals on marine herbivore communities' presence and abundance in shallow waters, where multiple studies show effects on Annelida, Echinodermata and Arthropoda (Budzałek et al., 2021). There have also been reports of negative effects from seaweed species on Mollusca, which are important in aquaculture (Budzałek et al., 2021).

Field studies with Rhodophyta indicate negative effects on certain coral species (Budzałek et al., 2021). For example, Tanner (1995) reports negative effects of red seaweeds (from the *Peyssonnelia* genus) on scleractinian corals. Negative effects occur due to contact, leading to retraction of the coral tissues. Additionally, necrosis has been observed in coral tissue due to contact with the Rhodophyta, which is explained by allelochemicals on their thalli (Tanner, 1995). A more recent study did similar tests on the coral specie *Porites rus* with four different algae: the green alga *Rhiphilia pencilloide* and the red algae *Phacelocarpus neurymenioides*, *Callophycus densus*, and *Plocamium pacificum* (Andras et al., 2012). All four of these algae resulted in significant bleaching of the coral, while the controls, *i.e.* plastic algae used to simulate the contact effect, showed no bleaching. Neurymenolide A was identified as the responsible allelochemical and was found on the thalli surface, but not in the water column (Andras et al., 2012). Given that coral bleaching is a result of the partial to total loss of its symbiotic microalgae, it is likely that the allelochemical resulting in bleaching either stresses the coral into expelling the microalgae or effects the microalgae directly (Douglas, 2003).

5.4.2 Allelopathy of macroalgae on microalgae

Following the ban on TBT-based antifouling paints, research has focused on finding non-toxic alternatives (Hellio et al., 2002). One promising approach is the use of natural antifouling compounds produced by marine organisms such as seaweeds, which themselves combat fouling by epibionts, organisms living attached to the macroalgal thallus (Hellio et al., 2002). Another reason to study allelopathy against microalgae is the need to control harmful algae blooms (HABs) such as the infamous red tides. This principle is similar to that used in agriculture, where allelochemicals have long been used for their weed controlling qualities (Cheema, Farooq, & Khaliq, 2013; Cheema, Farooq, & Wahid, 2013; Cheng & Cheng, 2015; Khamare et al., 2022).

Multiple papers report the inhibitory effects of macroalgae on microalgae (Hellio et al., 2002; Kakisawa et al., 1988; Macías et al., 2008; Thomas et al., 2023). For instance, Hellio et al. (2002) tested aqueous, ethanolic, and dichloromethane extracts from thirty macroalgae collected along the Northeast Atlantic coast against representative macro- and microalgal fouling species. Several extracts showed antifouling activity comparable to traditional biocides (e.g., TBTO and CuSO_4), but were non-toxic to invertebrate larvae such as oysters and sea urchins (Hellio et al., 2002). These findings highlight the potential of certain algal metabolites as effective, environmentally friendly antifoulants. Ideal candidates for commercial development should be active against a broad range of fouling organisms, function via chemical signaling rather than toxicity, and be suitable for large-scale production. At least twelve of the tested extracts met these criteria (Hellio et al., 2002). Another example is *Fucus vesiculosus* (Phaeophyceae) that was found to produce extracellular phenolic substances negatively impacting multiple microalgae of different taxonomic origin (J. McLachlan & J.S. Craigie, 1963). Moreover, *Ulva pertusa* successfully inhibited growth of 8 different microalgae at ecological relevant concentrations (Nan et al., 2004). Furthermore, An et al. (2008) tested 5 macroalgae for their potential to control the so-called red tides, a HAB created by the microalgae *Skeletonema costatum*. They reported *Porphyra* sp. to have the lowest EC_{50} , therefore highlighting the potential of Rhodophyta for allelopathy research. Ye & Zhang (2013) indicated a correlation between dried *Gracilaria tenuistipitata* and reduced photosynthesis resulting in growth inhibition of the microalga *Prorocentrum micans*. Moreover, allelopathic inhibition of the dinoflagellate (microalga) *Scrippsiella trochoidea* by *Gracilaria lemaneiformis* extracts was also observed by Ye et al. (2014).

These examples show the potential of macroalgae (especially Rhodophyta) to inhibit the growth of unwanted microalgae species. Despite the research that's already been conducted, many review papers on the subject report allelochemicals from macroalgae as an understudied field (Gross, 2003; Macías et al., 2008; Carpena et al., 2023; Semmouri et al., 2024).

5.4.3 Allelopathy in the North sea

Most research on macroalgal allelopathy is done in Australia, Mexico and the United States, as well as in parts of Asia and South America where aquaculture is an important sector (Budzałek et al., 2021). This suggests that Europe is already lacking behind on the subject. A Google Scholar search conducted in November 2024 using the terms “Rhodophyta,” “allelopathy,” and “North Sea” (searched anywhere in the article, with no date restrictions) yielded only two relevant publications: the experimental study by Lam et al. (2008) and the review by Semmouri et al. (2024). These were the only identified sources specifically addressing the allelopathic effects of red seaweeds on microalgae in the North Sea region. Lam et al. (2008) used macroalgae that were collected at Helgoland, an island off the North-West coast of Germany. They tested multiple extracts from species of Rhodophyta, as well as some Phaeophyceae and Chlorophyta on the attachment and proliferation of four diatom (microalgae) species commonly found in the North Sea. Another interesting part of their research consists of a selective electron microscopy of the algal thalli to see whether or not there were any diatoms living on the macroalgal surface. They found that mainly the red algae were densely populated with diatoms (Lam et al., 2008). It was therefore hypothesized that extracts from these seaweeds would have less inhibitory effects on the diatoms than the ones that showed no diatom population on their surface. However, after testing the extracts, they had to conclude that this hypothesis was incorrect. Extracts from *Corallina officinalis*, *Mastocarpus stellatus*, *Palmaria palmata*, and *Ulva sp.* inhibited both the attachment and proliferation of all three tested diatom species, whereas extracts from *Ceramium rubrum*, *Fucus vesiculosus*, and *Cladophora rupestris* exhibited inhibitory effects on only two of the diatoms. (Lam et al., 2008). They observed a species-specific interaction on attachment and proliferation, which potentially explains why macroalgae producing potent inhibitory extracts are not necessarily devoid of diatoms. Furthermore, it was observed that different microalgae have a different response to each extract, for example the brown alga *Fucus vesiculosus* inhibited two diatom species while promoting attachment of a third species and being neutral towards the fourth (Lam et al., 2008). Therefore, it was concluded that allelopathy of macroalgae is targeted and selective towards certain species. The paper also suggests that allelopathy of macroalgae on diatoms is still poorly understood (Lam et al., 2008). With the fact that this is the only research on the topic in the North Sea and studies about this subject in the BPNS are completely lacking, the objective for this thesis becomes clear.

6 Objectives

Macroalgal allelopathy represents a complex and underexplored ecological interaction that may play a significant role in shaping microalgal communities. Among the macroalgae, species belonging to the Rhodophyta (red algae) are known to produce a diverse array of bioactive compounds interesting for bioprospecting, as extensively discussed in Section 5.3. Yet, their allelopathic influence on benthic microalgae remains poorly understood. This thesis aims to explore the chemical basis and ecological relevance of Rhodophyta-derived allelochemicals and their effects on microalgal physiology. To achieve this, extracts from Rhodophyta were prepared using both polar and nonpolar solvents, with the goal of isolating distinct groups of bioactive compounds. The biological activity of these extracts was tested by exposing selected microalgal strains to a concentration series of each extract type. Growth inhibition assays were used to quantify the allelopathic impact, while additional measurements of oxidative stress potentially provide insight into the physiological mechanisms underlying any observed inhibitory effects. Furthermore, the nonpolar extracts were analyzed using gas chromatography-mass spectrometry (GC-MS) to identify fatty acid profiles of the red seaweeds and see if the fatty acid can be linked to the observed growth dynamics from the inhibition assays. With the same idea, the polar extracts were subjected to phycobiliprotein analysis to quantify pigment contents. The central objective of this research is to better understand the role of red macroalgae in regulating microalgal populations through the means of (secondary) metabolites. This work is guided by the following research questions:

1. **Do Rhodophyta extracts exhibit allelopathic effects on microalgal growth, and how does this vary between polar and nonpolar fractions?**
2. **Can fatty acids be associated with the inhibitory effects of the nonpolar extracts on microalgae?**
3. **How does the phycobiliprotein content in the polar extracts relate to their potential allelopathic activity?**

By addressing these questions, this thesis contributes to a deeper understanding of chemical interactions in marine benthic ecosystems and highlights the importance of macroalgal secondary metabolites in ecological dynamics.

7 Material and methods

7.1 Sea water origin and F/2 Medium

The seawater used for the cultivation and experiments in this thesis originated from the Belgian part of the North Sea. It was collected by SEA LIFE Blankenberge via a pipeline located at coordinates 51.321614, 3.143153 (latitude, longitude; World Geodetic System 1984), and

subsequently transported to the laboratory by truck. Upon arrival, the seawater was filtered using a 0.2 μm pore size filter (Sartobran P or Supor 47 mm PES, Pall Corporation) and stored in sterile containers. These included 2 L autoclaved Duran bottles and 2 L PET plastic bottles. Since the plastic bottles could not be autoclaved, they were sterilized through rinsing with ethanol (CAS: 64-17-5, 99.8%) followed by deionized water prior to filling, to minimize the risk of bacterial or microbial contamination.

The culture medium used was a modified version of the F/2 medium originally developed by Robert R. L. Guillard & John H. Ryther (1962) and Robert R. L. Guillard (1975). The exact composition of the medium used in this thesis is presented in Appendix A.2.

7.2 Test organisms

7.2.1 Microalgae

All tested microalgae species (listed in Table 2) were cultivated under constant environmental conditions, with a light irradiance of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a temperature of $20 \pm 1^\circ\text{C}$. Continuous irradiance (24 h light) was applied to maintain maximal growth rates. Cultures were inoculated at least once per week to prevent them from reaching stationary phase due to population saturation. Inoculation involved transferring a small volume of the existing culture into a sterile borosilicate glass Erlenmeyer (250 mL, SIMAX glassware or VWR Avantor) containing approximately 100 mL of fresh F/2 medium. This procedure was performed under sterile conditions next to a Bunsen burner. Fresh F/2 medium was added to a newly autoclaved Erlenmeyer flask, and the necks of both the old and new flasks were briefly passed through the flame to minimize the risk of contamination. A small amount of the old culture was then poured into the new flask.

Following inoculation, the original culture was stored at $15 \pm 1^\circ\text{C}$, where growth was slowed to allow for long-term maintenance. This culture served as a backup in case contamination occurred in the new inoculated cultures.

Table 2. Overview of test organisms (microalgae).

Microalgae	Phylum	Autor	Origin
<i>Tisochrysis lutea</i> *	Haptophyta	(Bendif et al., 2013)	ARC lab Ghent
<i>Phaeodactylum tricornutum</i>	Chrysophyta	(Joyce Lewin, 1958)	CCAP 1052/1A

* Formerly thought to be the same as *Isochrysis galbana* (Bendif et al., 2013).

7.2.2 Rhodophytes

The red macroalgae of whom the allelopathic properties were investigated in this thesis, as well as their origin, are presented in Table 3. In the laboratory, cultures were maintained vegetatively by transferring a fragment of the parent thallus into fresh F/2 medium. This method of propagation prevents sexual reproduction, thereby maintaining genetic consistency across

generations. Cultures were grown on a white bench to enhance light reflection and illuminated from above using a TL lamp at an intensity of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$, under a 16:8 h light:dark photoperiod. The cultures were kept in a temperature-controlled room at $15 \pm 1^\circ\text{C}$ and aerated continuously using glass Pasteur pipettes connected to a sterile air supply via bacterial filters. The procedures for making the extracts are discussed in Section 7.4.

Table 3. Overview of the used macroalgae.

Name	Phylum	Autor	Collection site
<i>Palmaria palmata</i>	Rhodophyta	Weber & Mohr (1805)	Fort d'Ambleteuse, France 50.8054035, 1.6005092 (WGS 84)
<i>Gracilaria gracilis</i>	Rhodophyta	Irvine & Farnham (1995)	Lago di Lèsina, Italy 41.8657348, 15.3541161 (WGS 84)
<i>Acrochaetium secundatum</i>	Rhodophyta	Nägeli (1858)	Fort d'Ambleteuse, France 50.8054035, 1.6005092 (WGS 84)

7.3 Test setup and procedure

In this thesis, 18 distinct growth inhibition assays with seaweed extracts were performed as shown in Figure 10: From each Rhodophyte (*Palmaria palmata*, *Gracilaria gracilis* and *Acrochaetium secundatum*), three types of extracts were prepared as discussed in Section 7.4. This resulted in 9 extracts, tested on each of the two test organisms *Tysochrisis lutea* and *Phaeodactylum tricornutum*. Later, two more growth inhibition tests are performed with eicosapentaenoic acid (EPA) instead of seaweed extracts.

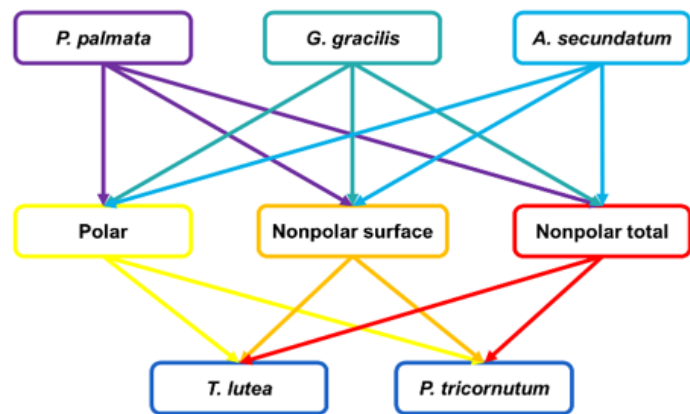


Figure 10. Overview of the performed tests, on the top level are the macroalgae of which the different extract types (second level) were made. At the bottom level the test organisms which were exposed to the extracts are shown.

The test setup used in this thesis was constant throughout the thesis and is visually represented in Figure 11. A concentration series was made depending on the extract used, but always with C0 representing the negative control (or the blank) and increasing extract concentrations from C1 to C6. For each concentration, three replicas (R1, R2 and R3 respectively) were included. Additionally, particle count flasks (PC) were implemented, containing the same amount of extract as the respective concentration series and medium, but no algae. These particle counts were designed to eliminate false positives within the cell counts, such as dust particles, salt crystals and other anomalies. Lastly, a solvent control (S) is implemented when the extract was made in a solvent that differs from the medium itself. The same amount of solvent is added as in the highest

concentration (C6), but without seaweed extract. As such, potential effects of the solvent (if present) can be distinguished from the effects of the extracts. For the inhibition assays, 100 mL borosilicate glass Erlenmeyers (SIMAX glassware or VWR Avantor) were used. These Erlenmeyers were closed off with Steristopfen caps and autoclaved at 121 °C for 20 minutes (VWR VAPOUR-line^{eco} vertical autoclave). All manipulations involving the removal of flask caps—whether during initial filling or subsequent sampling—are performed adjacent to a lit Bunsen burner on a work surface disinfected with Dettol. This precaution is taken to minimize the risk of microbial contamination.

To ensure optimal gas exchange and light penetration, Erlenmeyer flasks are filled to a total volume of 50 mL. Initially, 40 mL of F/2 medium is added to each flask. Culture densities are then measured, and the appropriate volume of algal culture is calculated to achieve a uniform starting density of 20,000 cells mL⁻¹ across all flasks. Based on this calculation and the required extract concentration, the remaining volume of F/2 medium to reach 50 mL is added, followed by the extract. Finally, the algal culture is added to each flask, ensuring that all samples are inoculated simultaneously and are immediately exposed to the designated extract concentration.

During the test, the algae were placed on a light bench (using Philips LED solutions, 400-700 nm) at an irradiance of 60 $\mu\text{mol s}^{-1} \text{m}^{-2}$ for 24 hours inside a temperature-controlled room at 20 ± 1 °C. Hence, no day/night routine is implemented to ensure maximal growth. Every 24 hours the erlenmeyers are shaken and their positions are switched randomly to eliminate possible impacts of small differences in light intensity.

At 72 and 96 hours of incubation, cell density was measured using the flow cytometer (CytoFLEX Beckman Coulter). From these cell densities, the growth rate (expressed in d⁻¹) is calculated using the difference between the natural logarithms at day 4 and day 0, divided by the amount of full days (4). The exact settings of the flow cytometer that were used are provided in Appendix A.1. For sampling, 200 μL of each Erlenmeyer was added to a VWR Tissue Culture Plates containing 96 wells and a flat bottom. The first three wells and each well in between different concentrations were filled with deionized water. For each treatment, the PC is measured first, followed by the three replicates. Due to technical issues, the flow cytometer could not always be used. In such cases, manual counting with a microscope (Medilux-12) was required for some tests. Sedgewick Rafter counters (chambers with dimensions 5 x 2 x 0.1 cm (length, width, depth)) were used, with the following protocol: Each chamber is filled with 1.100 mL consisting of a dilution of the sample and Lugol solution (depending on the amount of cells the dilution (Lugol:sample) is increased). After letting the cells settle to the bottom of the chamber for 10 minutes, each sample was counted five times. To obtain the microalgal density expressed in cells mL⁻¹, the average of these 5 counts was taken and multiplied by the dilution factor and a factor depending on the magnification of the microscope (factor 4000 for the used A20 lens). No PCs were used when

counting with a microscope, as the person counting could distinguish visually between cells and contamination.

$20 \pm 1 ^\circ C$ $60 \frac{\mu mol}{m^2 s}$	C0	C1	C2	C3	C4	C5	C6	S
R1								
R2								
R3								
PC								

Figure 11. Test setup. C0 is the blank, C1 - C6 are varying concentrations from low to high, S is the solvent control, R1-R3 are replicates and PC is the particle count in which no algae are added.

7.4 Macroalgae extracts

7.4.1 Polar extracts

Macroalgal thalli (Table 3) were freeze-dried for 48 hours using a Labconco FreezeZone 8L system and subsequently ground into a fine powder with an IKA A11 basic analytical mill. A stock solution with a concentration of 12 g dry weight per liter ($g_{DW} L^{-1}$) was prepared by dissolving 2.40 g of the powdered material in 200 mL of deionized water in a volumetric flask. The dry weight was measured using a precision balance with 0.01 g accuracy (Mettler PJ3600 DeltaRange). The solution was stirred for 30 minutes at room temperature using two small magnetic stir bars on a magnetic stirring plate set to 750 rpm. Following extraction, the solution was filtered through a 45 μm Supor PES membrane (47 mm diameter) using a KNF Neuberger Laboport vacuum pump. When extraction was performed prior to the day of the experiment, the filtered extract was stored at $-20 ^\circ C$ to prevent bacterial growth and degradation processes.

Table 4. Concentration series for polar extracts. C0 is the control, C1 to C6 the different concentrations (from low to high), while S is the solvent control in which only deionized water is added.

Name	C0	C1	C2	C3	C4	C5	C6	S
Concentration (g _{DW} L ⁻¹)	0	0.063	0.125	0.250	0.500	1.000	2.000	0

Deciding on ecologically relevant concentrations was quite difficult, as papers on the topic of algae coverage usually express the mass in terms of surface coverage (g m⁻² or in case of aquaculture harvests in g m⁻² d⁻¹) instead of g L⁻¹. Therefore, the concentration series was based on the research of Fu et al. (2022), who also worked with dry powder extracts of a Rhodophyta and did pre experiments to determine relevant concentrations. The concentration series is shown in Table 4.

7.4.2 Nonpolar extracts

For nonpolar extracts, the hexane dipping method was applied, similar to the method described in Lachnit et al. (2010) and De Nys et al. (1998). To obtain nonpolar extracts, 5 grams of fresh weight (g_{FW}) of the respective macroalgal thallus were submerged in 66.67 mL of hexane (CAS: 110-54-3, >99%) in a borosilicate glass beaker (VWR Avantor). Surface extracts were obtained by immersing macroalgal thalli in hexane for 30 seconds, allowing for the extraction of surface-associated compounds while minimizing cell lysis. For total extracts, the same procedure was applied, but the thalli were left in hexane for 5 minutes to promote cell membrane disruption and facilitate the release of intracellular compounds. After extraction, the seaweed material was removed and discarded.

For each macroalgal species, the extraction was performed twice, resulting in two hexane-based extracts. One extract was used for growth inhibition assays as described below, while the other was stored at -20 °C for subsequent GC-MS analysis, described in Section 7.5.2. For the inhibition assays, the hexane extract was transferred to a 250 mL borosilicate boiling flask (VWR Avantor) and concentrated using a rotary evaporator (Büchi R-100) equipped with an I-100 interface, V-100 vacuum pump, and B-100 heating bath. Evaporation was carried out at 264 mbar and 37 °C until only a minimal volume remained. The concentrated extract was then carefully transferred to a 16 mm rimless glass test tube (VWR Avantor) using a glass Pasteur pipette. The remaining solvent was subsequently evaporated until dry under a gentle stream of nitrogen gas (UN1066, Air Liquide). When no hexane remained, the extract was dissolved in F/2 medium and transferred to a 100 mL borosilicate volumetric flask (VWR Avantor). The glass tube was rinsed five times by adding a bit of F/2 medium and pipetting it into the volumetric flask. Lastly, the flask is filled to 100 mL with F/2.

Each time the extract was moved (*i.e.* from the beaker to the boiling flask and afterwards from the flask to the glass tube), the previous recipient was rinsed three times with hexane to make sure no extract is lost in the process. Assuming no extract dissolved or got lost during these processes, this results in a nonpolar extract of 5 g_{FW} dissolved into 100 mL F/2 medium, or a stock solution of 50 g_{FW} L⁻¹. Subsequently, a concentration series was made as shown in Table 5 and the test was set up as presented in Figure 11, but without solvent control (S) as the solvent used is the F/2 medium itself.

Table 5. Concentration series for apolar extracts. . C0 is the control, C1 to C6 the different concentrations (from low to high).

Name	C0	C1	C2	C3	C4	C5	C6
Concentration (g _{FW} L ⁻¹)	0	0.188	0.375	0.750	1.500	3.000	5.000

To determine ecologically relevant concentrations for the total extracts, the red alga *Palmaria palmata* was used as a reference species to facilitate comparison. The same concentration series derived from *P. palmata* was subsequently applied to all Rhodophyte extracts to ensure consistency across inhibition assays. According to Garbary et al. (2012) According to Garbary et al. (2012), *P. palmata* biomass in Canadian coastal environments ranges between 400 ± 200 g m⁻² and 1200 ± 400 g m⁻². Assuming this biomass is evenly distributed within the first meter of water above the seabed, this corresponds to a seaweed concentration of approximately 0.2 g L⁻¹ (minimum) to 1.6 g L⁻¹ (maximum).

In a cultivation study conducted by Sanderson et al. (2012) in Northwest Scotland, yields of 347 ± 43 g fresh weight were reported per 80 cm of rope over the period from January 25 to June 22, 2005. Given a cultivation setup with approximately four ropes per cubic meter, this results in a biomass density of 1388 ± 172 g_{FW} m⁻³, equivalent to approximately 1.4 g L⁻¹.

In contrast, land-based aquaculture systems can achieve much higher densities. For example, Corey et al. (2014) reported stocking densities of *P. palmata* ranging from 2.95 to 9.85 g L⁻¹. However, these values are considered less ecologically relevant due to the artificial nature of such systems. To capture the broad range of biomass concentrations observed in both natural and aquaculture contexts, the concentration series used in the inhibition assays was designed accordingly and are shown in Table 5.

7.5 Identification of potential allelopathic metabolites

7.5.1 Pigments

In the polar extracts, the main expected metabolites are the water soluble photosynthetic pigments, *i.e.*, the phycobiliproteins discussed in Section 5.2.3.2. To quantify phycobiliprotein

content, three replicates of 200 μL from each filtered polar extract (prepared as described in Section 7.4.1) were transferred into a 96-well flat-bottom tissue culture plate (VWR). The plate was then loaded into a Tecan Infinite M200 multi-mode microplate reader. Absorbance spectra of the stock solutions for each extract—*P. palmata*, *G. gracilis*, and *A. secundatum*—were subsequently recorded. A full visible light scan was performed across wavelengths ranging from 300 to 740 nm, along with specific absorbance readings at 564 nm and 620 nm, corresponding to the characteristic peaks of phycoerythrin and phycocyanin, the primary phycobiliproteins found in red algae (Dondald A. Bryant, 1982).

The phycobiliprotein concentrations were determined based on the assumption of a linear relationship between absorbance and concentration, as established through predetermined calibration curves supplied in Appendix A.5, Figure 28 for phycoerythrin ($R^2 = 0.9999$) and Figure 29 for phycocyanin ($R^2 = 0.9983$). PBP concentrations in the extract could be derived from the recorded absorbance value using the Beer-Lambert law. By dividing the obtained concentration in the stock solution by the concentration of the seaweed, the amount of pigment per gram of seaweed is obtained.

7.5.2 Fatty acids

Given that fatty acids are the primary metabolites expected in the nonpolar extracts, their quantification was prioritized using an established in-house protocol. A total of 35 fatty acids commonly found in seaweeds were targeted for analysis using gas chromatography–mass spectrometry (GC-MS). The analyses were carried out on a ThermoQuest CE Instruments system comprising an AS2000 autosampler, a Trace 2000 series gas chromatograph, and a Finnigan Trace DSQ mass spectrometer (Thermo Electron Corporation). Separation of components was achieved on a DB-225MS capillary column (Agilent Technologies 122-2932, Durabond), specifically suited for the analysis of fatty acid methyl esters. The injection was performed in splitless mode at 200 °C, with helium as the carrier gas at a constant flow rate of 1 mL min⁻¹. The GC oven was programmed to start at 50 °C (held for 1 minute), followed by a temperature increase of 7 °C per minute until reaching 185 °C, where it was held for 10 minutes. The temperature then increased at a rate of 15 °C per minute until it reached a final temperature of 230 °C, which was held for 14 minutes. The MS detector was operated in full scan mode, recording mass spectra from 40 to 440 m/z to allow detailed fragmentation patterns and optimal compound identification. The MS interface temperature was maintained at 250 °C. Data acquisition began after a retention time of 6.5 minutes to exclude the solvent peak from analysis.

Prior to GC-MS analysis, the nonpolar seaweed extracts (see Section 7.4.2) were subjected to a derivatization protocol to enable fatty acid identification. Extracts in hexane were first evaporated to dryness using a rotavapor and/or under a nitrogen stream at 50 °C. The dry extracts were transferred into 12 mL screw-cap test tubes. To minimize contamination, all glassware used for

transfer was rinsed with a small amount of hexane, which was subsequently added to the transferred extract. Before derivatization, 20 μL of an internal standard solution (500 ppm) was added to the dried extract, followed by vortexing. Subsequently, 3 mL of 3 M HCl in methanol was added. The samples were incubated at 60 °C for 15 minutes in a heating block (without nitrogen stream or oven) and then allowed to cool to room temperature.

Following derivatization, fatty acid methyl esters were extracted using 3 $\times$ 3 mL of hexane. The hexane fractions were pooled into a second test tube. If necessary, the combined extract was centrifuged (1500 rpm, 5 minutes) to remove any residual aqueous phase. The supernatant was concentrated to approximately 1 mL under a gentle nitrogen stream. After concentration, 10 μL of a recovery standard (RS; acenaphthene-d8) was added to each sample. The final solutions were transferred into GC vials and stored at 4 °C until GC-MS analysis. Two blank samples, consisting of empty test tubes processed identically, were included as procedural controls.

GC-MS quantification was performed in the ThermoScientific Xcalibur software (v4.1), which returns the results in concentrations expressed in $\mu\text{g mL}^{-1}$ (or ppm). Given that 1 mL is quantified, the concentration in $\mu\text{g g}_{\text{FW}}^{-1}$ is obtained by dividing by the total amount of fresh weight in the original seaweed extract.

7.6 Eicosapentaenoic acid tests

Following the same procedure described in Section 7.3, one more test was performed on both test organisms. Given that during the fatty acid analysis, eicosapentaenoic acid (EPA) was found in high concentrations compared to other fatty acids, this fatty acid was tested separately for its inhibitory effects. Extracts are made by dissolving 18.75 mg cis-5,8,11,14,17-Eicosapentaenoic acid (CAS: 10417-94-4) in 100 mL ethanol. Small amounts of this stock solution are added into each Erlenmeyer to obtain the concentration series provided in Table 6.

Table 6. Concentration series of eicosapentaenoic acid.

Name	C0	C1	C2	C3	C4	C5	C6
Concentration (mg L^{-1})	0	0.019	0.038	0.075	0.150	0.300	0.600

A maximum concentration of 112.34 μg EPA per gram of fresh seaweed weight was found in the total nonpolar extract of *P. palmata*. In the nonpolar extract tests, the highest concentration corresponded to 5 g_{FW} , equating to 0.562 mg EPA. To ensure consistency with previous seaweed extract tests, the concentration series (C0–C6) was aligned with the extract concentrations used in those experiments, albeit round up for the ease of making the extracts.

7.7 Oxidative stress

Antioxidant activity in the polar extracts was recorded as well, as PBP's are known for their antioxidant properties (see Section 5.3.3.1). Therefore, in order to explore whether there was a link with oxidative stress and the results of the inhibition tests, antioxidant activity was assessed using a DPPH assay, which is based on the reduction of the stable DPPH radical by antioxidants, resulting in a measurable decrease in absorbance. The protocol of Bertalaníč et al. (2012) was followed. Briefly, a 200 μM DPPH solution was prepared in ethanol for *A. secundatum* and in demineralized water for *P. palmata* and *G. gracilis*. The selection of these solvents is based on previous studies by the promotor identifying these as optimal for each species. Reactions were carried out in 2 mL microcentrifuge tubes at 25 °C. After 60 minutes of incubation in the dark, absorbance at 520 nm was measured. Results were expressed as the decrease in absorbance relative to blanks, and antioxidant capacity was quantified by comparison to a Trolox standard curve. Therefore, the final results are relative to the antioxidant activity of Trolox and expressed as milligram Trolox equivalent per gram dry weight ($\text{mgTe g}_{\text{DW}}^{-1}$).

7.8 Statistical analysis

7.8.1 Dose-Response Inhibition Tests

Dose-response relationships were assessed for six polar extract treatments for the two benthic microalgal species (*Tisochrysis lutea* and *Phaeodactylum tricornutum*), resulting in twelve unique concentration-response datasets. Data processing and analysis were conducted in R (v4.3.1) using the drc package (v3.0.1) for dose-response modeling and readxl (v1.4.5) for data import. For each treatment, multiple nonlinear models were fit, including log-logistic (LL.2, LL.3, LL.4, LL.5), Weibull (W1.2, W2.4), and Brain-Cousens models (BC.4, BC.5). Model selection was based on the lowest Akaike Information Criterion (AIC), and convergence diagnostics were checked to ensure model validity. From the best-fitting models, effective concentrations causing 10% inhibition (EC_{10}) and 50% inhibition (EC_{50}) were estimated using the ED() function, using the control group as 0% effect and a growth rate of 0 d^{-1} as 100% effect. When EC_{10} could not be estimated due to poor model fit or lack of observable inhibition, values were reported as not available (NA).

For visual representation, model predictions were plotted with 95% confidence intervals, illustrated using shaded bands. EC_{10} and EC_{50} estimates were highlighted with vertical dashed and dotted lines, color-coded to match treatment curves. Figures were generated with base R functions. To compare treatment effects statistically, additional dose-wise analyses were performed. For each seaweed extract and microalgal combination (18 in total), data were subjected to one-way analysis of variance (ANOVA), using aov() to evaluate differences in microalgal growth rate across concentrations. Assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. If assumptions were

met, post-hoc comparisons were conducted using Tukey's Honest Significant Difference (HSD). If assumptions were violated, Kruskal-Wallis tests were performed followed by Dunn's post-hoc tests (with Bonferroni correction) using the FSA package. Additional pairwise comparisons between lowest and highest concentrations were conducted via independent t-tests. NOEC and LOEC were manually determined based on significance compared to the control, with NOEC as the highest non-significant concentration and LOEC as the lowest significant one. If NOEC equaled the highest concentration, LOEC was marked as NA.

7.8.2 Phycobiliprotein absorbance analysis

Absorbance data were imported from Excel, transposed, and reshaped into long format using the dplyr (v1.1.4), tidyr (v1.3.1), and readxl (v1.4.5) packages. Mean absorbance and standard deviation (SD) were calculated per wavelength and group. Absorbance spectra were plotted using ggplot2 (v3.5.1), with ribbons representing ± 1 SD. Local maxima corresponding to phycobiliprotein peaks were identified within three target ranges (490–510 nm, 550–570 nm, 610–630 nm) and an additional unique peak for *P. palmata* (320–340 nm). These maxima were annotated directly on the plots.

To determine the concentrations of phycoerythrin and phycocyanin, blank-corrected absorbance values at 564 nm and 620 nm were converted to concentration values. For each seaweed species, three biological replicates were analyzed. Statistical analysis was conducted using one-way ANOVA with the base R function aov() to assess whether pigment concentrations differed significantly among species. Post hoc pairwise comparisons were carried out using Tukey's Honest Significant Difference (HSD) test (TukeyHSD()). Visualization of the data was performed using the ggplot2 (v3.5.1) and dplyr (v1.1.4) packages, which were used to generate boxplots with overlaid data points and customized aesthetics for each species.

7.8.3 Fatty Acid Composition Analysis

Fatty acid profiling was performed for surface and total lipid extracts of *G. gracilis* using targeted GC-MS data processed in Excel. Data were imported using readxl (v1.4.5), and bar plots of individual fatty acid concentrations (in $\mu\text{g g}_{\text{FW}}^{-1}$) were generated using ggplot2 (v3.5.1).

7.8.4 DPPH assays

A one-way Analysis of Variance (ANOVA) was conducted to determine whether there were significant differences in measured values among the three seaweed species (*Acrochaetium secundatum*, *Gracilaria gracilis*, and *Palmaria palmata*). To identify which groups differed, a post hoc Tukey's Honest Significant Difference (HSD) test was performed.

8 Results

8.1 Microalgal growth inhibition

Control growth rates varied between experiments and species, with *Phaeodactylum tricornutum* consistently growing faster than *Tisochrysis lutea*. Notable intra-species variation was observed, with control growth rates ranging from 0.72 to 1.25 d⁻¹ for *T. lutea* and to 0.88 to 1.29 d⁻¹ for *P. tricornutum*. These differences likely reflect variation in culture age and proximity to carrying capacity at experiment start. However, since each test used the same culture and results are normalized to controls, these factors do not affect data interpretation.

8.1.1 Polar extracts

The effects of the polar seaweed extracts on microalgal growth are presented as dose-response curves in Figure 12, with the shaded area visualizing the 95% confidence levels. The best fitted models for these plots are presented in Table 9, Appendix A.7. Where applicable, the 96 hour EC₁₀ and EC₅₀ values are indicated by dashed and dotted vertical lines, respectively. Additionally, the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined and are reported in Table 7 in Appendix A.4.

For optimal comparison between treatments, growth rates were normalized by dividing each data point by the maximum growth rate observed in that specific test. The non-normalized growth data, including statistical comparisons between treatments and controls, are provided in Appendix A.3 (Figure 21 for *T. lutea* and Figure 22 for *P. tricornutum*).

T. lutea

For *T. lutea*, significant growth inhibition was observed at the highest tested concentration (2 g_{DW} L⁻¹) for all three seaweed species (ANOVA, $p < 0.05$). Notably, the *P. palmata* extracts with concentrations below 2 g_{DW} L⁻¹ resulted in a significant growth stimulation (instead of inhibition) of *T. lutea* compared to control (Tukey HSD, adjusted $p < 0.05$). The strongest inhibitory effect occurred with *A. secundatum*, which was the only extract yielding both an EC₅₀ (1.014 g_{DW} L⁻¹) and an EC₁₀ (0.044 g_{DW} L⁻¹). Only an EC₁₀ could be derived for *G. gracilis* (1.530 g_{DW} L⁻¹) and *P. palmata* (1.755 g_{DW} L⁻¹).

P. tricornutum

In contrast, *P. tricornutum* exhibited a more limited sensitivity to the extracts. A significant decrease in growth was observed with the highest concentration (2 g_{DW} L⁻¹) of *A. secundatum* (ANOVA, $p < 0.05$). No significant inhibitory or stimulatory effects on growth were observed for either *G. gracilis* or *P. palmata* extracts across the tested concentration range. Only one EC₁₀ value could be calculated, corresponding to the *A. secundatum* extract (1.283 g_{DW} L⁻¹).

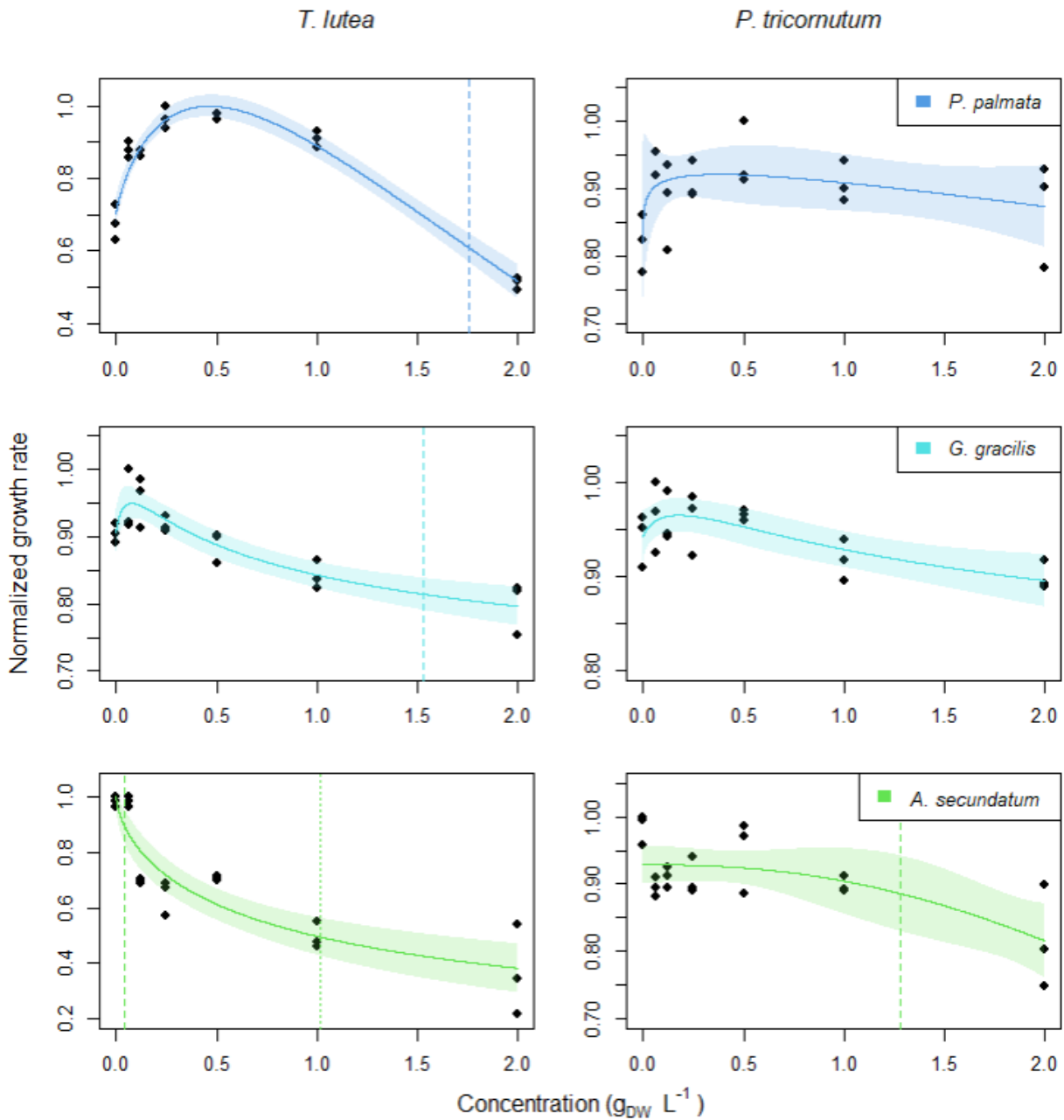


Figure 12. Dose response plots with 95% confidence intervals (shaded area) of the polar extracts. The left column shows growth rates at 96 hours of *Tisochrysis lutea*, the right column of *Phaeodactylum tricornutum*. The rows display the effects of the seaweed species: *Palmaria palmata* (top row, dark blue); *Gracilaria gracilis* (middle row, light blue) and *Acrochaetium secundatum* (bottom row, green). The concentrations (expressed in gram dry weight per liter ($\text{g}_{\text{DW}} \text{L}^{-1}$)) are shown on they x-axis and the normalized growth rate on the y-axis. The calculated 96 hour EC_{10} values are displayed with dashed vertical lines, while the 96 hour EC_{50} is displayed as a dotted line. Note that the y-axis is set to best fit the data and is therefore not equal for all plots. Plots are generated in RStudio with the drc package.

8.1.2 Nonpolar surface extracts

Dose–response curves for the nonpolar surface extracts with their 95% confidence intervals (shaded area) are presented in Figure 13, depicting the normalized growth rates of *Tisochrysis lutea* and *Phaeodactylum tricornutum* in response to increasing extract concentrations (expressed in $\text{g}_{\text{FW}} \text{L}^{-1}$). The best fitted models for these plots are supplied in Table 9, Appendix A.7. Where calculable, EC_{10} values (after 96 hours exposure) are indicated with dashed vertical lines. No EC_{50} values could be determined for these results. Statistical analyses of the non-normalized data were performed using ANOVA or, when assumptions of normality or homoscedasticity were violated, the Kruskal–Wallis test. This more conservative test sometimes indicated no significance, although a strong difference between higher concentrations compared to the control could be seen in boxplots. Therefore, when using Kruskal–Wallis, an extra t-test was performed between the control and highest concentration. Full statistical results are provided in Appendix A.3 (Figure 23 for *T. lutea*, Figure 24 for *P. tricornutum*). An overview of the EC, NOEC and LOEC values for all treatments are included in Table 7, Appendix A.4.

T. lutea

For *T. lutea*, all three seaweed extracts induced a concentration-dependent inhibition of growth (ANOVA, $p < 0.05$). The strongest inhibitory effect was observed for *P. palmata*, where significant inhibition was observed from concentrations $\geq 3 \text{ g}_{\text{FW}} \text{L}^{-1}$ (Tukey HSD, adjusted $p < 0.05$). *G. gracilis* also resulted in a significant reduction in growth rate at the same concentrations ($\geq 3 \text{ g}_{\text{FW}} \text{L}^{-1}$, Tukey HSD, adjusted $p < 0.05$). A reliable EC_{10} could be derived for both *P. palmata* ($1.431 \text{ g}_{\text{FW}} \text{L}^{-1}$) and *G. gracilis* ($1.666 \text{ g}_{\text{FW}} \text{L}^{-1}$). The *A. secundatum* test did not satisfy ANOVA criteria and the Kruskal–Wallis test did not reveal a significant trend, a supplementary t-test comparing the control and the highest concentration ($5 \text{ g}_{\text{FW}} \text{L}^{-1}$) indicated a statistically significant inhibitory effect ($p < 0.05$). Despite the significant growth inhibition, no EC_{10} value could be derived.

P. tricornutum

For *P. tricornutum*, the inhibitory responses were generally less pronounced than those observed for *T. lutea*. Similarly to *T. lutea* tests, extracts from *P. palmata* and *G. gracilis* induced the strongest response. Although the assumptions for ANOVA were not met and the Kruskal–Wallis test did not detect significance across concentrations for *P. palmata*, a t-test comparison at the highest concentration ($5 \text{ g}_{\text{FW}} \text{L}^{-1}$) confirmed a significant reduction in growth ($p < 0.05$). Moreover, an EC_{10} of $0.416 \text{ g}_{\text{FW}} \text{L}^{-1}$ was derived based on the fitted model. *G. gracilis* elicited a mild, concentration-dependent decrease in growth, and even though no EC_{10} could be derived, it showed significant inhibition for concentrations $\geq 0.375 \text{ g}_{\text{FW}} \text{L}^{-1}$ (Tukey HSD, adjusted $p < 0.05$). *A. secundatum* showed no significant effect on *P. tricornutum* growth across the concentration range.

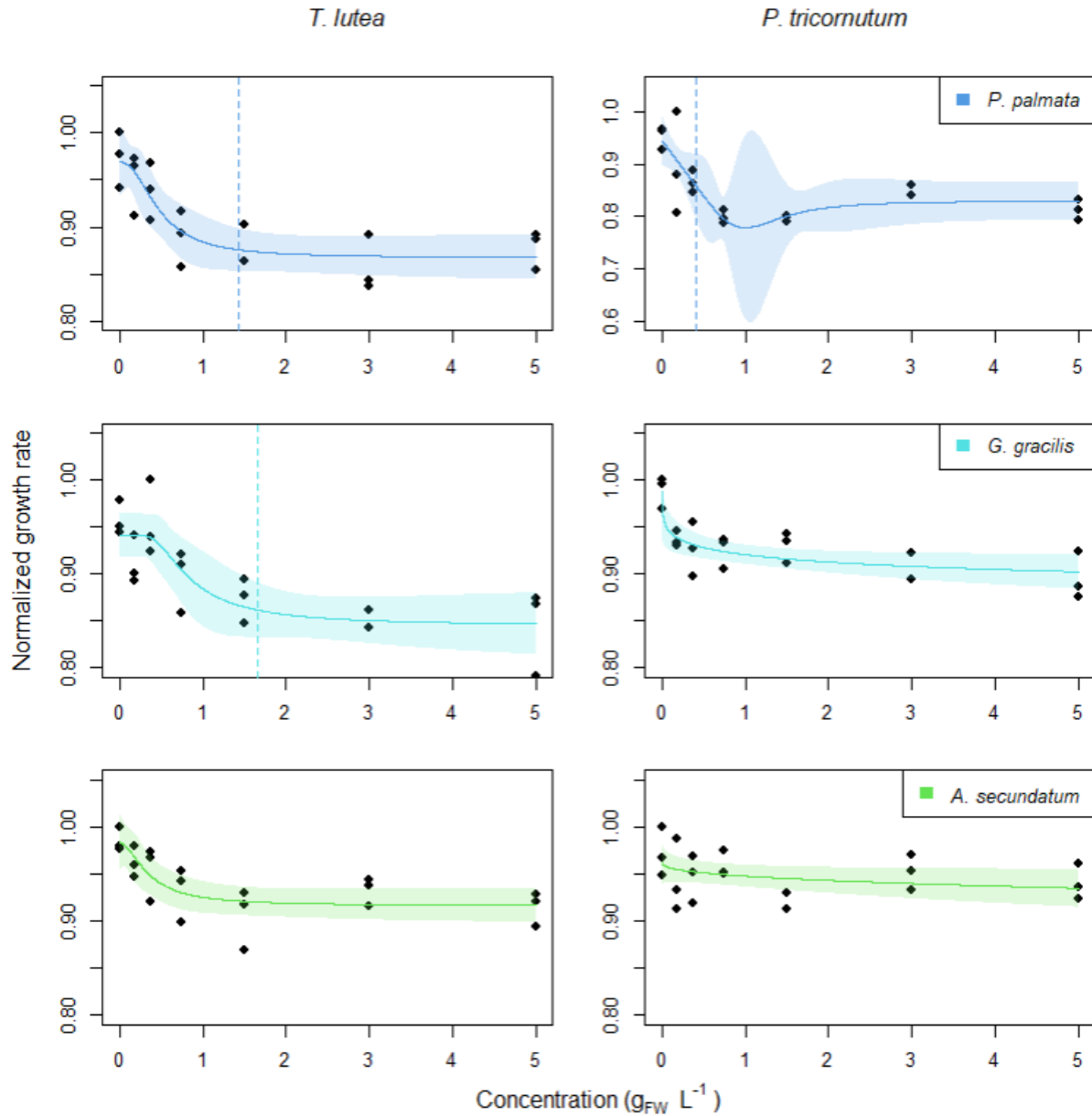


Figure 13. Dose response plots with 95% confidence intervals (shaded area) of the nonpolar surface extracts. The left column shows growth rates at 96 hours of *Tisochrysis lutea*, the right column of *Phaeodactylum tricornutum*. The rows display the effects of the seaweed species: *Palmaria palmata* (top row, dark blue); *Gracilaria gracilis* (middle row, light blue) and *Acrochaetium secundatum* (bottom row, green). The concentrations (in gram dry weight per liter (g_{DW} L⁻¹)) are shown on they x-axis and the normalized growth rate on the y-axis. The calculated 96 hour EC₁₀ values are displayed with dashed vertical lines, EC₅₀ values could not be calculated for these results. Plots are generated in RStudio with the drc package.

8.1.3 Nonpolar total extracts

Figure 14 presents the dose–response curves and their 95% confidence intervals for the nonpolar total extracts of *Palmaria palmata*, *Gracilaria gracilis*, and *Acrochaetium secundatum* on the growth of *Tisochrysis lutea* and *Phaeodactylum tricornutum*. The best fitted models for these plots are presented in Table 9, Appendix A.7. Dashed and dotted vertical lines represent the EC₁₀ and EC₅₀ values, respectively, calculated after 96 hours of exposure. Statistical significance of growth inhibition, based on non-normalized growth rates, is provided in Appendix A.3 (Figure 25 for *T. lutea* and Figure 26 for *P. tricornutum*). EC values as well as NOEC and LOEC values for all treatments are presented in Table 7, Appendix A.4.

T. lutea

T. lutea displayed high sensitivity to the total extracts of *P. palmata*. Exposure to *P. palmata* resulted in a steep, concentration-dependent inhibition of growth. A significant reduction in growth rate was observed at every concentration tested (ANOVA, $p < 0.05$), with both EC₁₀ (0.082 g_{FW} L⁻¹) and EC₅₀ (1.098 g_{FW} L⁻¹) values clearly defined by the fitted model. *G. gracilis* also significantly inhibited growth at concentrations ≥ 0.375 g_{FW} L⁻¹ (Tukey HSD, adjusted $p < 0.05$). However, the response was less steep than that of *P. palmata* and no EC values could be derived. Exposure to *A. secundatum* extracts did not produce a significant growth reduction across the tested concentrations. A slight increase in growth rate was observed at intermediate concentrations, though this effect was not statistically significant. Therefore, no EC values could be obtained.

P. tricornutum

Compared to *T. lutea*, *P. tricornutum* showed a more moderate response to the nonpolar total extracts. The *P. palmata* extract caused a significant reduction in growth starting from concentrations ≥ 0.75 g_{FW} L⁻¹ (Tukey HSD, adjusted $p < 0.05$), and a gradual inhibitory trend was observed across the full range. Moreover, an EC₁₀ value of 0.706 g_{FW} L⁻¹ was calculated. The extract from *G. gracilis* resulted in inhibition, but did not follow a dose-dependent trend like all other tests, with a significant reduction noted at 1.5 and 3 g_{FW} L⁻¹ but no effect at the highest concentration of 5 g_{FW} L⁻¹ (Tukey HSD, adjusted $p < 0.05$). *A. secundatum* did not significantly affect *P. tricornutum* growth.

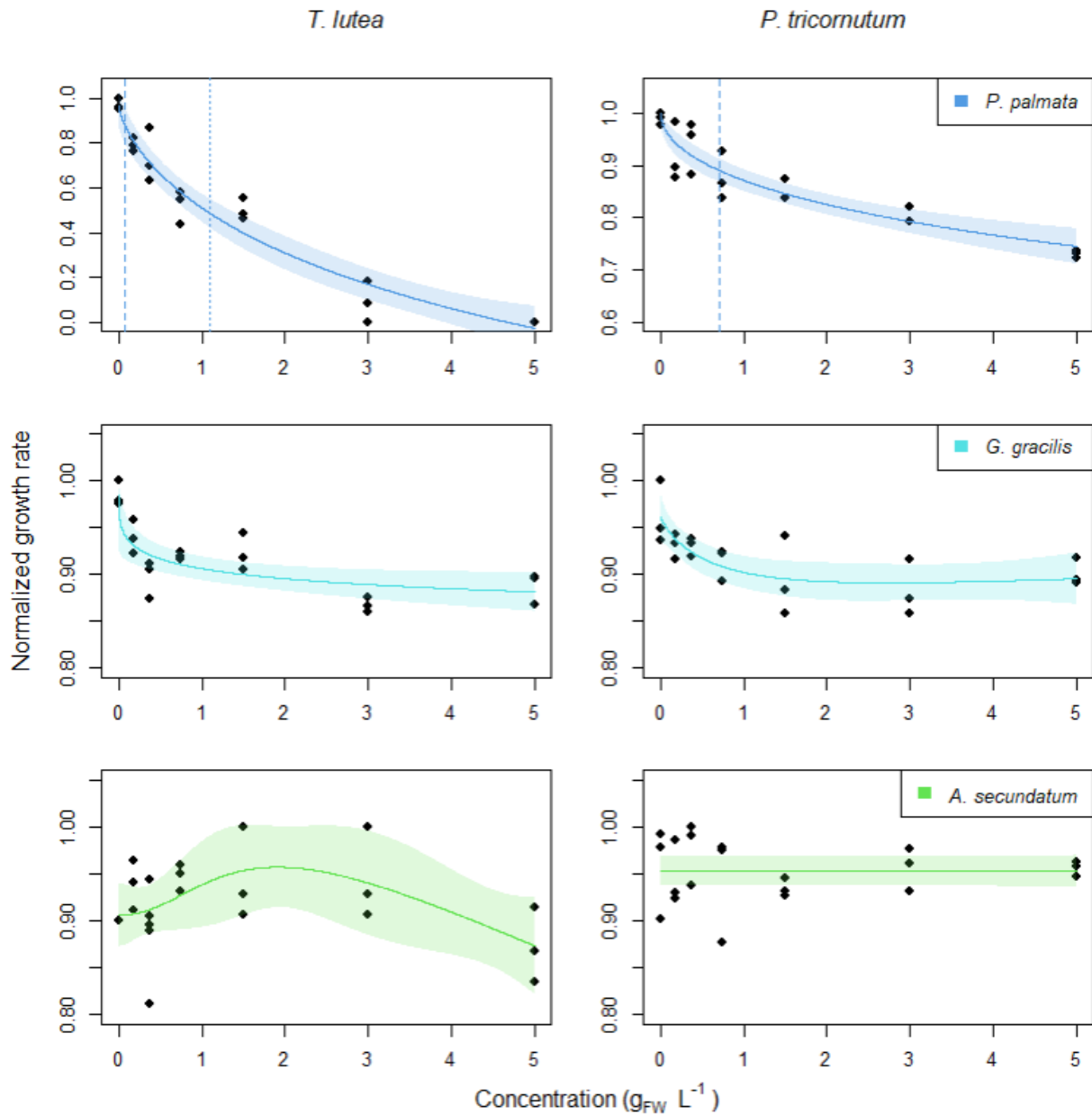


Figure 14. Dose response plots with 95% confidence intervals (shaded area) of the nonpolar total extracts. The left column shows growth rates at 96 hours of *Tisochrysis lutea*, the right column of *Phaeodactylum tricornutum*. The rows display the effects of the seaweed species: *Palmaria palmata* (top row, dark blue); *Gracilaria gracilis* (middle row, light blue) and *Acrochaetium secundatum* (bottom row, green). The concentrations (in gram dry weight per liter ($\text{g}_{\text{DW}} \text{L}^{-1}$)) are shown on they x-axis and the normalized growth rate on the y-axis. The calculated 96 hour EC_{10} values are displayed with dashed vertical lines, while the 96 hour EC_{50} is displayed as a dotted line. Note that the y-axis is set to best fit the data and is therefore not equal for all plots. Plots are generated in RStudio with the drc package.

8.2 Phycobiliprotein analysis

Absorbance spectra of the polar extracts from each seaweed species are shown in Figure 15. All extracts display local maxima at approximately 498, 564, and 614 nm. An extra peak at 326 nm is uniquely observed for *P. palmata*.

The absorbance of phycoerythrin and phycocyanin at 564 nm and 620 nm, respectively, was quantified using compound-specific calibration curves (Appendix A.5, Figure 28 and Figure 29). Both the figure and the exact concentrations derived from the calibration curves indicate that *A. secundatum* contains significantly higher levels of phycobiliproteins.

Among the three seaweed species, *A. secundatum* showed the highest pigment concentrations, with an average phycoerythrin content of $17.56 \pm 0.79 \text{ mg L}^{-1}$ and phycocyanin content of $5.14 \pm 0.23 \text{ mg L}^{-1}$. This is visualized in Figure 16. Both of these were significantly higher than the *G. gracilis* and *P. palmata* extracts, while there was no significance observed between these two (Tukey HSD, adjusted $p < 0.05$). *G. gracilis* exhibited intermediate values, with phycoerythrin at $11.47 \pm 0.39 \text{ mg L}^{-1}$ and phycocyanin at $2.82 \pm 0.12 \text{ mg L}^{-1}$. *P. palmata* had the lowest concentrations, showing a phycoerythrin content of $10.31 \pm 0.02 \text{ mg L}^{-1}$ and phycocyanin content of $2.62 \pm 0.07 \text{ mg L}^{-1}$. These results indicate a clear variation in pigment composition among the species, with *A. secundatum* being significantly more pigment-rich for both pigments (ANOVA, $p < 0.05$).

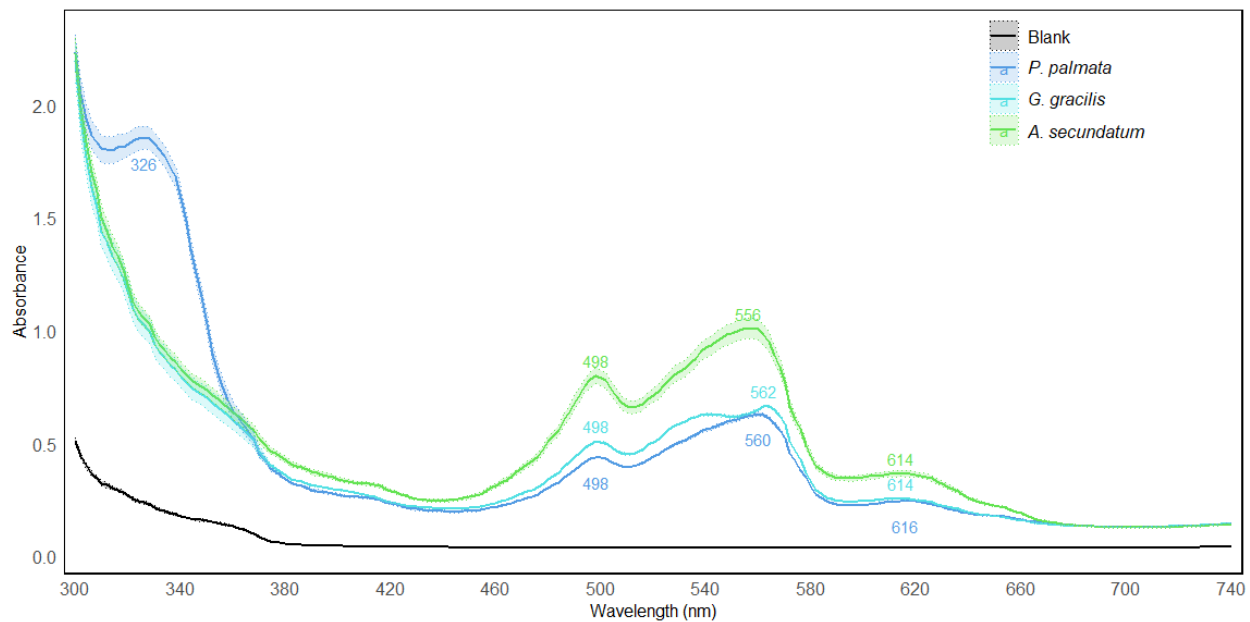


Figure 15. Full absorbance scan of visual light (wavelengths 300-740 nm) for the different polar seaweed extracts. *Acrochaetium secundatum* (green) shows the highest absorbance peaks at 498, 556 and 614 nm. *Gracilaria gracilis* and *Palmaria palmata* show a similar curve but with less expressed absorbance peaks at these wavelengths. *P. palmata* has a unique peak at 326 nm. The blank (black) is the absorbance of deionized water.

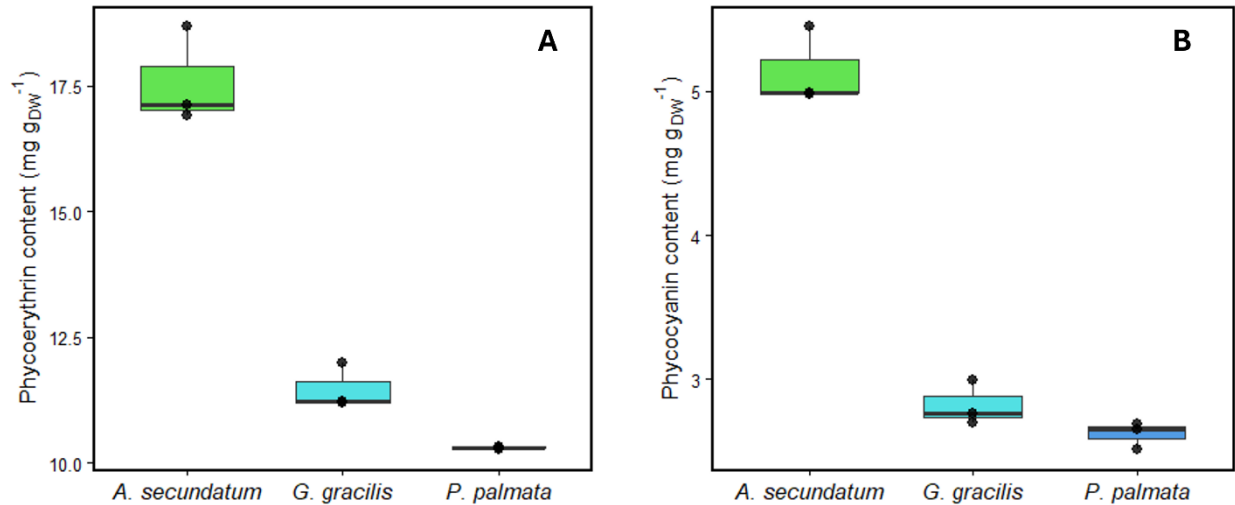


Figure 16. Phycoerythrin (A) and phycocyanin (B) content in the three Rhodophyta, *Acrochaetium secundatum* (green), *Gracilaria gracilis* (light blue) and *Palmaria palmata* (dark blue). No significant difference between *P. palmata* and *G. gracilis* is observed, while significant difference between *A. secundatum* with both other seaweeds was found for phycoerythrin (ANOVA, $p < 0.0001$) and phycocyanin (ANOVA, $p < 0.00001$).

8.3 Fatty acid analysis of nonpolar extracts

The fatty acid profiles of the nonpolar seaweed extracts, quantified via GC-MS, are summarized in Figure 17. Among all analyzed extracts, the total extract of *Palmaria palmata* (abbreviated as NTP) exhibited a markedly higher absolute concentration of fatty acids compared to any other sample, with a total of $147.08 \mu\text{g g}_{\text{FW}}^{-1}$. Notably, this extract was dominated by eicosapentaenoic acid (EPA; cis-5,8,11,14,17-eicosapentaenoate, C20:5), which alone accounted for $112.34 \mu\text{g g}_{\text{FW}}^{-1}$, approximately 76% of the total fatty acid content. This high absolute and relative EPA content clearly distinguishes the NTP extract as the most lipid-rich of the tested extracts, being particularly enriched in long-chain polyunsaturated fatty acids.

A similar, albeit less pronounced, pattern was observed in the total extract of *Gracilaria gracilis* (NTG), which contained a total fatty acid content of $22.32 \mu\text{g g}_{\text{FW}}^{-1}$. EPA was again the most abundant fatty acid in this extract, present at $9.13 \mu\text{g g}_{\text{FW}}^{-1}$, corresponding to 40.90% of the total fatty acid content. The total extract of *Acrochaetium secundatum* (NTA), in contrast, contained very low overall levels of fatty acids ($0.50 \mu\text{g g}_{\text{FW}}^{-1}$), with EPA making up 16.32% of this modest total.

Surface extracts (NSP, NSG, and NSA) displayed distinct profiles in both concentration and composition. The surface extract of *P. palmata* (NSP) exhibited a total fatty acid content of $21.84 \mu\text{g g}_{\text{FW}}^{-1}$, a value comparable to that of the *G. gracilis* total extract. However, the compositional profile differed significantly. In NSP, palmitic acid (C16:0) was the most abundant fatty acid at $8.26 \mu\text{g g}_{\text{FW}}^{-1}$, comprising 37.83% of the total, while EPA was present in much lower concentrations ($1.28 \mu\text{g g}_{\text{FW}}^{-1}$, 5.86%). This suggests that *P. palmata* allocates EPA predominantly

within its intracellular (total) fraction, while surface-associated lipids are more saturated in nature.

The surface extract of *G. gracilis* (NSG) contained $4.89 \mu\text{g g}_{\text{FW}}^{-1}$ total fatty acids, of which EPA contributed 12.79%. Interestingly, this extract was unique in containing dihomo- γ -linolenic acid (DGLA), which accounted for 14.24% of the total fatty acid profile. Arachidonic acid (AA) was also present in NSG at 10.15%, but absent from most other extracts. These findings suggest a distinct surface lipid composition for *G. gracilis*, potentially reflecting specific ecological or physiological roles of these surface-associated fatty acids.

In *A. secundatum*, surface (NSA) and total (NTA) extracts both showed minimal fatty acid concentrations, with $0.89 \mu\text{g g}_{\text{FW}}^{-1}$ and $0.50 \mu\text{g g}_{\text{FW}}^{-1}$, respectively. Despite these low totals, the relative compositions were informative. Palmitic acid was again the major surface fatty acid (35.61%), while stearic acid (C18:0) showed notable enrichment in both fractions, comprising 16.63% in NSA and 18.66% in NTA. EPA was present in moderate proportions (NSA: 15.17%, NTA: 16.32%) despite the overall low lipid yield.

A more detailed comparison of the six most abundant fatty acids across all extracts, expressed as a percentage of the total fatty acid content within each extract, is presented in Figure 18. This relative analysis reveals clear compositional distinctions between extract types (surface vs. total) and among species. In general, total extracts were more enriched in long-chain polyunsaturated fatty acids such as EPA, particularly in *P. palmata* and *G. gracilis*, while surface extracts tended to show higher proportions of saturated fatty acids such as palmitic acid and stearic acid.

For example, palmitic acid was the dominant fatty acid in all surface extracts, with relative abundances ranging between 35% and 45%, whereas its presence was substantially lower in the total extracts of the same species. This suggests a functional partitioning of fatty acid types between surface and intracellular (or membrane-bound) lipid fractions. Oleic acid (C18:1) was found at its highest level in the total extract of *P. palmata* (15.76%), but was present only in minor quantities in all other extracts. Similarly, as mentioned earlier, DGLA and AA were uniquely associated with the surface extract of *G. gracilis*, reinforcing the notion that this species may produce or retain certain fatty acids on its surface for specific ecological functions (*e.g.*, defense or signaling).

A full overview of all identified fatty acids, including short names, systematic names, and retention times, is provided in Table 8 (Appendix A.6). For extended compositional comparisons beyond the six most dominant fatty acids, readers are referred to Figure 30 (Appendix A.6), which presents the complete fatty acid profiles across all extracts and species.

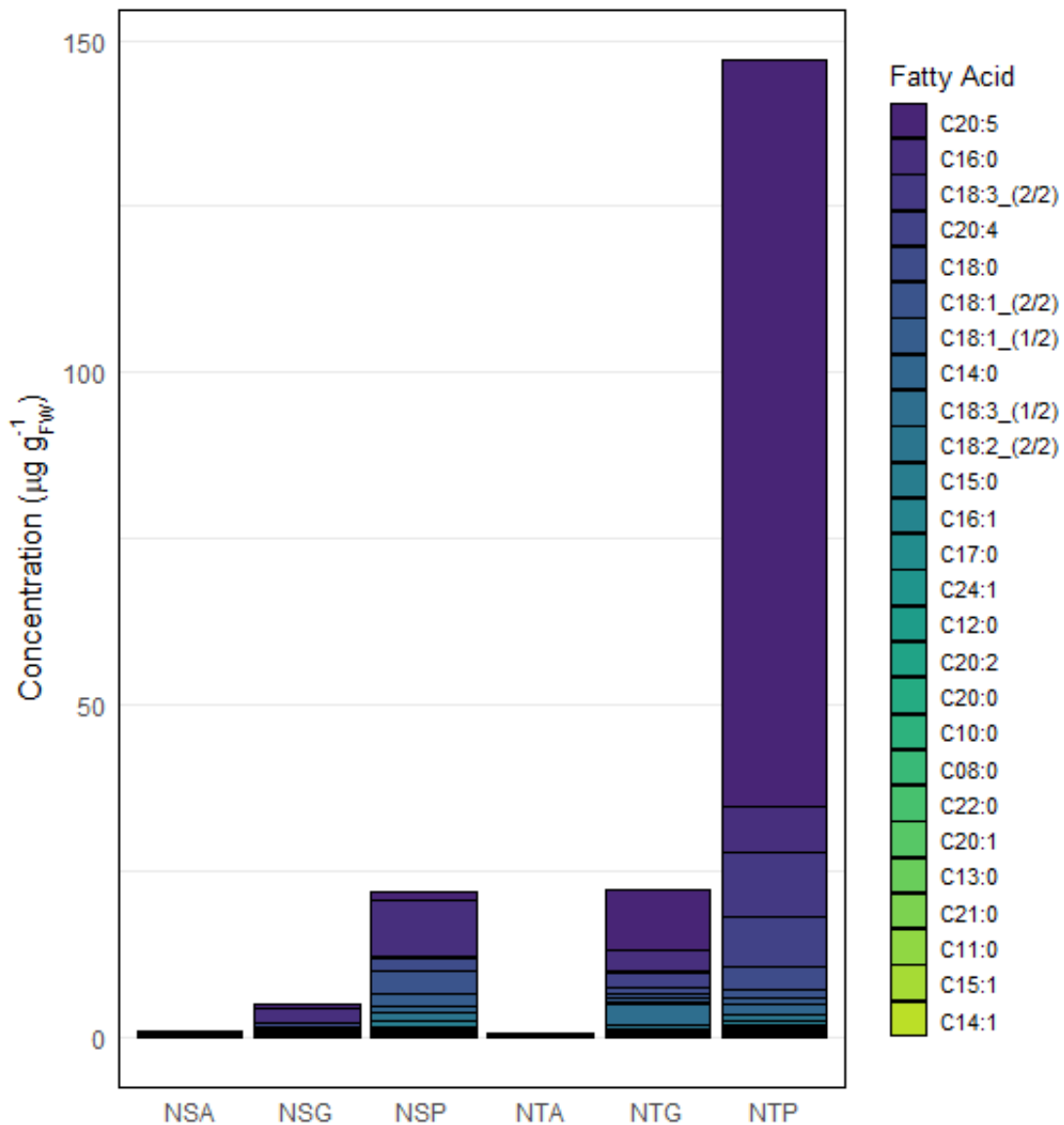


Figure 17. Fatty acid content in nonpolar extracts as quantified with GC-MS. The x-axis displays six sample types: nonpolar surface extracts of *Acrochaetium secundatum* (NSA), *Gracilaria gracilis* (NSG), and *Palmaria palmata* (NSP), as well as nonpolar total extracts from the same species—NTA, NTG, and NTP, respectively. The y-axis represents fatty acid concentrations, expressed in micrograms per gram of fresh weight ($\mu\text{g gFW}^{-1}$).

Across all samples, the main fatty acids found in relatively high concentrations are C16:0 (methyl palmitate), C18:0 (methyl stearate), and C20:5 (methyl cis-5,8,11,14,17-eicosapentaenoate). Among these, C16:0 is especially dominant in both the surface and total fractions of *A. secundatum*, whereas C20:5 is particularly abundant in the total fractions of *G. gracilis* and *P. palmata*. In the surface layers of *G. gracilis* and *P. palmata*, relatively high levels of C18:2 (methyl linoleate) are also observed. Meanwhile, the total fractions of *G. gracilis* and *P. palmata* are notable for higher levels of C20:4 (methyl cis-5,8,11,14-eicosatetraenoate) and C18:3 isomers—specifically C18:3_(1/2) (methyl gamma-linolenate) in *G. gracilis* and C18:3_(2/2) (methyl linolenate) in *P. palmata*.

Several fatty acids were consistently undetected (*i.e.*, quantified as 0) across all samples and were therefore excluded from the plots. These include: C17:0, C18:2_(1/2), C20:3_(1/2), C20:3_(2/2), C22:1, C22:2, C23:0, C22:6, and C24:0.

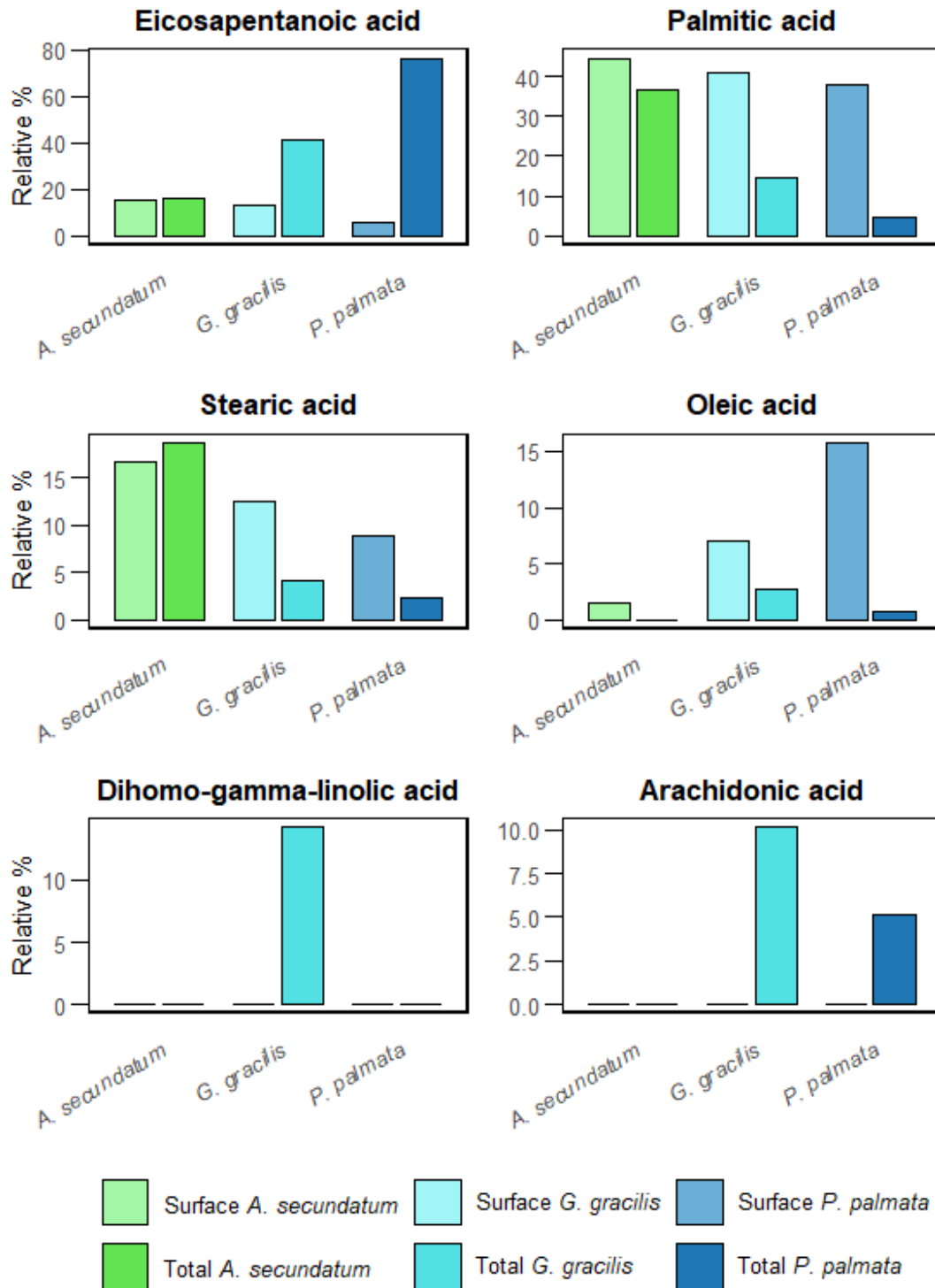


Figure 18. Relative percentage (%) of the six most dominant fatty acids in the Rhodophyta *Acrochaetium secundatum* (green), *Gracilaria gracilis* (light blue) and *Palmaria palmata* (dark blue). The total extracts are darkened, whereas the surface extract are a lighter shade.

8.4 Eicosapentaenoic acid tests

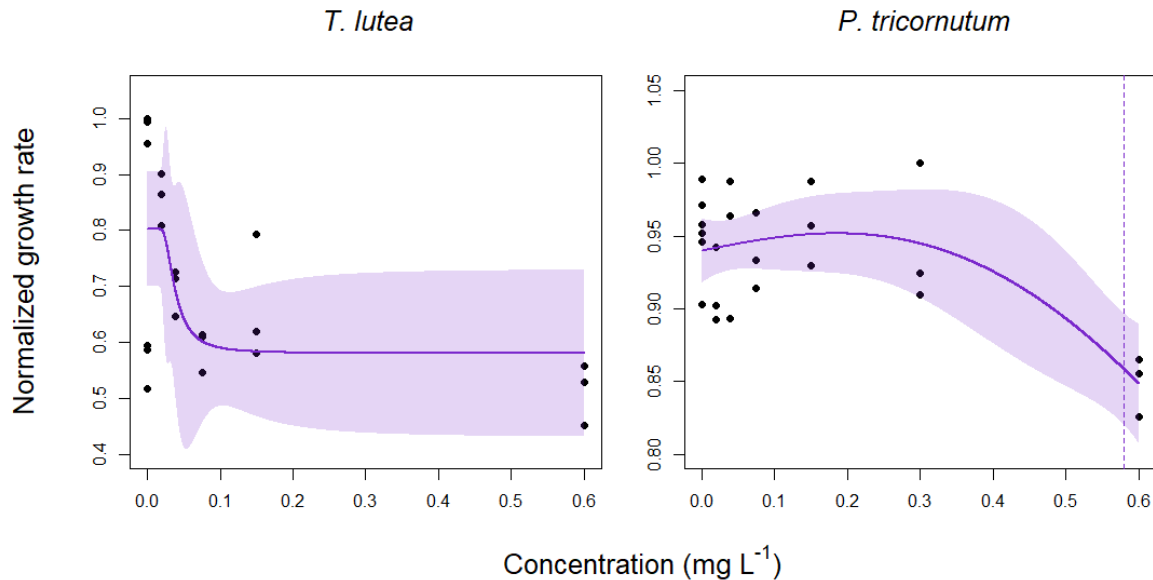


Figure 19. Dose response plots with 95% confidence intervals (shaded area) of eicosapentaenoic acid (EPA). The left column shows growth rates at 96 hours of *Tisochrysis lutea*, the right column of *Phaeodactylum tricornutum*. The concentrations (expressed in milligram EPA per liter (mg L⁻¹)) are shown on the x-axis and the normalized growth rate on the y-axis. The calculated EC₁₀ values are displayed with dashed vertical lines. No EC₅₀ could be determined. Note that the y-axis is set to best fit the data and is therefore not equal for all plots. Plots are generated in RStudio with the drc package. In *T. lutea*, the concentration at 0.019 mg L⁻¹ is a significantly higher than that of 0 mg L⁻¹, giving a false impression of growth inhibition due to limits of best fitted model (W2.4), similar problems arose with the next-best model (LL.4).

Dose-response curves for EPA exposure are presented in Figure 19, showing the normalized growth rates of *Tisochrysis lutea* and *Phaeodactylum tricornutum* in response to increasing concentrations of EPA. EC₁₀ values, where applicable, are indicated by dashed vertical lines. Statistical analyses were conducted on raw growth rate data using ANOVA. Further details are provided in Appendix A.3, Figure 27.

For *T. lutea*, the growth rate at 0 mg L⁻¹ (solvent control) was as low as that observed at the highest concentration (0.6 mg L⁻¹) tested, indicating a possible solvent-related suppression of growth. The t-test between the blank and the solvent control indicated significant effects of the solvent, as shown in Appendix A.3, Figure 27. Therefore, no clear dose-dependent response could be found, and no EC₁₀ value was obtained.

In *P. tricornutum*, growth was generally stable across lower EPA concentrations. However, a statistically significant reduction in growth rate was only observed at the highest tested concentration (0.6 mg L⁻¹, ANOVA, $p < 0.05$). In this case, the t-test indicated no difference between the solvent control and the blank-treatment. An EC₁₀ value could be derived and is presented together with NOEC and LOEC values in Table 7, Appendix A.4.

8.5 DPPH assay

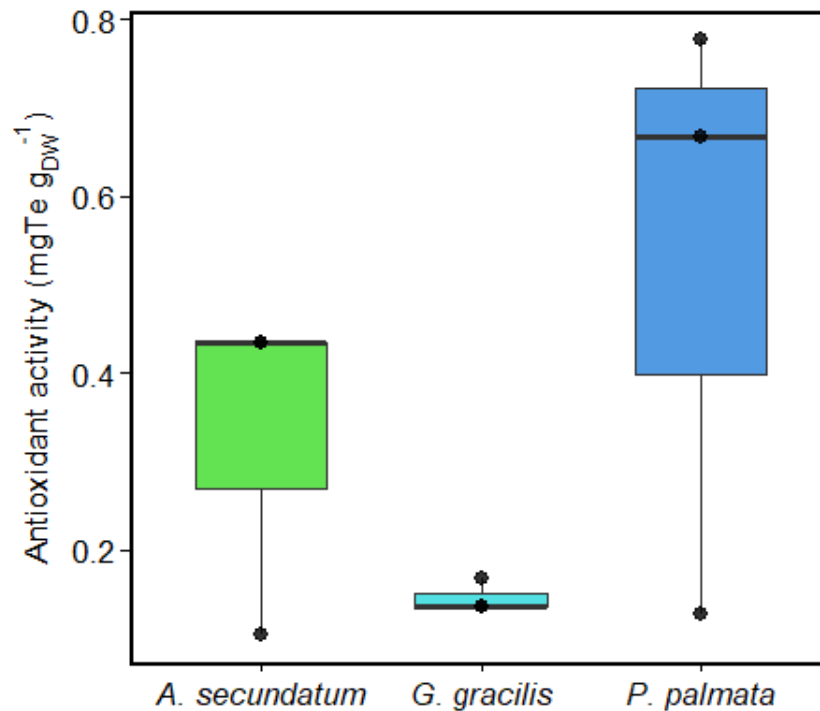


Figure 20. DPPH assay results of dry powder from seaweed species *Acrochaetium secundatum*, *Gracilaria gracilis* and *Palmaria palmata*, expressed in mg Trolox equivalent per gram dry weight (mgTe g_{DW}⁻¹). There are no significant differences between seaweed species (ANOVA, $p < 0.05$). The used solvent for *G. gracilis* and *P. palmata* was demineralized water, while that of *A. secundatum* was ethanol.

The DPPH assay results are shown in Figure 20, the average radical scavenging capacity expressed in Trolox equivalent per gram dry weight was highest for *P. palmata* (0.52 ± 0.35), followed by *A. secundatum* (0.32 ± 0.19) and with *G. gracilis* (0.15 ± 0.02) the lowest average antioxidant activity. However, the differences are not significant.

8.6 pH measurements

The pH was measured at the beginning and end of each test to monitor potential changes associated with algal growth. For *T. lutea*, the average pH increased from 8.2 ± 0.1 at the start to 8.7 ± 0.6 at the end of the exposure period. Similarly, for *P. tricornutum*, pH values rose from 8.3 ± 0.1 to 9.4 ± 0.8 . A potential correlation between cell density and pH was hypothesized, given the expected influence of photosynthetic activity on medium alkalinity. However, no significant relationship was detected based on Spearman's rank correlation analysis conducted in R version 4.4.1 ($p > 0.05$). Visual representations of the pH and cell density data are provided in Figure 31, Appendix A.8.

9 Discussion

This study evaluated the inhibitory effects of Rhodophyta extracts on the growth of two marine microalgae, *Tisochrysis lutea* and *Phaeodactylum tricornutum*, using a combination of dose–response assays, chemical characterization, and complementary bioactivity tests. The observed biological responses varied significantly across extract types, seaweed species, and test organisms, highlighting complex interactions driven by both the chemical composition of the extracts and the physiological traits of the microalgae.

9.1 Species specific growth inhibition

Across all treatments, *Tisochrysis lutea* consistently exhibited greater sensitivity to the seaweed extracts than *Phaeodactylum tricornutum*, as indicated by stronger dose-dependent responses and lower EC_{10}/EC_{50} values. This interspecific variation was already discussed briefly in Section 5.4.2 and aligns with findings from several studies, which have shown species-specific responses among microalgae (Franz et al., 2008; Gallo et al., 2020; Karseno et al., 2009; Levy et al., 2007, 2008; Magnusson et al., 2010; Orvos et al., 2002; Q. Zhao et al., 2018). For instance, Q. Zhao et al. (2018) demonstrated highly variable EC_{50} values across three microalgae exposed to 15 different sponge-, gorgonia- and coral-derived compounds, with no single species consistently being most sensitive. Similarly, exposure to a cyanobacteria-derived phycoerythrin-like compound led to a twelvefold difference in IC_{50} values between two green microalgal species (Karseno et al., 2009). Moreover, diatoms (such as *P. tricornutum*) have generally shown higher tolerance to organic compounds, such as herbicides and antibiotics (Franz et al., 2008; Magnusson et al., 2010; Orvos et al., 2002), while being more sensitive to metals like copper (Levy et al., 2007, 2008).

Given the highly variable responses of microalgae to different inhibitory compounds, identifying a definitive cause for the observed sensitivity differences between *T. lutea* and *P. tricornutum* remains challenging. One possible explanation for the higher sensitivity of *T. lutea* is its smaller size and more spherical morphology compared to the elongated oval shape of *P. tricornutum*. (Bendif et al., 2013; Martino et al., 2007). This suggests that *T. lutea* possesses a higher specific surface area, potentially providing greater interface for interaction with, or absorption of, the active compounds present in the extracts.

9.2 Linking Chemical Composition to Bioactivity

The inhibition assays identified the most potent extract fractions in terms of growth suppression, while chemical analyses offered insight into potential bioactive compounds responsible for these effects. The following sections provide a detailed discussion of each extract.

9.2.1 Polar extracts

Among the extract types, polar extracts exhibited a broader range of effects, including significant stimulation at sub-inhibitory concentrations (notably for *P. palmata* and *G. gracilis*) and strong inhibition at higher doses, especially in the case of *A. secundatum*. The inhibitory potency of *A. secundatum*'s polar extract was the highest observed, yielding both EC_{10} and EC_{50} values for *T. lutea* (0.044 and $1.014 \text{ g}_{\text{DW}} \text{ L}^{-1}$, respectively), and a measurable EC_{10} for *P. tricornutum* ($1.283 \text{ g}_{\text{DW}} \text{ L}^{-1}$).

9.2.1.1 Phycobiliprotein analysis

Notably, PBP content was highest in *A. secundatum*, with phycoerythrin and phycocyanin concentrations of $17.56 \pm 0.79 \text{ mg g}_{\text{DW}}^{-1}$ and $5.14 \pm 0.23 \text{ mg g}_{\text{DW}}^{-1}$, respectively, substantially exceeding those in *G. gracilis* and *P. palmata*. This pattern aligns with the treatments exhibiting the strongest inhibitory effects, suggesting a clear association between phycobiliprotein content and microalgal growth suppression. In the PBP absorption spectrum, distinct peaks were observed at 564 nm and 614 nm, corresponding to phycoerythrin and phycocyanin, respectively (Nobel, 2020). The peak at 498 nm is a shoulder peak from phycoerythrin as well (Bryant, 1982; Rossano et al., 2003).

A distinct absorption peak at 326 nm was observed in the analysis of *P. palmata* extracts, which may indicate the presence of mycosporine-like amino acids (MAAs). This interpretation is supported by Beach et al. (1997), who reported that macroalgal UV-absorbing maxima between 317.5 and 344.5 nm typically comprise multiple components, with derivative peaks commonly occurring between 317.5 and 365 nm. These features were attributed to the presence of MAAs (Beach et al., 1997). These MAAs are commonly seen as photoprotectors to protect cell organelles from UV-radiation (Beach et al., 1997; Sinha et al., 2000). The protection from UV-light could explain the observed stimulation in *P. palmata* for low extract concentrations, assuming that the effect is overshadowed at higher concentrations, for example by the reduced light availability due to PBP absorption of potential allelopathic interactions. A slight stimulation is also observed for *G. gracilis*, although MAAs were not measured in the absorbance scan. However, species of the *Gracilariales* are commonly reported to also contain MAAs (Barceló-Villalobos et al., 2017; Sinha et al., 2000; Torres et al., 2016).

9.2.1.2 Growth inhibition

Fu et al. (2022) evaluated growth inhibition of *Ulva prolifera* after exposure to dry powder of the red seaweed *Neopyropia yezoensis*. The dry powder was directly added into the enriched seawater medium, as such resulting in a polar extract. They report significant growth inhibition from concentrations exceeding $1.2 \text{ g}_{\text{DW}} \text{ L}^{-1}$ after nine days (Fu et al., 2022). This value is remarkably close to the obtained results in this thesis, where *A. secundatum* led to inhibition at $2 \text{ g}_{\text{DW}} \text{ L}^{-1}$, but not for $1 \text{ g}_{\text{DW}} \text{ L}^{-1}$.

Fu et al. (2022) concluded that, after ruling out environmental variables as potential confounding factors, the most plausible explanation for the observed inhibition is the presence of bioactive compounds, suggesting an allelopathic mode of action.

In this thesis study, all experimental conditions used in the control groups, including growth medium composition, temperature, salinity, and light regime, were identical to those used in the treatments with seaweed extracts. Therefore, these factors cannot account for the observed growth inhibition. While pH may fluctuate over the course of the assay due to the natural increase associated with microalgal photosynthetic activity, often exceeding values of 8.5, microalgae are generally tolerant to such elevated pH levels (Beltrán-Rocha et al., 2021; Goldman et al., 1982). Consequently, pH variation is unlikely to have contributed significantly to the inhibitory effects observed.

However, it should be noted that the polar extracts, particularly at higher concentrations of *A. secundatum*, resulted in a noticeable pink coloration to the medium. This discoloration may have reduced light availability in the Erlenmeyer flasks, potentially affecting microalgal growth. Additionally, as PBPs are specialized for light absorption, their presence in the water column could attenuate wavelengths essential for photosynthesis, thereby contributing to growth inhibition. The pigments in *P. tricornutum* have absorption peaks between 400 and 500 nm and include chlorophyll a, chlorophyll c, fucoxanthin, β -carotene, diatoxanthin, diadinoxanthin, violaxanthin, antheraxanthin and zeaxanthin (Kuczynska et al., 2015). For *T. lutea* the main photosynthetic pigments are chlorophyll *a*, carotenoid and to a lesser extend chlorophyll *c* (Alkhamis & Qin, 2016). While chlorophyll *a* and carotenoids also have their absorption maxima between 400 and 500 nm, chlorophyll *c* absorption is in the 500 to 600 nm range (Dougherty et al., 1970; Guidi et al., 2017). The main absorption peaks in the PBP extracts were 498, 564, and 614 nm. The absorption spectra of the PBP and both *P. tricornutum* and *T. lutea* pigments show some overlap, although not much, except for chlorophyll *c*. This suggests that the reduced light availability due to PBP could indeed be the cause of growth inhibition, the considerable overlap between absorbance of chlorophyll *c* and the PBP could also explain the enhanced sensitivity of *T. lutea*.

As discussed, the PBP seems to fit the observed trends well, but due to their light absorbing qualities, it is difficult to exclude reduced light availability as a possible source of the inhibition. In their investigation of allelochemicals produced by cyanobacteria and their inhibitory effects on the growth of the green microalgae *Chlorella fusca* and *Chlamydomonas reinhardtii*, Karseno et al. (2009) demonstrated that *Oscillatoria* sp. and *Scytonema* sp. contain and possibly secrete an extracellular compound, referred to as OsPP (*Oscillatoria* pink pigment), which significantly suppresses algal growth in a concentration-dependent manner. Analytical characterization of OsPP revealed it to be a phycoerythrin-like phycobiliprotein. Based on the strong chemical similarity, the authors propose that phycoerythrin itself may exhibit comparable inhibitory properties (Karseno et al., 2009). Since the PE content in *A. secundatum* is significantly higher

than that of *P. palmata*, this could also explain the inhibitory characteristics of the seaweed extracts.

Other polar metabolites should also be considered as potential reasons for the observed inhibition. Macías et al. (2008) suggests phenolic compounds to be the most commonly acclaimed allelochemicals originating from aquatic organisms. Phenolic compounds are present across all three major seaweed groups, though they have been most extensively studied in brown seaweeds, which uniquely produce phlorotannins (Cotas, Leandro, Monteiro, et al., 2020; Sadeghi et al., 2024). In contrast, phenolics from red seaweeds have received comparatively limited attention, despite the fact that bromophenols are predominantly associated with this group (Cotas, Leandro, Monteiro, et al., 2020; Sadeghi et al., 2024). Additionally, several red algal species have been reported to exhibit relatively high total phenolic contents in polar extracts. (Chakraborty et al., 2015). Most papers on the allelochemical potential of phenols focus on cyanobacteria as test organism. For example, Q. Zhao et al. (2023), found that quercetin (a flavonoid) inhibits *Microcystis aeruginosa* growth with 40 mg L⁻¹ resulting in an inhibition of over 90% at 96 hours. Furthermore, they found a negative relation between chlorophyll *a* content with increasing doses of quercetin. Similarly, Huang et al. (2020) indicate inhibitory effects of the phenolic compounds pyrogalllic acid and gallic acid by inhibition of photosystem II. Moreover, they concluded that polyphenols released at low concentrations negatively affect phytoplankton growth (Huang et al., 2020).

In contrast, Chukwuma et al. (2024) concluded that phenolic compounds were unlikely to be the primary drivers of the growth inhibition observed in their allelopathy experiments, despite the fact that the seaweed extracts contained considerable amounts of phenolic acids, flavonoids, and phlorotannins in the case of brown seaweeds. Their conclusion was based on the observation that extracts with relatively lower phenolic content induced stronger inhibitory effects, suggesting that other bioactive constituents may play a more significant role in the allelopathic activity. Considering this, the PBP seems the most likely allelochemical in the polar extracts of this thesis.

9.2.1.3 Application potential

Assuming PE is the sole inhibitory compound, its required concentration for algal growth suppression can be estimated. Based on an EC₅₀ value of 1.014 g_{DW} L⁻¹ for *T. lutea* inhibition and a PE content of 17.56 mg g_{DW}⁻¹ in the *A. secundatum* extract, a concentration of 17.81 mg L⁻¹ of PE would be necessary to achieve 50% growth inhibition. These concentrations are relatively high for practical applications in marine environments. For instance, inhibiting *T. lutea* growth in a 1 m³ tank would require 17.81 g of PE, corresponding to approximately 1014 g_{DW} of *A. secundatum*. Assuming optimized aquaculture practices, a yield of 1 kg of seaweed per square meter annually can be achieved, this would equate to a cultivation area requirement of 1.01 m² per treatment (World Bank Group, n.d.). However, considering the degradation rate of PE—99 days at 4 °C and 23 days at 25 °C (Kannaujiya & Sinha, 2016)—a continuous supply would necessitate a larger cultivation area, estimated between 4 and 16 m² per cubic meter of treated water. Although, *A.*

secundatum can be easily grown vertically on land, thereby saving space, but creating new impracticalities associated with land-based aquaculture. Moreover, due to the polar nature of PE, application in open marine systems is likely impractical, as the compound would rapidly dilute, significantly reducing its efficacy.

For this reason, nonpolar allelochemicals present greater potential for application as algicidal agents in marine environments, such as their incorporation into antifouling coatings on ship hulls. This potential is further supported by recent findings from Chukwuma et al. (2024), who reported that less polar extracts—obtained using ethyl acetate and hexane—exhibited significantly stronger algal growth inhibition compared to more polar extracts derived using water and methanol.

9.2.2 Nonpolar extracts

In general, the surface extracts exhibited significant growth inhibition at higher concentrations, with the notable exception of the *A. secundatum* extract on *P. tricornutum*, which showed no significant inhibitory effect. Nonpolar surface extracts induced only mild inhibition, insufficient to determine EC_{50} values. This suggests either a limited potency of the bioactive compounds or a low abundance of active substances at the surface.

A similar pattern was observed for the total extracts, though with generally stronger effects. For instance, the total extract of *P. palmata* on *T. lutea* produced both a lower EC_{10} ($0.082 \text{ g}_{FW} \text{ L}^{-1}$ vs. $1.431 \text{ g}_{FW} \text{ L}^{-1}$ for the surface extract) and an EC_{50} of $1.098 \text{ g}_{FW} \text{ L}^{-1}$. Interestingly, although the EC_{10} of the total *P. palmata* extract on *P. tricornutum* was slightly lower than that of the surface extract, it resulted in a greater overall reduction in growth. The initial decline in growth was more gradual, but the inhibition progressed more consistently with increasing concentration, indicating a clearer concentration-dependent response. In contrast, no EC_{10} could be calculated for the total extract of *G. gracilis* on either test species, although its surface extract did yield an EC_{10} in the *T. lutea* assay.

In summary, *A. secundatum* demonstrated no clear inhibitory effect in either nonpolar extract, while *G. gracilis* exhibited only slight inhibition in both surface and total extracts, with no major differences between the two. Notably, the total extracts of *P. palmata* appear to be considerably more potent than the corresponding surface extracts.

9.2.2.1 Fatty acid analysis and EPA inhibition test

GC-MS analysis revealed clear differences in fatty acid content and composition across the nonpolar extracts of the three red algae species. Overall, total extracts were more enriched in polyunsaturated fatty acids (PUFAs), especially EPA, while surface extracts were dominated by saturated and monounsaturated fatty acids, such as palmitic acid. *P. palmata* exhibited the highest total fatty acid content, particularly in its total extract ($147.08 \mu\text{g g}_{FW}^{-1}$), with EPA as the dominant component (76.36% relative abundance). Similar fatty acid profiles were reported by Schmid et al. (2014), who made an extraction from dry weight and also report EPA and palmitic

acid as the two most dominant fatty acids across all the Rhodophyta they tested (including *P. palmata* and *G. gracilis*). Furthermore, they also report arachidonic acid as uniquely higher in *G. gracilis*. Moreover, Sohrabipour et al. (2019) indicate that EPA is typically found in high relative percentages in Rhodophyta, but not in Chlorophyta or Phaeophyceae.

Given the strongest observed growth inhibition was associated with the total extract of *P. palmata*, and the notable EPA content in this extract, EPA was subsequently evaluated for its potential inhibitory qualities. The dose-response analysis revealed species-specific sensitivity to EPA exposure. *T. lutea* showed no clear dose-dependent inhibition, likely due to significant growth suppression by the solvent itself, as confirmed by statistical comparisons with the blank (t-test, $p < 0.05$). As a result, no EC_{10} value could be determined for this species. In contrast, *P. tricornutum* exhibited stable growth at lower EPA concentrations, with a significant reduction only at the highest concentration tested (0.6 mg L^{-1} ; ANOVA, $p < 0.05$). In this case, the absence of solvent-related effects confirmed the validity of the observed response, enabling the determination of an EC_{10} value of 0.579 mg L^{-1} . Although no EC_{50} value could be derived in this thesis, Wu et al. (2006) reported EC_{50} values of 100 mg L^{-1} and 125 mg L^{-1} for EPA after 24 hours of exposure in *Monoraphidium contortum* and *Chlorella vulgaris*, respectively.

The concentration of 0.6 mg L^{-1} was found in the highest concentration of *P. palmata* total extract ($5 \text{ g}_{\text{FW}} \text{ L}^{-1}$). As discussed in Section 7.4.2, this concentration is only relevant in land-based aquaculture and not found in the marine environment, which suggests that both species show limited sensitivity at environmentally relevant concentrations. Moreover, these total extracts were made by inflicting cell lysis in the seaweed, which will not typically happen to entire seaweeds at once. Therefore, EPA alone does not seem to explain the observed inhibition. However, given the observed solvent effects and the fact that EPA is a nonpolar compound which was exposed in a polar medium, therefore potentially limiting contact with the test organisms, a better test setup is required to arrive at a clear conclusion. For example, Kamaya et al. (2003) used dimethyl sulfoxide (DMSO) as solvent instead of ethanol. Furthermore, the observed dominance of palmitic acid on the surface suggests that algae cover their thalli with this fatty acid for a reason, so this might also prove an interesting area of future research.

In contrast, surface extracts across species generally contained lower EPA levels and were richer in palmitic acid, suggesting a structural or protective role for saturated fatty acids at the algal surface. This matches the observations of Papazian et al. (2019), who analyzed the leaf surface from seagrass *Zostera marina*, also indicating palmitic acid to be the most abundant fatty acid together with myristic acid, forming a protective layer covering the entire leaf. Notably, Wu et al. (2006) reported 24-hour EC_{50} values for palmitic acid that were approximately tenfold lower for *Monoraphidium contortum* (9.2 mg L^{-1}) and twofold lower for *Chlorella vulgaris* (59.1 mg L^{-1}), compared to the corresponding values for eicosapentaenoic acid.

Furthermore, the surface extract of *G. gracilis* contained fatty acids such as dihomog- γ -linolenic acid and arachidonic acid, which were not found in other extracts, indicating species-specific metabolic features. This highlights functional and compositional differentiation between extract types and species.

9.2.2.2 Growth inhibition

A search of the literature on Google Scholar (May 2025) using the keywords “Rhodophyta, growth inhibition, microalgae,” and including the term “non-polar” or “nonpolar” yielded no relevant publications. This suggests that studies specifically addressing the inhibitory effects of non-polar Rhodophyta extracts on microalgae are currently lacking. However, some growth inhibition assays with metabolites from other marine sources or on different test organisms exist. For example, Kakisawa et al. (1988) found the FA stearidonic acid (18:4 n-3), extracted from the brown seaweed *Cladosiphon okamuranus*, to hold allelopathic qualities against multiple microalgae, including the HAB *Chattonella antiqua*. Stearidonic acid is an intermediate in the conversion of alpha-linoleic acid to EPA (Calder, 2021). Moreover, it is reported in the Rhodophyta FA analysis of Schmid et al. (2014), where they quantified stearidonic acid in *P. palmata*, but did not find it in *G. gracilis*. This may help explain the pronounced growth inhibition caused by *P. palmata* observed in this thesis. However, this fatty acid was not identified in the present fatty acid analysis, indicating that further investigation is necessary to evaluate this hypothesis.

Additionally, Rocha et al. (2011) made both dichloromethane and hexane extracts from the red seaweed *Centroceras clavulatum*. They report that both extracts inhibit fungal growth of *Cladosporium cladosporioides* and *C. sphaerospermum*. Additionally, GC-MS analysis revealed that hexadecanoic acid and pentadecanoic acid were the predominant fatty acids present in both extracts. EPA was also detected, but ranked as the fifth most abundant compound. Even more closely related to this thesis, Abdul Malik et al. (2020) reports that during the cultivation of the red alga *Halymenia floresii* in an integrated multi-trophic aquaculture (IMTA) system, researchers observed an absence of biofouling by green algae or sessile invertebrates. This suggested that *H. floresii* may release allelopathic surface metabolites that prevent the settlement and growth of fouling organisms (Abdul Malik et al., 2020).

To investigate this, surface-associated compounds were extracted using a dip method with n-hexane, and whole-cell metabolites were extracted using the same solvent over 24 hours. Surface extracts (600 ng cm⁻²) and whole-cell extracts (4.5 μ g mg⁻¹) were chemically profiled using LC-MS and Mass Bank searches. Bioactivity tests showed that surface extracts interfered with bacterial cell to cell communication (*quorum sensing*), particularly in bacterial strains isolated from *H. floresii*'s surface, such as *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, and *Ruegeria* (Abdul Malik et al., 2020). Abdul Malik et al. (2020) suggest that compounds like flavonoids, brominated substances, and volatile halogenated organic compounds (VHOCs) may play a role in *H. floresii*'s chemical defense. This interference with bacterial communication, rather than direct

antimicrobial action, may reduce biofouling and the risk of resistant strain development in aquaculture systems. The study highlights the red seaweed *H. floresii* as a promising model for sustainable biofouling control in IMTA, with future work planned on investigating surface defenses in chemically undefended or bleached individuals (Abdul Malik et al., 2020).

Next to the fatty acids discussed above, other metabolites have been reported as allelochemicals in Rhodophyta. For example, *Corallina pilulifera* produces bromoform to eliminate epiphytic organisms, especially microalgae, from its thallus (Ohsawa et al., 2001). Moreover, numerous volatile halogenated organic compounds (VHOCs) have been reported to be released by Rhodophyta. These include brominated compounds such as polybromomethanes, bromoform, and dibromochloromethane; iodinated compounds such as diiodomethane, sec-butyl iodide, butyl iodide, methyl iodide, and chloriodomethane; and other halogenated compounds including alkyl monohalides, dihalides, chloroform, perchloroethylene, methylchloroform, carbon tetrachloride, and trichloroethylene (Garcia-Jimenez et al., 2013; Gschwend et al., 1985; Mtolera et al., 1996; Ohsawa et al., 2001). According to Gschwend et al. (1985) these VHOCs can be released in concentrations up to milligrams per day by some macroalgae. However, Ohsawa et al. (2001) reported that bromoform emission rates from *C. pilulifera* and *Lithophyllum yessoense* did not significantly inhibit microalgal growth. They found a threshold of $0.09 \text{ ng min}^{-1} \text{ cm}^{-2}$ for microalgal growth inhibition, but production rates in *C. pilulifera* and *L. yessoense* are 0.06 and $0.02 \text{ ng min}^{-1} \text{ cm}^{-2}$, respectively. Notably, the production rates suggested by Gschwend et al. (1985) could be high enough to reach this threshold. Therefore, further research towards VHOCs is required to eliminate or identify these metabolites as allelochemicals.

Lastly, flavonoids are also reported to be potential allelochemicals (Abdul Malik et al., 2020; Chukwuma et al., 2024). Sudatti et al. (2020) extracted elatol (a sesquiterpenoid, flavonoid) from *Laurencia dendroidea*, which they reported to inhibit photosynthetic activity of *L. dendroidea* itself when exposed to it. In their book, Hellio et al. (2009) extensively investigated marine natural products for the use of antifouling activities. For Rhodophyta, elatol is the most extensively discussed bioactive molecule. It inhibits bacterial growth at MIC values comparable to commercial antibiotics and prevents mussel byssal attachment (da Gama et al., 2008; Hellio et al., 2009; Santhanaraju Vairappan et al., 2001).

In summary, although Rhodophyta derived (nonpolar) metabolites have not been tested as allelochemicals for the use against microalgae, their allelopathic potential has been reported multiple times and is therefore worth further investigation.

9.2.2.3 Application potential

The application potential of nonpolar allelochemicals derived from *P. palmata* presents a notable advantage over polar metabolites: their hydrophobic nature allows for surface application

without rapid dilution in aquatic environments. This characteristic is particularly relevant for antifouling applications, such as preventing microalgal biofilm formation on ship hulls or submerged surfaces.

Although this study did not succeed in identifying a single metabolite as the primary inhibitory compound, the total nonpolar extract exhibited a measurable inhibitory effect, with an EC_{50} value of $1.098 \text{ g}_{FW} \text{ L}^{-1}$. For practical application, such as incorporation into an antifouling paint, only surface-level inhibition is required. While surface-based bioassays were beyond the scope of this work, a theoretical estimation of biomass requirements can be made. To do so, concentrations in mass per surface area are more relevant and often used for surface extracts, as Abdul Malik et al. (2020) did in their research. However, estimating the surface area of *G. gracilis* and *A. secundatum* will prove very difficult due to their morphology.

Assuming a paint formulation containing the extract concentration equivalent to the EC_{10} ($1.098 \text{ g}_{FW} \text{ L}^{-1}$) and a moderate application rate of 0.3 L m^{-2} , the resulting biomass requirement would be $0.329 \text{ g}_{FW} \text{ m}^{-2}$. Given that the wetted surface area of a typical containership is approximately 6761 m^2 (Bonfim et al., 2024), a full coating would require an estimated 2.2 kg_{FW} of *P. palmata*.

Production data from *P. palmata* cultivation suggest this biomass requirement is feasible. J. Matos et al. (2006) report yields of $241 \text{ g}_{FW} \text{ m}^{-2} \text{ day}^{-1}$ under optimal conditions, while Sanderson et al. (2012) estimate yields of $180 \text{ t}_{FW} \text{ ha}^{-1} \text{ year}^{-1}$, equivalent to $49.3 \text{ g}_{FW} \text{ m}^{-2} \text{ day}^{-1}$. Based on the former, a cultivation area of one square meter could theoretically produce sufficient biomass for a full ship coating within approximately nine days. An additional benefit of the nonpolar extract is its complete transparency, which may enable the development of antifouling coatings for transparent surfaces such as aquarium windows, where visual clarity is essential.

9.2.3 DPPH assays

No statistical significant differences in DPPH radical scavenging are observed between the seaweed extracts. Heffernan et al. (2015) also performed DPPH assays on five Rhodophyta, but with different endpoints (not expressed in Trolox equivalent). Similar to this thesis, they indicate *G. gracilis* to have the lowest antioxidant activity. However, Nova et al. (2023) reports equal DPPH radical scavenging for *P. palmata* and *G. gracilis* with 0.127 ± 0.010 and $0.115 \pm 0.005 \text{ mgTe g}_{DW}^{-1}$, respectively. This is within the same order of magnitude as was found in this thesis, with also no significant difference.

A search on Google Scholar yielded no published results specifically addressing DPPH antioxidant activity in *Acrochaetium secundatum* (containing the words: “DPPH *Acrochaetium secundatum*”, searched in May 2025).

9.2.4 pH

Although an increase in pH was observed at the end of each test, no clear correlation between cell density and pH was found in this thesis. This contrasts with the data of Schmidt & Hansen (2001), who observed similar increases in pH at the end of each test but also reported more clearly defined correlations between algal density and pH.

10 Future research

This study highlights the promising yet underexplored potential of metabolites derived from Rhodophyta as allelochemicals against microalgae. Future work should prioritize the isolation and identification of specific bioactive compounds from potent extracts—particularly those of the nonpolar extracts, given their high application potential. Candidates such as EPA, palmitic acid, stearidonic acid, brominated compounds, and flavonoids like elatol warrant targeted analysis through advanced GC-MS and LC-MS techniques, especially for compounds not detected in the current study. Furthermore, the clear differences in fatty acid profiles between surface and total extracts suggest distinct ecological roles, with surface-associated metabolites like palmitic acid potentially involved in antifouling or microbial defense. Further research should examine these functional roles, focusing on species-specific metabolite production and the physical location of bioactive compounds.

To reduce the influence of solvent-related artifacts and improve the delivery of hydrophobic compounds, inhibition tests should be repeated using alternative solvents such as DMSO instead of ethanol. In addition, metabolite fractionation and combinatorial bioassays could help determine whether observed effects are due to individual compounds or synergistic interactions.

Beyond the inhibition assays performed in this thesis, future studies should include surface-based inhibition assays and small-scale field or co-culture experiments to assess the ecological feasibility and long-term effectiveness of nonpolar extracts. Special consideration should be given to their application in antifouling coatings, including formulation stability, transparency, and potential impacts on non-target organisms. The optically clear nature of these extracts may also allow for innovative uses on transparent surfaces, such as aquarium glass or underwater monitoring equipment.

Overall, advancing our understanding of the chemical diversity, ecological roles, and practical applications of nonpolar Rhodophyta metabolites will be key to unlocking their full biotechnological potential.

11 Conclusion

This study demonstrates that Rhodophyta extracts exert species-specific and extract-type-specific inhibitory effects on marine microalgae, with *Tisochrysis lutea* generally displaying greater sensitivity than *Phaeodactylum tricornutum*. Among the different extract types, both polar and nonpolar fractions elicited biological responses, though with distinct characteristics and implications for ecological function and potential application. Notably, polar extracts—particularly those from *Acrochaetium secundatum*—showed considerable inhibitory activity, which can likely be attributed to their high phycobiliprotein (PBP) content, especially phycoerythrin. Spectral analysis supported the presence of these pigments, aligning with the observed bioactivity. However, it remains challenging to disentangle direct biochemical inhibition from potential indirect effects, such as reduced light availability due to pigment absorption. Additional polar compounds, such as phenolics, may also contribute to the observed inhibition, though their role appears secondary in this context. Despite their potency, the practical use of polar compounds like PBPs in antifouling or algicidal applications is limited by their rapid dilution in open aquatic systems and the relatively high concentrations required to achieve inhibitory effects.

In contrast, nonpolar extracts—especially the total extract of *Palmaria palmata*—exhibited the strongest and most consistent inhibitory effects across both microalgal species, as evidenced by low EC_{10} and EC_{50} values. Fatty acid profiling revealed a high content of eicosapentaenoic acid (EPA) in this extract, which is known for its potential allelopathic properties. However, EPA alone could not fully account for the extent of growth inhibition observed, suggesting that additional allelochemicals—possibly including flavonoids, fatty acid derivatives, or volatile halogenated organic compounds (VHOCs)—are likely involved. Surface extracts generally exhibited weaker activity but displayed a distinct fatty acid profile dominated by palmitic acid, indicating functional differentiation between nonpolar extract types and potentially different ecological roles. The literature supports the allelopathic potential of several nonpolar metabolite classes, though their specific mechanisms of action on marine microalgae remain underexplored.

The practical application potential of *P. palmata* nonpolar extracts appears promising, particularly in antifouling strategies, due to their efficacy at moderate concentrations, optical transparency, and the scalable nature of *P. palmata* biomass production in aquaculture. Antioxidant activity, as measured by DPPH radical scavenging capacity, was also highest in *P. palmata*, further supporting its bioactive potential. Interestingly, pH fluctuations observed during the growth inhibition tests did not reliably correlate with biomass reduction, contrasting with previous studies and suggesting that pH shifts may not be a consistent indicator of allelopathic activity in this context.

Together, these findings highlight the ecological complexity and biotechnological potential of Rhodophyta-derived compounds. They underscore the importance of integrating chemical profiling with targeted bioassays to identify both functional allelochemicals and their modes of action. Future work should aim to isolate and characterize the specific active constituents responsible for inhibition and explore their ecological relevance in natural assemblages, as well as their feasibility in applied contexts such as biofouling control or microalgal population management.

12 References

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13 Appendix

A.1 Flow cytometer settings

Option	Setting
Events to display	1000
Events to record	10000
Time to record	60 sec
Sampling speed	Medium (30 $\mu\text{L min}^{-1}$)
<i>T. Lutea</i> definition	0 – 200*10 ⁴ FSC-A, 10 ⁴ – 10 ⁶ FITC-A
<i>P. tricornutum</i> definition	0 – 360*10 ⁴ FSC-A, 1.1*10 ³ – 1.5*10 ⁴ FITC-A

A.2 F/2 medium

Component	Stock solution concentration (g L ⁻¹)	Volume of stock into F/2 medium (mL L ⁻¹)	Final concentration In F/2 medium (g L ⁻¹)
NaNO ₃	75	1	75 * 10 ⁻³
NaH ₂ PO ₄ ·H ₂ O	5	1	5 * 10 ⁻³
Na ₂ CO ₃	30	1	30 * 10 ⁻³
Trace metals:		1	
CuSO ₄ ·5H ₂ O	9.8 * 10 ⁻³		9.8 * 10 ⁻⁶
Na ₂ MoO ₄ ·H ₂ O	6.3 * 10 ⁻³		6.3 * 10 ⁻⁶
ZnSO ₄ ·7H ₂ O	22 * 10 ⁻³		22 * 10 ⁻⁶
CoCl ₂ ·6H ₂ O	10 * 10 ⁻³		10 * 10 ⁻⁶
MnCl ₂ ·4H ₂ O	0.18		0.18 * 10 ⁻³
Vitamins:		0.5	
Thiamine HCl (vit. B1)	0.2		0.1 * 10 ⁻³
Biotin (vit. H)	10 ⁻⁴		50 * 10 ⁻⁹
Cyanocobalamin (vit. B12)	10 ⁻³		0.5 * 10 ⁻⁶
Antibiotic:		0.2	
Chloramphenicol	1		0.2 * 10 ⁻³

A.3 Non normalized plots

Polar extracts on *Tisochrysis lutea*

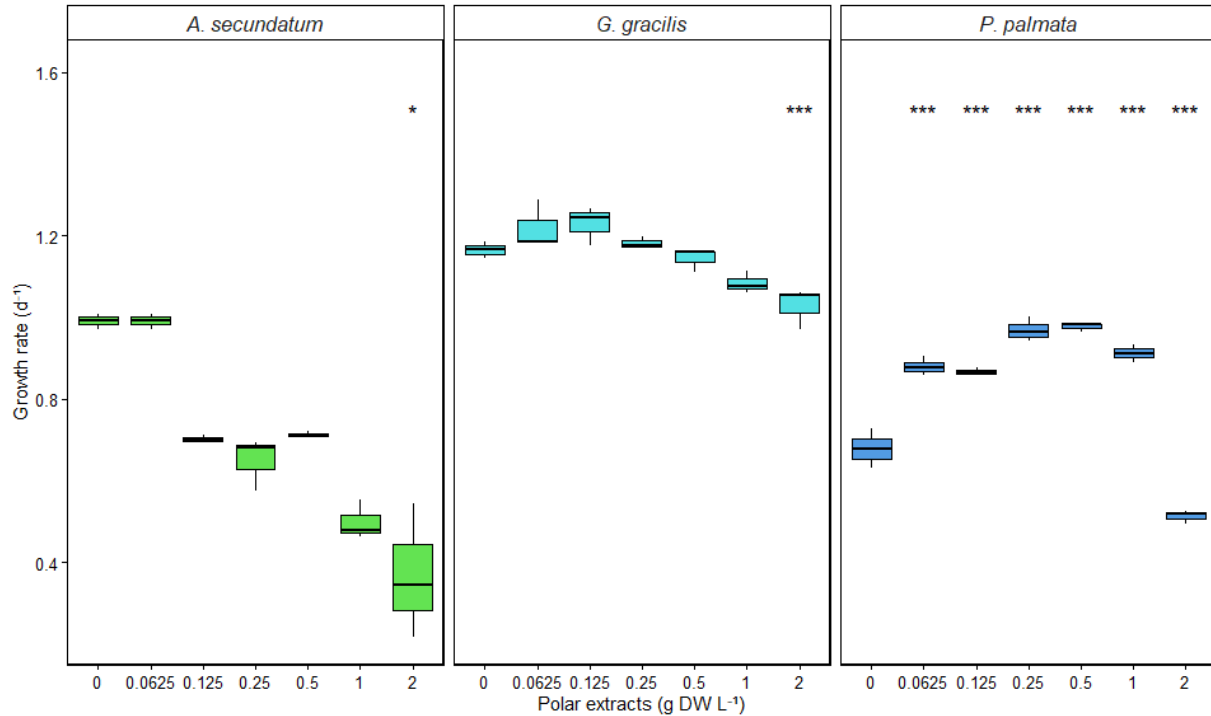


Figure 21. Polar Rhodophyte extracts' growth inhibiting effects on *Tisochrysis lutea*. The concentrations (expressed in gram dry weight per liter (g_{DW} L⁻¹)) are shown on they x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (***) for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). *G. gracilis* and *P. palmata* are tested using ANOVA. The data of *A. secundatum* extracts do not satisfy the homogeneity criterium with the Levene's test and are therefore tested with Kruskal-Wallis, a more conservative non-parametric test.

Polar extracts on Phaeodactylum tricornutum

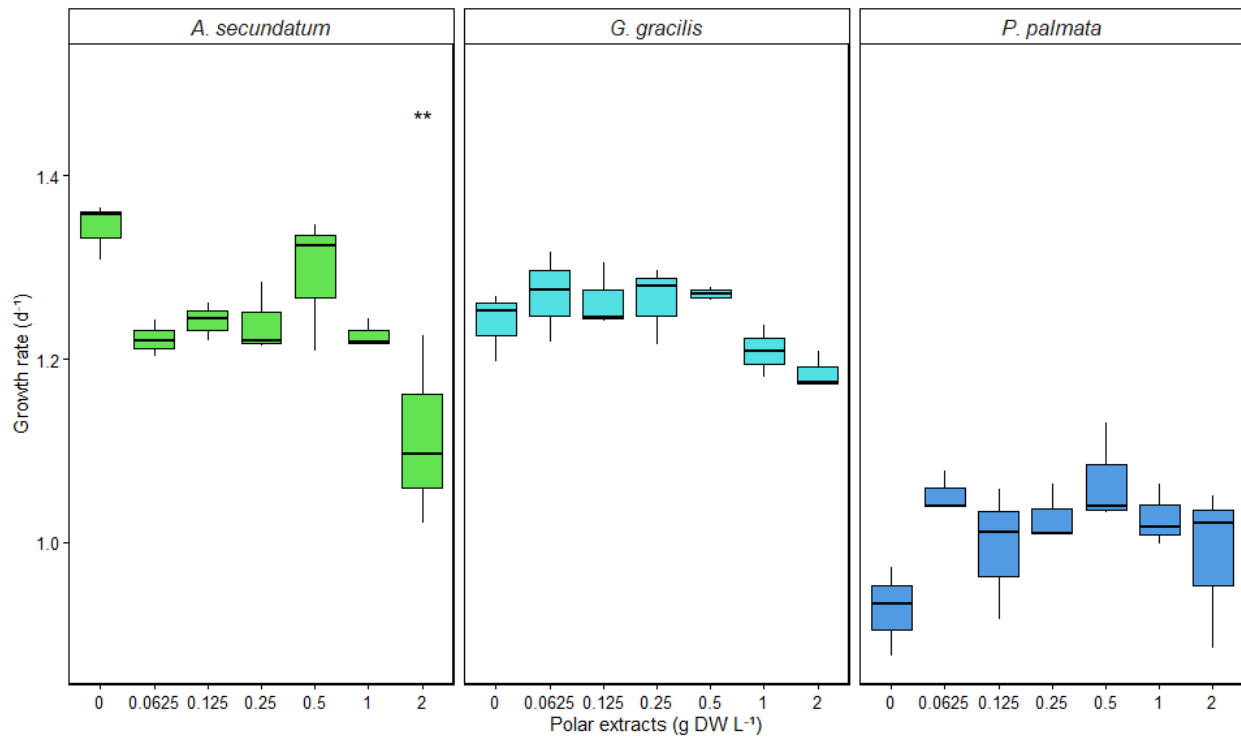


Figure 22. Polar Rhodophyte extracts' growth inhibiting effects on *Phaeodactylum tricornutum*. The concentrations (expressed in gram dry weight per liter (g_{DW} L⁻¹)) are shown on they x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (***) for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance.

*Nonpolar surface extracts on *Tisochrysis lutea**

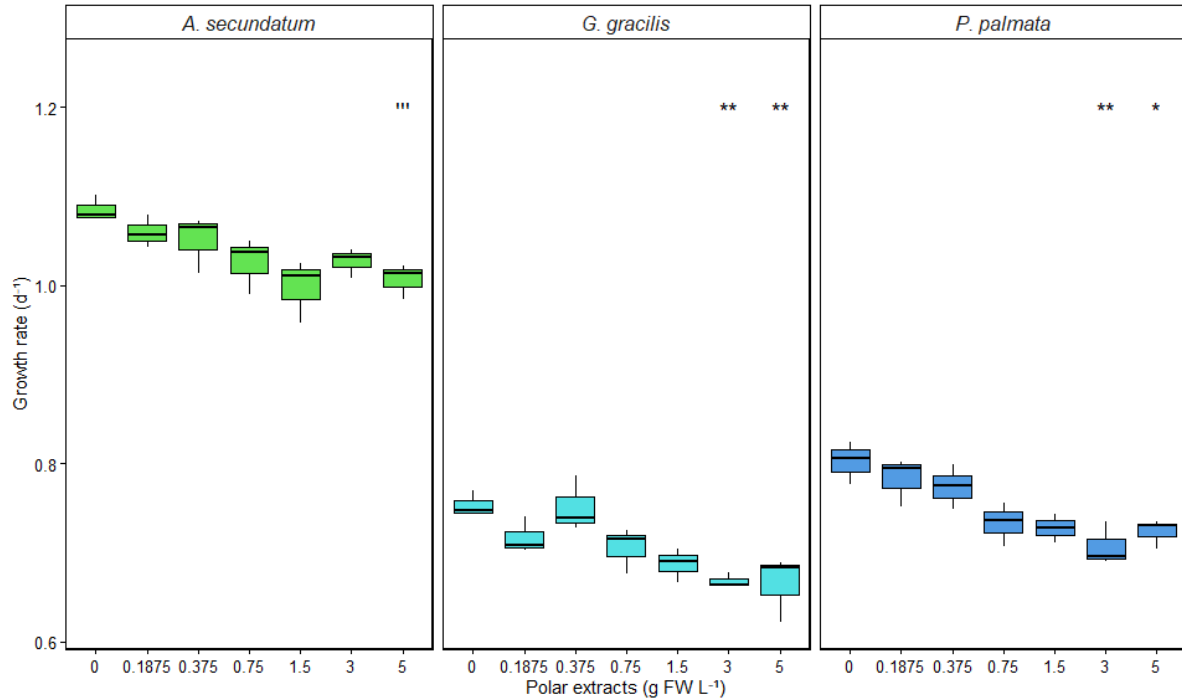


Figure 23. Nonpolar surface Rhodophyte extract effects on *Tisochrysis lutea*. The concentrations (expressed in gram dry weight per liter (g_{FW} L⁻¹)) are shown on they x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (*** for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance. The data of *A. secundatum* extracts do not satisfy the homogeneity criterium with the Levene's test and are therefore tested with Kruskal-Wallis, a more conservative non-parametric test. An extra T-test was performed between the control and highest concentration because of the conservative nature of the Kruskal-Wallis test. The same significance levels are used, but shown as a prime symbol (') instead of an asterisk.

Nonpolar surface extracts on *Phaeodactylum tricornutum*

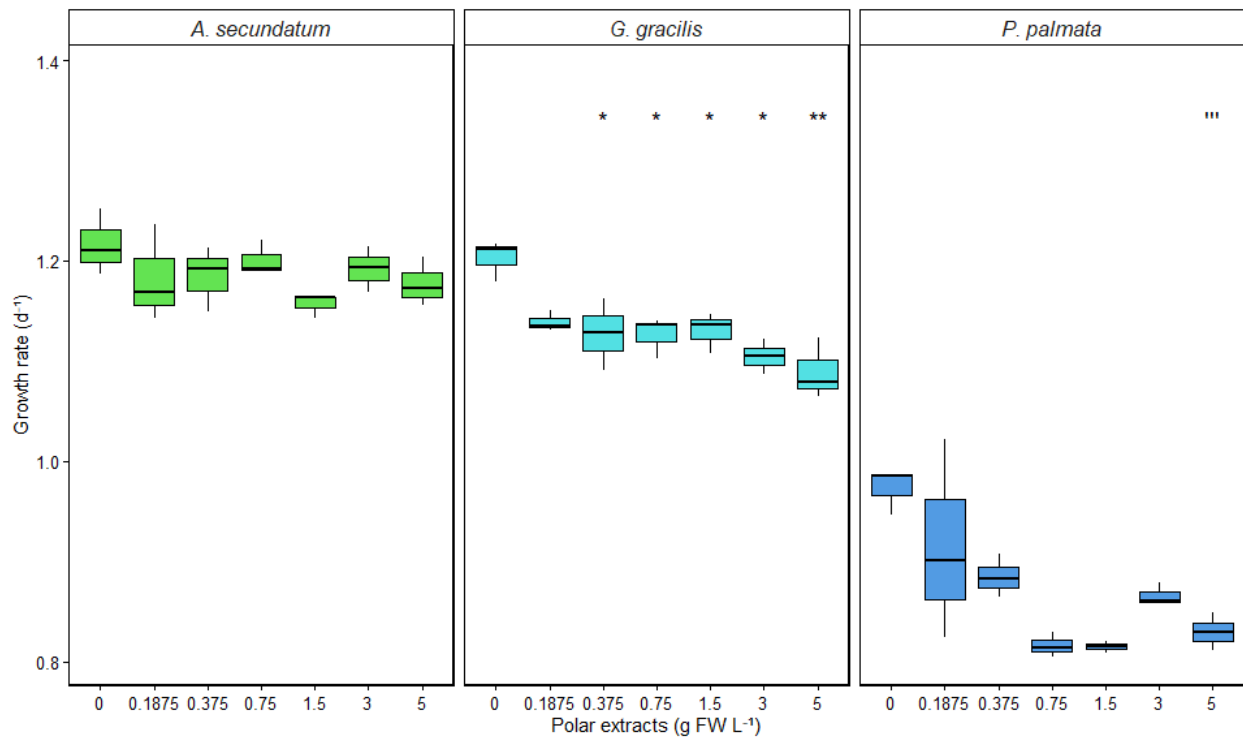


Figure 24. Nonpolar surface Rhodophyte extract effects on *Phaeodactylum tricornutum*. The concentrations (expressed in gram dry weight per liter (g_{FW} L⁻¹)) are shown on they x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (***) for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance. The data of *A. secundatum* extracts do not satisfy the homogeneity criterium with the Levene's test and are therefore tested with Kruskal-Wallis, a more conservative non-parametric test. An extra T-test was performed between the control and highest concentration because of the conservative nature of the Kruskal-Wallis test. The same significance levels are used, but shown as a prime symbol (') instead of an asterisk.

Nonpolar total extracts on Tisochrysis lutea

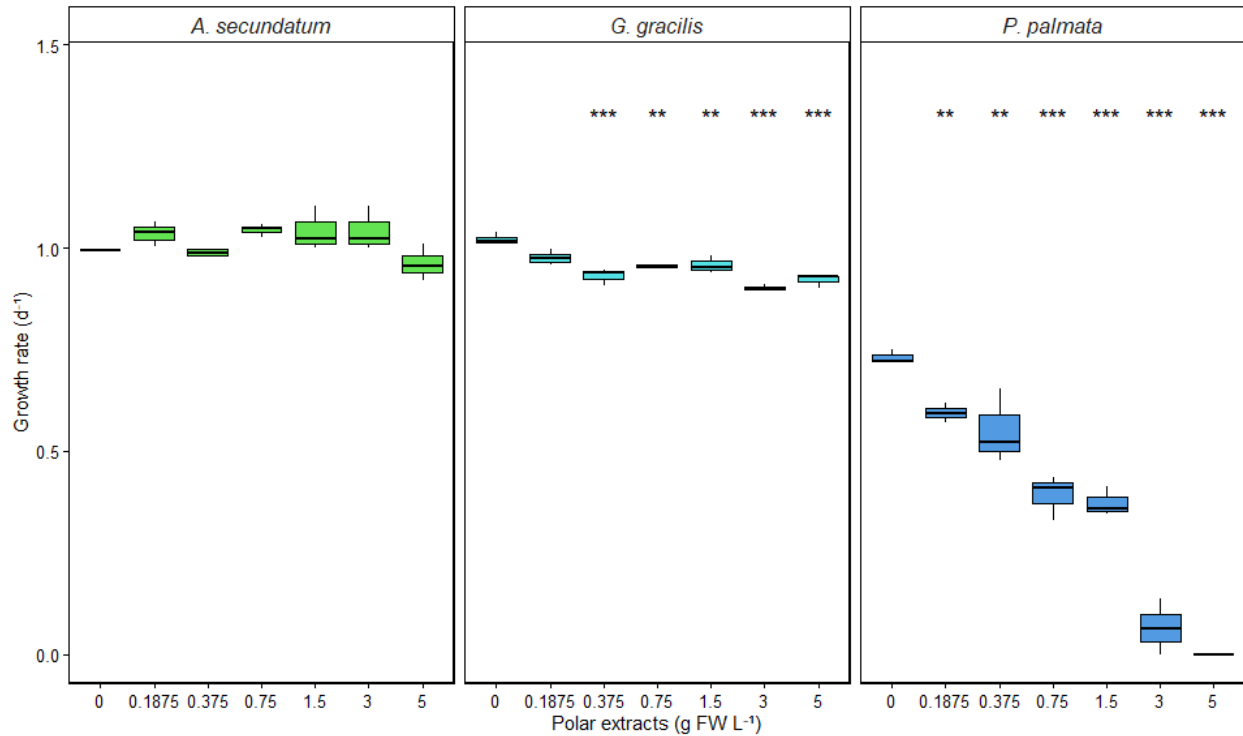


Figure 25. Nonpolar total Rhodophyte extracts' growth inhibiting effects on *Tisochrysis lutea*. The concentrations (expressed in gram dry weight per liter ($\text{g}_{\text{FW}} \text{L}^{-1}$)) are shown on the x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (***) for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance.

Nonpolar total extracts on *Phaeodactylum*

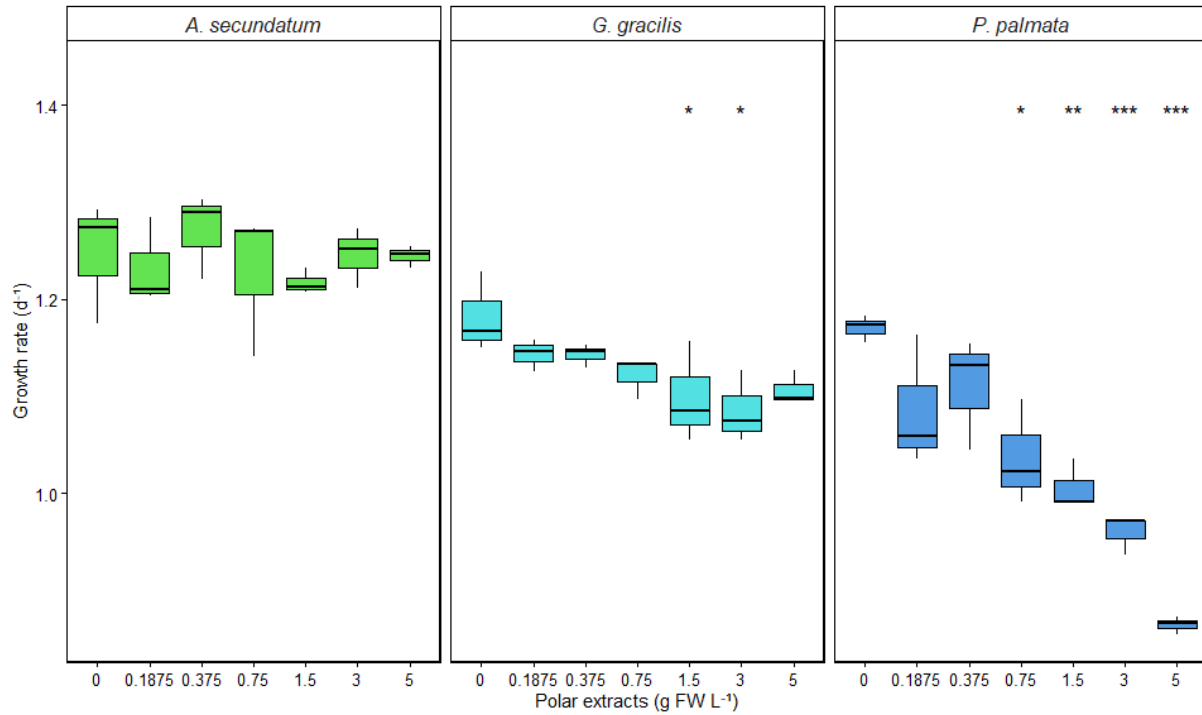


Figure 26. Nonpolar total Rhodophyte extracts' growth inhibiting effects on *Phaeodactylum tricornutum*. The concentrations (expressed in gram dry weight per liter ($\text{g}_{\text{FW}} \text{L}^{-1}$)) are shown on they x-axis and the normalized growth rate on the y-axis. The left plot (green) are the effects of *Acrochaetium secundatum*, in the middle (light blue) are the ones of *Gracilaria gracilis* and on the right (dark blue) those of *Palmaria palmata*. Significance levels are displayed atop the respective concentration using the following code (***) for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance.

Eicosapentaenoic acid boxplots

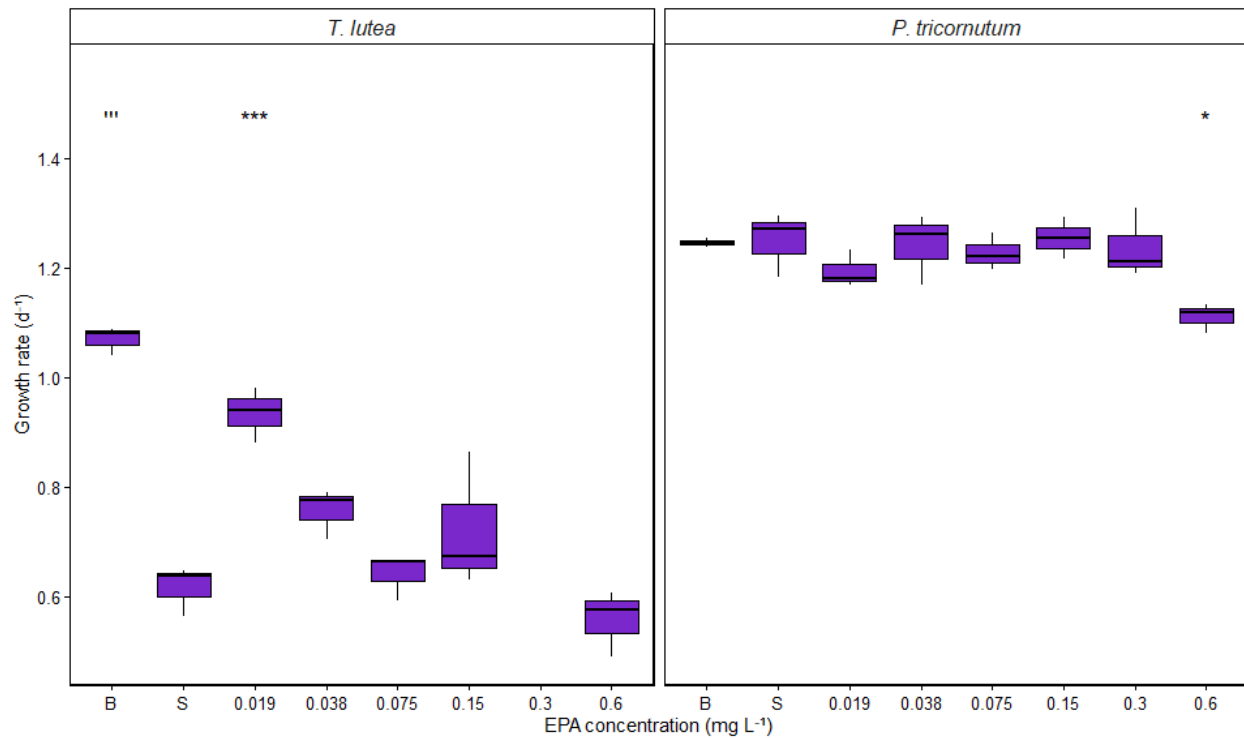


Figure 27. Inhibiting effects of eicosapentaenoic acid (EPA). The concentrations (expressed in milligram EPA per liter (mg L⁻¹)) are shown on the x-axis and the normalized growth rate on the y-axis. On the left, the results of the inhibition test on *Tisochrysis lutea* are shown, on the right those on *Phaeodactylum tricornutum*. Significance levels are displayed atop the respective concentration using the following code (*** for $p < 0.001$, ** for $0.001 < p < 0.01$, * for $0.01 < p < 0.05$). ANOVA was used to check for significance between each concentration and the solvent control (S). The significance between the blank (B) and S is checked with a T-Test. The same significance levels are used, but shown as a prime symbol (') instead of an asterisk.

A.4 NOEC, LOEC, EC10 and EC50

Table 7. The NOEC, LOEC, EC10 and EC50 of the tests, when not calculable this is shown as NA (not applicable).

Extract type	Seaweed species	Test organism	EC10	EC50	NOEC	LOEC	unit
Polar	<i>P. palmata</i>	<i>T. lutea</i>	1.755	NA	0	0.0625	g _{DW} L ⁻¹
Polar	<i>P. palmata</i>	<i>P. tricornutum</i>	NA	NA	2	NA	g _{DW} L ⁻¹
Polar	<i>G. gracilis</i>	<i>T. lutea</i>	1.530	NA	1	2	g _{DW} L ⁻¹
Polar	<i>G. gracilis</i>	<i>P. tricornutum</i>	NA	NA	2	NA	g _{DW} L ⁻¹
Polar	<i>A. secundatum</i>	<i>T. lutea</i>	0.044	1.014	1	2	g _{DW} L ⁻¹
Polar	<i>A. secundatum</i>	<i>P. tricornutum</i>	1.283	NA	1	2	g _{DW} L ⁻¹
Nonpolar surface	<i>P. palmata</i>	<i>T. lutea</i>	1.431	NA	1.5	3	g _{FW} L ⁻¹
Nonpolar surface	<i>P. palmata</i>	<i>P. tricornutum</i>	0.416	NA	3	5	g _{FW} L ⁻¹
Nonpolar surface	<i>G. gracilis</i>	<i>T. lutea</i>	1.666	NA	1.5	3	g _{FW} L ⁻¹
Nonpolar surface	<i>G. gracilis</i>	<i>P. tricornutum</i>	NA	NA	0.188	0.375	g _{FW} L ⁻¹
Nonpolar surface	<i>A. secundatum</i>	<i>T. lutea</i>	NA	NA	3	5	g _{FW} L ⁻¹
Nonpolar surface	<i>A. secundatum</i>	<i>P. tricornutum</i>	NA	NA	5	NA	g _{FW} L ⁻¹
Nonpolar total	<i>P. palmata</i>	<i>T. lutea</i>	0.082	1.098	0	0.188	g _{FW} L ⁻¹
Nonpolar total	<i>P. palmata</i>	<i>P. tricornutum</i>	0.706	NA	0.375	0.750	g _{FW} L ⁻¹
Nonpolar total	<i>G. gracilis</i>	<i>T. lutea</i>	NA	NA	0.1875	0.375	g _{FW} L ⁻¹
Nonpolar total	<i>G. gracilis</i>	<i>P. tricornutum</i>	NA	NA	0.750	3	g _{FW} L ⁻¹
Nonpolar total	<i>A. secundatum</i>	<i>T. lutea</i>	NA	NA	5	NA	g _{FW} L ⁻¹
Nonpolar total	<i>A. secundatum</i>	<i>P. tricornutum</i>	NA	NA	5	NA	g _{FW} L ⁻¹
EPA	/	<i>T. lutea</i>	NA	NA	0.6	NA	mg L ⁻¹
EPA	/	<i>P. tricornutum</i>	0.579	NA	0.3	0.6	mg L ⁻¹

A.5 Phycobiliprotein analysis

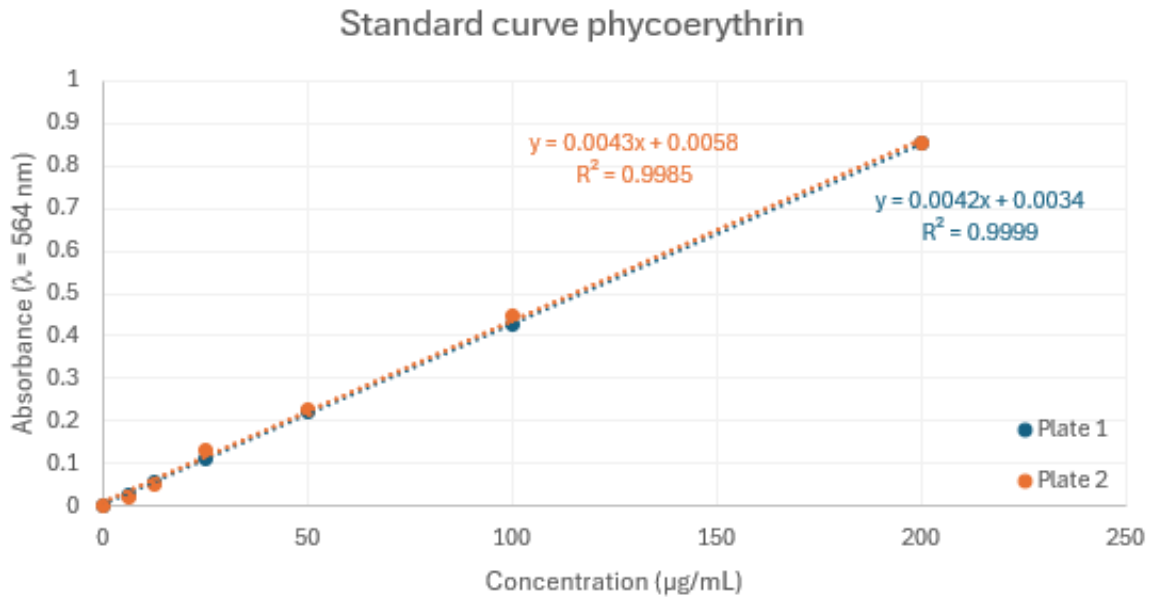


Figure 28. Calibration curve of phycoerythrin. The linear relation between phycoerythrin concentration in $\mu\text{g mL}^{-1}$ and the absorbance at 564 nm is given by the function $y = 0.0042x + 0.0034$ with y absorbance and x concentration.

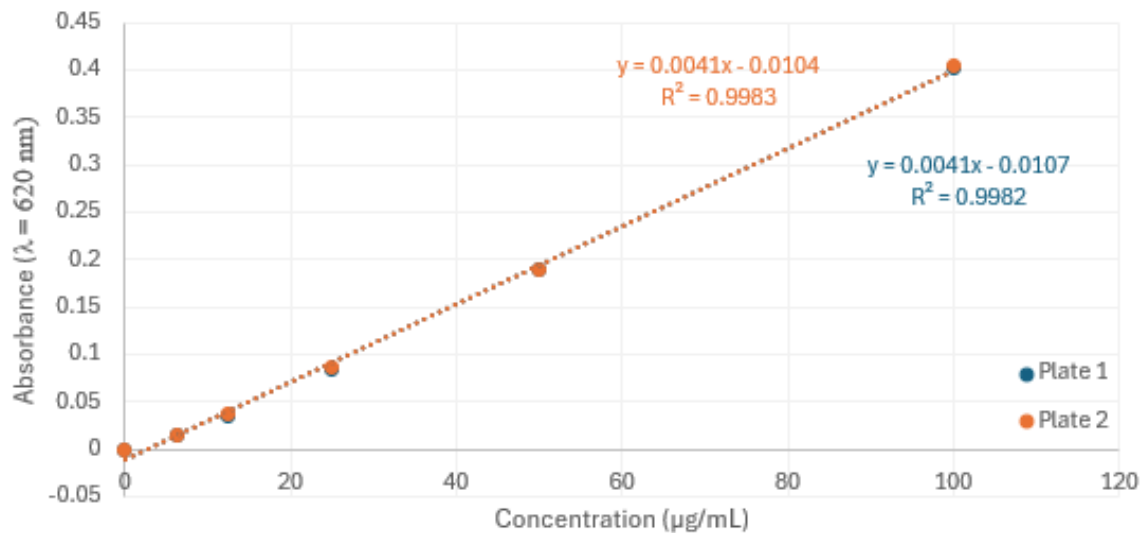


Figure 29. Calibration curve phycocyanin. The linear relation between phycocyanin concentration in $\mu\text{g mL}^{-1}$ and the absorbance at 620 nm is given by the function $y = 0.0041x + 0.0104$ with y absorbance and x concentration.

A.6 Fatty acids

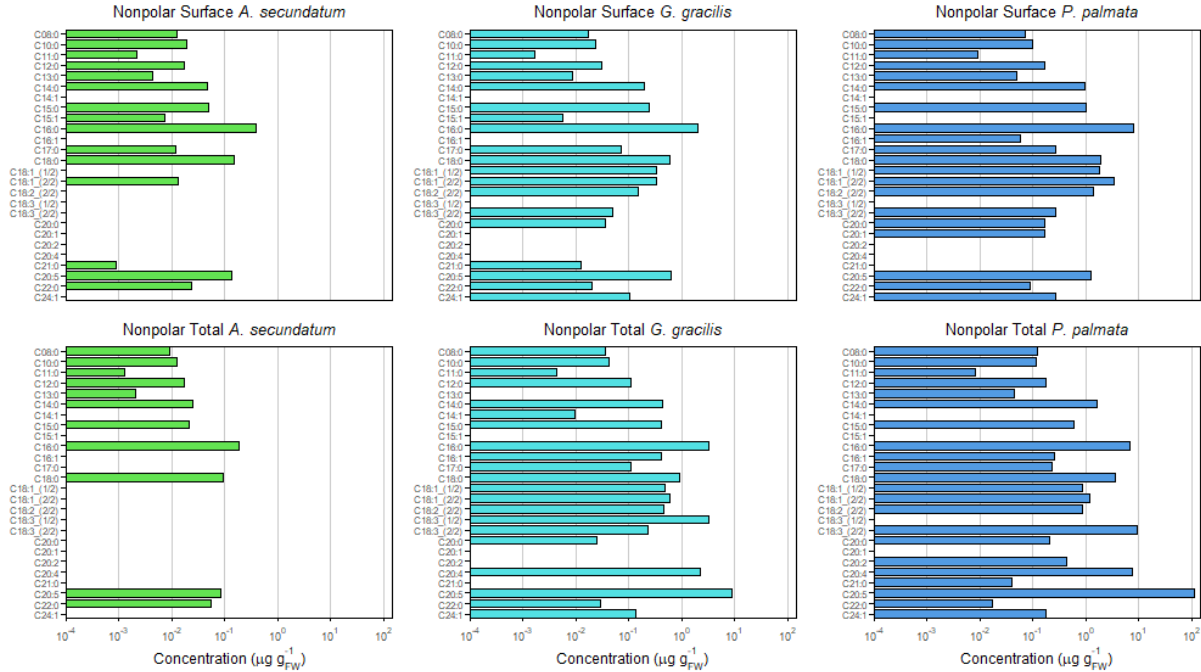


Figure 30. Fatty acid contents in the nonpolar extracts measured with GC-MS. The concentration is expressed as microgram fatty acid per gram of fresh weight ($\mu\text{g g}_{\text{FW}}^{-1}$) on a logarithmic x-axis. The top row presents the results of the surface extracts, while the bottom row displays the total extracts. The plots on the left (green) correspond to *Acrochaetium secundatum*, those in the center (light blue) to *Gracilaria gracilis*, and those on the right (dark blue) to *Palmaria palmata*. *P. palmata* and *G. gracilis* have a more diverse fatty acid profile compared to *A. secundatum* as well as higher concentrations. The total extracts have higher concentrations compared to the surface extracts. In the surface extracts C16:0 is the dominant fatty acid, while in the total extracts C20:5 becomes more dominant in *G. gracilis* and *P. palmata*.

Table 8. Fatty acid names and retention times.

Short name	Full name	Retention time (GC)
C8:0	Methyl octanoate	10.07
C10:0	Methyl decanoate	13.66
C11:0	Methyl undecanoate	15.33
C12:0	Methyl laurate	19.92
C13:0	Methyl tridecanoate	18.43
C14:0	Methyl myristate	19.86
C14:1	Methyl myristoleate	20.27
C15:0	Methyl pentadecanoate	21.37
C15:1	Methyl cis-10 pentadecanoate	21.89
C16:0	Methyl palmitate	23.28
C16:1	Methyl palmitoleate	23.73
C17:0	Methyl heptadecanoate	25.84
C17:1	Methyl cis-10 heptadecanoate	26.49
C18:0	Methyl stearate	29.35
C18:1_(1/2)	Methyl trans-9 eladiate	29.82
C18:1_(2/2)	Methyl cis-9 oleate	29.94
C18:2_(1/2)	Methyl linolelaidate	30.97
C18:2_(2/2)	Methyl linoleate	31.17
C18:3_(1/2)	Methyl-gamma-linolenate	31.58
C18:3_(2/2)	Methyl linolenate	32.19
C20:0	Methyl arachidate	33.73
C20:1	Methyl cis-11-eicosanoate	33.94
C20:2	Methyl cis-11,14-eicosadienoate	34.50
C20:3_(1/2)	Methyl cis-8,11,14-eicosatrienoate	34.76
C20:4	Methyl cis-5,8,11,14-eicosatetranoate	34.89
C21:0	Methyl heneicosanoate	35.25
C20:3_(2/2)	Methyl cis-11,14,17-eicosatrienoate	35.27
C20:5	Methyl cis-5,8,11,14,17-eicosapentaenoate	35.69
C22:0	Methyl behenate	37.01
C22:1	Methyl erucate	37.38
C22:2	Methyl cis-13,16-docosadienoate	38.21
C23:0	Methyl tricosanoate	39.20
C22:6	Methyl cis-4,7,10,13,16,19-docosahexaenoate	40.45
C24:0	Methyl lignocerate	41.92
C24:1	Methyl nervonate	42.57

A.7 Statistical models

Table 9 . Best fitted models for dose response plots. LL refers to the log-logistic model, commonly used to describe sigmoidal responses. BC stands for the Brain-Cousens model, which accounts for hormesis (low-dose stimulation). W denotes the Weibull model, another flexible option for asymmetric dose-response curves. The number indicates how many parameters the model includes.

Extract type	Seaweed species	Test organism	Best fitted model
Polar	<i>P. palmata</i>	<i>T. lutea</i>	BC.5
Polar	<i>P. palmata</i>	<i>P. tricornutum</i>	BC.5
Polar	<i>G. gracilis</i>	<i>T. lutea</i>	BC.4
Polar	<i>G. gracilis</i>	<i>P. tricornutum</i>	BC.4
Polar	<i>A. secundatum</i>	<i>T. lutea</i>	LL.2
Polar	<i>A. secundatum</i>	<i>P. tricornutum</i>	LL.3
Nonpolar surface	<i>P. palmata</i>	<i>T. lutea</i>	LL.4
Nonpolar surface	<i>P. palmata</i>	<i>P. tricornutum</i>	LL.4
Nonpolar surface	<i>G. gracilis</i>	<i>T. lutea</i>	W2.4
Nonpolar surface	<i>G. gracilis</i>	<i>P. tricornutum</i>	LL.4
Nonpolar surface	<i>A. secundatum</i>	<i>T. lutea</i>	LL.4
Nonpolar surface	<i>A. secundatum</i>	<i>P. tricornutum</i>	LL.3
Nonpolar total	<i>P. palmata</i>	<i>T. lutea</i>	BC.4
Nonpolar total	<i>P. palmata</i>	<i>P. tricornutum</i>	LL.2
Nonpolar total	<i>G. gracilis</i>	<i>T. lutea</i>	LL.4
Nonpolar total	<i>G. gracilis</i>	<i>P. tricornutum</i>	BC.4
Nonpolar total	<i>A. secundatum</i>	<i>T. lutea</i>	BC.5
Nonpolar total	<i>A. secundatum</i>	<i>P. tricornutum</i>	LL.3
EPA	/	<i>T. lutea</i>	W2.4
EPA	/	<i>P. tricornutum</i>	BC.4

A.8 pH measurements

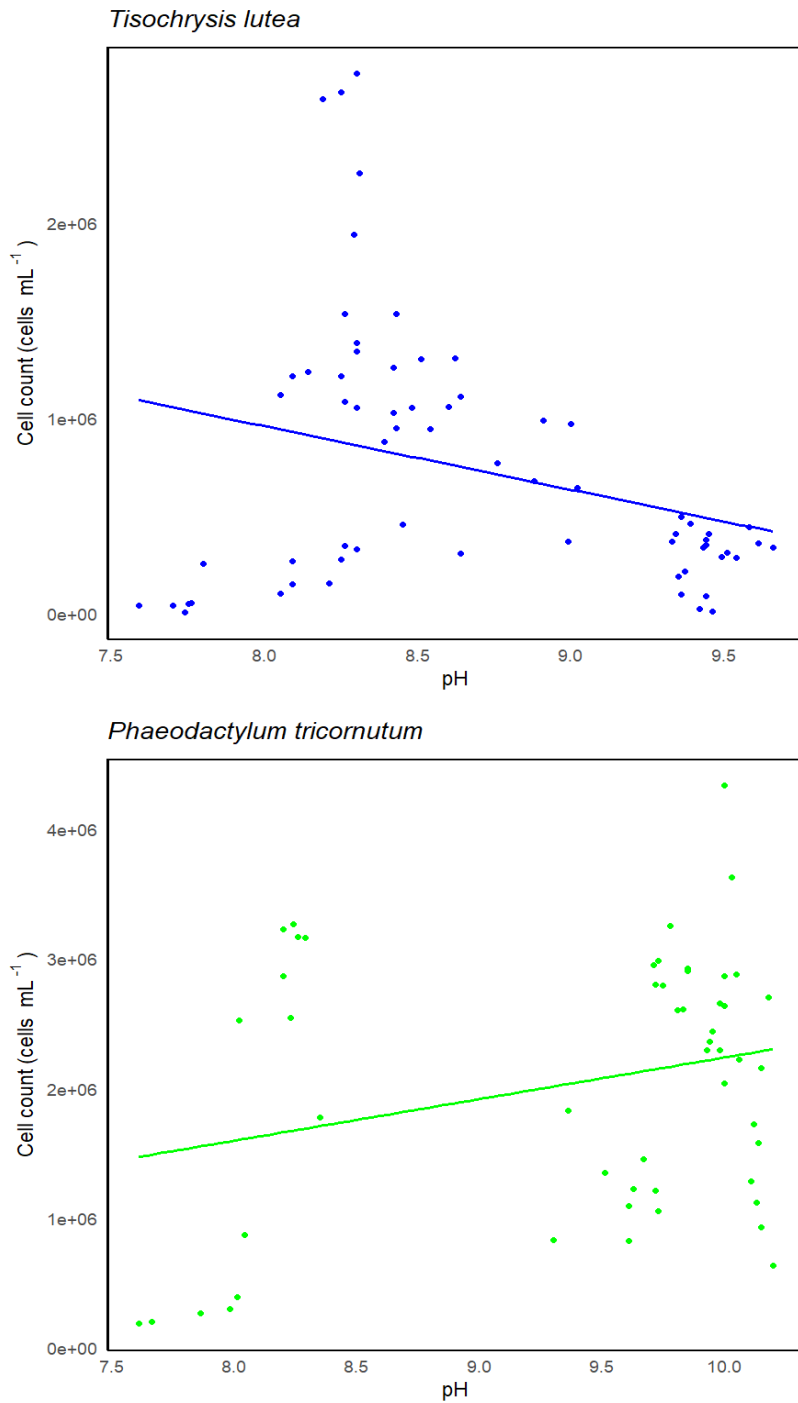


Figure 31. Scatter of cell counts (expressed in cells mL⁻¹) and pH. Correlation was tested with Spearman's rank correlation in R4.4.1, both correlations depicted are insignificant ($p > 0.05$). No clear correlation between the pH and the cell count is observed.