Allelopathic Interactions of Marine Diatoms

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Abbreviations

AHL	N-acyl homoserine lactones
CSP	coomassie blue stainable particle
DMSP	dimethylsulfoniopropionate
GC	gas chromatography
Gyr	gigayears
MS	mass spectrometry
MWCO	molecular weight cut off
Myr	megayears
NMR	nuclear magnetic resonance spectroscopy
PSP	paralytic shellfish poising toxin
PUA	polyunsaturated aldehydes
QS	quorum sensing
RT-PCR	real time polymerase chain reaction
TEP	transparent exoploymer particles
T-RFLP	terminal-restriction fragment length polymorphism
UPLC	ultra performance liquid chromatography

Zusammenfassung

Als Bestandteil des eukaryotischen Phytoplanktons bilden Diatomeen die Grundlage des marinen Nahrungsnetzes und tragen außerordentlich zur weltweiten Kohlenstofffixierung bei. Die Zusammensetzung dieser Diatomeengemeinschaft wird jedoch nicht ausschließlich durch abiotische Faktoren wie Nährstoff- oder Temperaturbedingungen geformt. Vielmehr wird angenommen, dass biotische Einflüsse einen Effekt auf die pelagische Phytoplankton Gemeinschaft haben. Derartige biotische Interaktionen der Diatomeen, welche durch chemische Signalmoleküle vermittelt werden, existieren unter anderem wahrscheinlich zwischen Bakterien oder anderen Diatomeen. Für beide Interaktionen existiert jedoch nur ein limitiertes Verständnis.

Für die Untersuchungen der Diatomeen-Bakterien Interaktionen habe ich mich auf das Bakterium *Kordia algicida* konzentriert, welches bekanntermaßen die Diatomee *Skeletonema costatum* lysieren kann. Die aktiven Substanzen sowie regulatorische Mechanismen dieser Interaktion wurden bisher jedoch nicht aufgeklärt. Mit einer Bioassay-geleiteten Fraktionierung und Inhibitor Experimenten konnte ich zeigen, dass es sich bei den aktiven Substanzen um Proteasen handelt. Die Ausschüttung der Protease wurde nicht durch die Präsenz der lysierten Diatomee induziert. Jedoch konnte die Proteasefreisetzung durch die Zugabe eines sterilen konditionierten Mediums dichter Bakterienkulturen initiiert werden, was auf die Anwesenheit autoinduzierender Substanzen hindeutet. Dieses Phänomen ist von quorum sensing regulierten Prozessen bekannt.

Interessanterweise wurde nicht bei allen Diatomeen das Wachstum durch die Proteasen inhibiert und ein spezies-spezifischer Effekt wurde sichtbar. Die Diatomee *Chaetoceros didymus* zeigte eine physiologische Antwort in Form einer Freisetzung von eigenen Proteasen, welche durch die bakteriellen Proteine hervorgerufen wurde. Dieser Mechanismus kann möglicherweise die Resistenz dieser Alge gegenüber *K. algicida* erklären.

Frühere Studien konnten zeigen, dass Diatomeen die bakterielle Gemeinschaft durch die Freisetzung von Fettsäurederivaten, bekannt als polyungesättigte Aldehyde (PUA),

beeinflussen können. Die Ergebnisse wurden allerdings oft in Laborversuchen mit unnatürlich hohen PUA Konzentrationen erzielt. In einem Mesokosmosversuch, der natürliche Bedingungen wesentlich besser repräsentiert als Laborversuche, haben wir ökologisch relevante PUA Konzentrationen verwendet, um ihren Effekt auf die mikrobielle Gemeinschaft zu überprüfen. Mit verschiedenen *S. marinoi* Isolaten, einer Seewasserkontrolle und einer Seewasserkontrolle mit PUA Zusatz, war es möglich den Effekt von PUA zu evaluieren, während andere Parameter kontrolliert wurden. Die PUA Konzentrationen in beiden *S. marinoi* Isolaten und in der PUA Kontrolle waren geringer als 1 nM, während in der reinen Seewasserkontrolle nahezu kein PUA detektiert wurde. Die Bakterienabundanz sowie die Bakteriendiversität, welche durch Fingerprint Analysen ermittelt wurde, zeigten keinen Unterschied zwischen den einzelnen Behandlungen. Somit ist es unwahrscheinlich, dass PUA die Bakteriengemeinschaft formen. Trotzdem beherbergten zeitweise die Isolate spezifische bakterielle Gemeinschaften. Die Faktoren, welche diese spezifischen Bakterienpopulationen hervorrufen, konnten noch nicht identifiziert werden.

Diatomeen produzieren nicht nur chemische Signale, mit denen Bakterien beeinflusst werden. Komplexe Interaktionen können ebenso zwischen verschiedenen Diatomeenarten auftreten. Wir konnten Bromocyan identifizieren, welches von *Nitzschia* cf. *pellucida* freigesetzt wird, um konkurrierende Algen zu töten. Die Biosynthese dieses neuen Naturstoffs konnte nicht im Detail beschrieben werden, jedoch ist wahrscheinlich die Oxidation von Bromiden mittels einer Bromoperoxidase und Wasserstoffperoxid involviert.

Neben der Freisetzung einzelner bioaktiver Substanzen, können Diatomeen auf eine komplexere Art und Weise mit ihrer Umwelt interagieren. Vielmehr hängt der gesamte extra- und intrazelluläre Metabolismus von konkurrierenden Diatomeen und Bakterien ab. Um solche Phänomene zu untersuchen, habe ich einen Co-Kultivierungsaufbau entwickelt und optimiert, bei dem zwei verschiedene Organismen Infochemikalien austauschen können, jedoch kein direkter Zellkontakt vorliegt. Diese Kultivierungstechnik in Verbindung mit extra- und intrazellulären metabolischen Studien auf Basis von Ultraleistungsflüssigchromatographie-Massenspektrometrie (UPLC-MS) zeigte, dass die chemische Umgebung einer Diatomee stark von der Präsenz anderer Diatomeen abhängt. Zusätzlich zeigte eine Diatomee ein verstärktes

Wachstum, wenn es den Exsudaten des Interaktionspartners ausgesetzt war. Diese Ergebnisse deuten darauf hin, dass Stoffströme einen Einfluss auf die Algengemeinschaft haben können.

In einer weiteren Co-Kultur mit der Diatomee *Thalassiosira pseudonana* und dem Bakterium *Dinoroseobacter shibae* zeigte sich eine Aktivierung des zellulären Algenmetabolismuses in Gegenwart der Bakterien. So wurde eine erhöhte Produktion verschiedener Substanzen, vor allem von zellulären Aminosäuren, in Anwesenheit von *D. shibae* festgestellt.

Zusammenfassend zeigen die Ergebnisse dieser Dissertation, dass Diatomeen stark von co-existierenden Organismen beeinflusst werden. Dieser Einfluss kann offensichtlich sein, zum Beispiel wenn das Wachstum einer Alge durch inhibierende oder stimulierende chemische Signale von anderen Organismen beeinflusst wird. Der Einfluss kann allerdings auch wesentlich subtiler sein, zum Beispiel wenn andere Organismen eine Veränderung interner oder externer Metabolite bewirken. Diese Resultate heben die Signifikanz chemischer Signalstoffe für die Funktionsweise des Ökosystems hervor.

Summary

Diatoms comprise a diverse group of eukaryotic phytoplankton that build up the base of the marine food web and contribute greatly to world-wide carbon fixation. The composition of the diatom community is not exclusively shaped by abiotic factors such as nutrient or temperature conditions but rather biotic influences are also believed to have an effect on the pelagic phytoplankton community. These biotic interactions of diatoms, transmitted by chemical cues released by one interaction partner, are likely to be between bacteria or other diatoms but for both cases only a limited knowledge exists.

For the investigation of diatom-bacteria interactions I focused on the bacterium *Kordia algicida* which is known to lyse the diatom *Skeletonema costatum* even though the active compounds and the regulatory mechanism have not been elucidated to date. Bioassay guided fractionation and inhibitor experiments revealed that the active substances exuded by the bacteria are proteases. Protease release was not induced by the presence of the target diatom. Rather bacterial cultures exposed to sterile filtered medium from dense bacterial cultures released proteases significantly earlier than control cultures demonstrating the presence of autoinducing substances, a hallmark for quorum sensing.

Interestingly, not all tested diatoms were inhibited by the protease, suggesting a species-specific effect. The diatom *Chaetoceros didymus* showed an induced physiological response, a release of algal proteases, due to the presence of the bacterial proteins, which might explain the resistance of the alga to the bacterial proteases.

Previous studies have indicated that diatoms may also shape the bacterial community, through release of one class of fatty acid derived substances called polyunsaturated aldehydes (PUA). However, these results were often based on concentrations of PUA used in laboratory experiments which are much higher than in natural conditions. In a mesocosm approach, much closer to the natural situation than laboratory cultures, we used ecologically relevant concentrations of PUA to test their effects in an environment close to field conditions. Using different *S. marinoi* strains, a seawater control and a seawater treatment supplemented with PUA it was possible to

evaluate the effect of PUA while controlling for other variables. PUA concentrations in both the *S. marinoi* treatments and in the PUA treatment were less than 1 nM while the control contained nearly no detectable PUA. However, bacterial abundance and bacterial diversity obtained by fingerprinting analysis were not different between different treatments at natural and elevated PUA concentrations. These results indicate that PUA are unlikely to shape bacterial communities. Still, algae harbored at least temporarily strain-specific bacterial communities. Nonetheless, the factors that cause specific bacterial communities to be associated with individual algal strains or blooms have still not been identified.

Diatoms may not only produce chemical signals that affect bacteria but a complex signaling may also exist between different diatom species. Here we identified the novel natural product cyanogen bromide which is released by *Nitzschia* cf. *pellucida* to kill competing diatom species. Although it has not been elucidated in detail, the biosynthesis of BrCN most likely involves the oxidation of bromide with the aid of a bromoperoxidase and cellular hydrogen peroxide.

Diatoms are likely to interact with their environment using mechanisms more complex than the release of single metabolites and changing the growth of competitors. In fact, diatom's whole intra- and extracellular metabolism may react to the presence of competing diatoms or bacteria. Therefore I developed and optimized co-culture devices that facilitate the co-culturing of two different organisms that are able to exchange potential infochemicals without having direct cell contact. The use of these culturing techniques in combination with an ultra performance liquid chromatography-mass spectrometry (UPLC-MS) metabolic profiling of extra- and intracellular metabolites revealed that the chemical space surrounding a diatom strongly depends on the presence of other diatom species. In addition, one diatom species showed an enhanced growth when exposed to the chemicals produced by another diatom, suggesting that metabolites fluxes can result in changes of the diatom community composition.

In another experiment, co-culturing of the diatom *Thalassiosira pseudonana* with the bacterium *Dinoroseobacter shibae* suggests that diatom cellular metabolism is more active in the presence of the bacteria. The presence of *D. shibae* increased diatom production of several compounds, especially cellular amino acids.

Taken together, the results in this thesis illustrate that diatoms are strongly influenced by co-existing organisms. The influence can be obvious, for example when diatom growth is inhibited or stimulated by chemical signals produced by other organisms. However, the influence can also be more subtle, as when the presence of other organisms results in a shift in internal or external diatom metabolites. All these results emphasize the importance of chemical cues for the ecosystem functioning.

1 Introduction

Diatoms, global players in various habitats

Established in 1897, the term "phytoplankton" describes a group of typically single-celled, photosynthetic active organisms that drift with currents in fresh and marine waters (Falkowski and Raven 1997). The phytoplankton plays an essential role in many elemental biogeochemical cycles due to their uptake, transformation, incorporation and release of elements during physiological processes such as photosynthesis (Redfield 1958). These elements include, among others, carbon (Falkowski *et al.* 2000), nitrogen (Arrigo 2005), sulfur (Andreae and Crutzen 1997), silicon (Treguer *et al.* 1995) and iron (Morel and Price 2003). The impact of phytoplankton is most remarkable for the oxygenation of the Earth's atmosphere which started roughly 2.3 Gyr ago (Bekker *et al.* 2004). Indeed, although they contribute less than 1% to the Earth's biomass, the phytoplankton is responsible for approximately 50% of the annual carbon fixation (Field *et al.* 1998). In contrast to terrestrial plants, which are dominated by only one clade (Embryophyta), the phytoplankton community is characterized by a noteworthy diversity consisting of a minimum of eight major divisions or phyla representing about 25,000 morphologically defined forms (Falkowski *et al.* 2004). The most diversified group of this phytoplankton community with more than 200,000 species (Kooistra *et al.* 2007) is the group of diatoms (Bacillariophyceae) which evolved between the Proterozoic and the Jurassic (650-140 Myr) (Sims *et al.* 2006). Diatoms perform approximately 20% of the world wide primary production by themselves, and are thus responsible for about 40% of the carbon fixation in marine ecosystems (Field *et al.* 1998; Nelson *et al.* 1995). One of the most important characteristic feature of diatoms is their use of dissolved silicic acid (SiO_2 nH_2O) for the construction of their delicate cell walls (Drum and Gordon 2003).

Diatoms inhabit different types of the marine environments. Pelagic diatoms live in the photic zone of the open ocean and in well-mixed costal and upwelling areas (Bowler *et al.* 2010). Diatoms often dominate the phytoplankton community. When the environmental conditions are favorable, for instance after nutrient input during spring,

exponential cell growth can occur and algal blooms can be formed (**Figure 1A**). In this case the available nutrients are depleted, the cell abundance will decrease rapidly, and cells will usually sediment rather than being consumed by grazers (Kiørboe 1993; Legendre 1990).

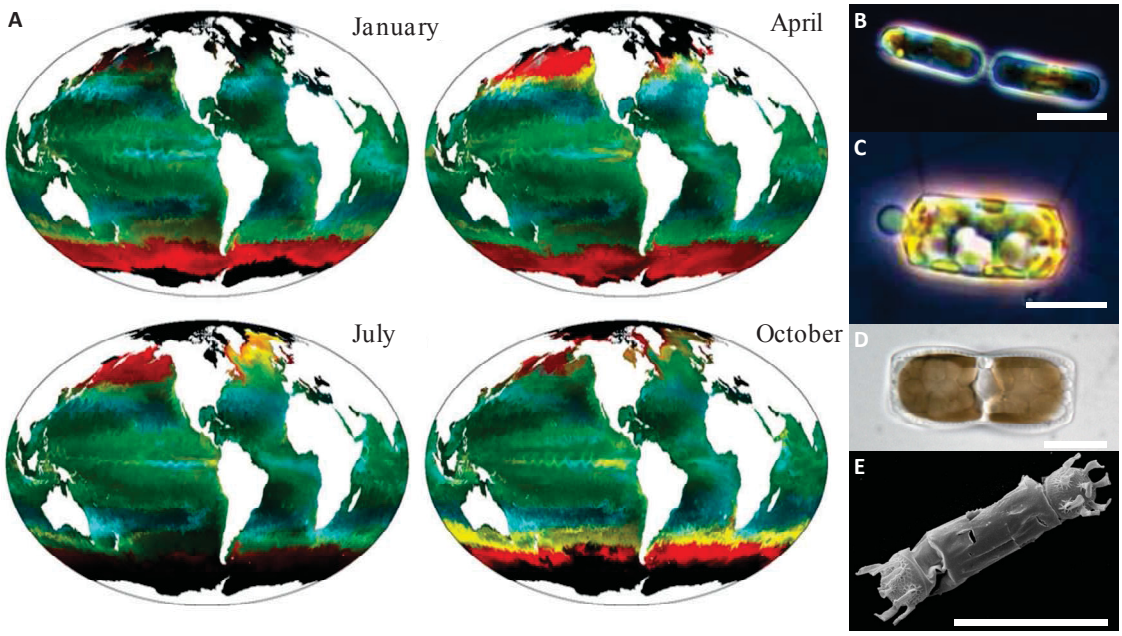

Figure 1: Distribution und morphology of diatoms. (A) Model of global distribution of four phytoplankton types including diatoms. The modeled organisms are diatoms (red), Prochlorococcus *(green), picoplankton (blue) and other large phytoplankton (yellow). The color shading reflects the group while the color intensity reflects the abundance of the particular group (Bowler* et al. *2010). Light microscopic picture of (B) the world-wide distributed chain forming diatom* Skeletonema costatum*, (C) the single cellular* Thalassiosira weissflogii*, (D) the benthic diatom* Nitzschia *cf.* pellucida*. and (E) a scanning electron microscopic picture of* S. costatum*. Scale bar in B-E: 10 µm.*

Additionally benthic diatoms can live on sediment surfaces in estuarine and other shallow water ecosystems where diatom biofilms are an important carbon source for benthic heterotrophic organisms (Middelburg *et al.* 2000). The ecological relevance of benthic diatoms can partially be explained by the colonization of diatoms and subsequent settlers on natural and manmade surfaces, a process known as biofouling. Such biofilms cause severe economic losses and attempts have been made to control the biofouling of several surfaces (Molino and Wetherbee 2008).

Interestingly, diatoms in these habitats do not grow homogenously but often have a patchy distribution which cannot be explained only by the influence of abiotic factors (Saburova and Polikarpov 1995).

Importance of chemically mediated interactions

Seawater is an isotropic and mostly unstructured environment without well defined ecological niches as known from terrestrial ecology. Still diatoms live in surprisingly complex communities with many different species co-existing next to each other. This phenomena has been recorded for several decades and was termed "the paradox of the plankton" (Hutchinson 1961). Lately, there is an increasing appreciation that chemically mediated interactions (summarized in **Figure 2**) are essential for the structure of these ecosystems (Ianora *et al.* 2011) which is evidenced by a number of recent reviews on the subject (Ianora *et al.* 2011; Leflaive and Ten-Hage 2009; Paul and Pohnert 2011; Pohnert 2010; Sieg *et al.* 2011; Van Donk *et al.* 2011).

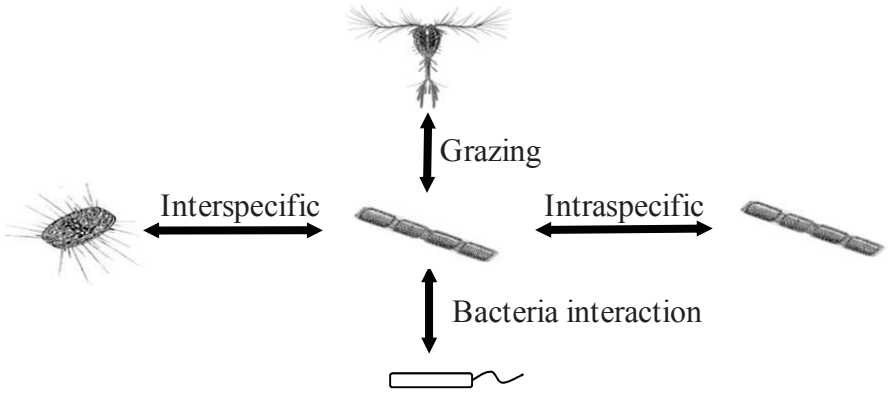

Figure 2: Schematic illustration of potential chemically mediated interactions of diatoms excluding abiotic influences. Diatoms can interact with diatoms on the same trophic level, either conspecifics or with diatoms of from other species. Additionally, diatoms can interact with higher trophic levels such as copepods as well as with highly abundant bacteria. Viruses, even though important for diatom performance, are excluded from the graph. Diatom and copepod pictures are courtesy of Wim van Egmond.

Chemical signals may be used by diatoms to interact with conspecifics. For example, there is evidence that the diatom *Pseudostaurosira trainorii* regulate sexual reproduction by the excretion of pheromones with an identity which remains elusive

(Sato *et al.* 2011). Phytoplankton, including diatoms, might also interact with grazers by the production of feeding deterrents. For instance, diatoms of the genus *Pseudo-nitzschia* are known to produce the neurotoxin domoic acid (Bates *et al.* 1989). Even though the potential role of that toxin as an anti-grazer defense is still under debate, there is some evidence that krill exposed to high domoic acid concentrations had significantly reduced feeding rates (Bargu *et al.* 2006), although other studies could not attribute domoic acid an effect on copepods feeding behavior (Olson *et al.* 2006). Additionally, the ubiquitous plankton metabolite dimethylsulfoniopropionate (DMSP) is known to be involved in chemically mediated interactions. DMSP which can be cleaved to dimethylsulfide (DMS) and acrylate by several microalgae (Wolfe *et al.* 2002) inhibits the ingestion of protists while the cleavage products had no feeding deterrent activity (Strom *et al.* 2003) emphasizing the multifaceted chemically mediated interactions of diatoms. Diatoms can also intercept the chemical signals of other species. For example, grazers do have an influence on diatom morphology by the release of chemical cues as shown for the diatom *Skeletonema costatum* (Bergkvist *et al.* 2012). In the following sections I will focus on allelopathic interactions of diatoms including diatom-microalgae as well as diatom-bacteria interactions.

Allelopathic interactions of diatoms I: Diatom-microalgae interactions

Allelopathy derived from the Greek *allelon* meaning “of each other” and *pathos* “to suffer” has several varying definitions. One definition uses that term strictly and considers only deleterious interactions between primary producers and between primary producers and microorganisms (Rice and Leon 1974). However, because the term was originally intended to be used for both inhibitory and stimulatory interactions between either primary producers or between primary producers and microorganisms (Molisch 1937), I will apply this definition during this thesis.

Research on allelopathic interactions of microalgae has been primarily motivated by the neurotoxic effects of certain algae to humans, especially dinoflagellates, which can form harmful algal blooms (Cembella 2003). Even though the identification of active allelochemicals could not be realized by most studies, there is an expanding knowledge on allelopathic interactions of microalgae in general. For instance, dinoflagellates of the genus *Alexandrium*, capable of producing paralytic shellfish poising toxins (PSPs), are

among the best studied examples. This alga appears to be broadly allelopathic against a range of different phytoplankton species including the diatom *Thalassiosira weissflogii* (Tillmann *et al.* 2007). However, the allelopathic activity cannot be attributed to PSPs (Tillmann and John 2002). Thus the identity of the active principle remains elusive even though preliminary characterization attempts have been performed (Ma *et al.* 2009). Similarly, the dinoflagellate *Karenia brevis* inhibits the growth of competing algal species such as the diatom *Skeletonema costatum* with as yet unidentified substances (Kubanek *et al.* 2005; Prince *et al.* 2008a; Prince *et al.* 2010). However, interestingly this diatom developed strategies to undermine the allelopathic effect indicated by less active cell-free filtrates of *S. costatum* and *K. brevis* co-cultures (Prince *et al.* 2008b) highlighting complex regulating mechanism in plankton interactions. *S. costatum* growth was also inhibited by partially characterized polysaccacharide-protein complexes released from the raphidophyte *Heterosigma akashivo* (Yamasaki *et al.* 2009).

Investigations dealing with nontoxic species in diatom-diatom interactions are by far less abundant. Early studies report that the diatom *T. pseudonana* had reduced growth due to allelopathic substances from *Phaeodactylum tricornutum* (Sharp *et al.* 1979). More recently it was shown that the diatom *Haslea ostrearia* produces a blue-green pigment called marennine which can negatively affect the growth of several diatom species including *S. costatum* (Pouvreau *et al.* 2007). Otherwise, there is only a limited knowledge about the chemically mediated interactions between members of this important group of microalgae. Thus, this thesis aims to contribute to a better understanding of diatom-diatom interactions with an approach that will be discussed later on of this chapter.

Allelopathic interactions of diatoms II: Microalgae-bacteria interactions

Bacteria are the most abundant living organisms in the ocean having an average concentration of approximately 10^6 cells mL^{-1} (Cole 1982) with a huge metabolic potential. Bacteria play also a crucial role for the ecosystem functioning e.g. by the energy transfer to higher trophic levels via the microbial loop (Azam *et al.* 1983). In contrast to diatom-diatom interactions, there are more investigations performed for diatom-bacteria interactions. Molecular techniques have been applied to investigating

the effect diatoms have on the bacterial community. Researchers have shown that diatoms harbor distinct bacterial communities which change over the course of a diatom bloom (Grossart *et al.* 2005; Riemann *et al.* 2000). The effects certain bacteria can have on diatoms are very diverse. In several systems, symbiotic relationships in which bacteria can support the growth of microalgae by the allocation of growth promoting factors such as vitamins have been reported (Cole 1982; Croft *et al.* 2005). In contrast, bacteria can also inhibit the growth of diatoms by direct or indirect contact (Mayali and Azam 2004). So far, there is only a limited knowledge about the active compounds involved. Still, there are limited studies that describe proteins as bioactive substances in these interactions (Lee *et al.* 2000; Mitsutani *et al.* 2001). Interestingly, often different species of diatoms are differently affected by these bacteria (Lovejoy *et al.* 1998; Mayali and Azam 2004) but the underlying mechanism for that are so far obscure. The reason for that observation can be diverse. For example, differently affected diatoms might have a different evolutionary history or do not share the same target of the bacterial compounds. However, an increasing knowledge on these mechanisms can help to better understand the species diversity in the oceans.

Besides their effect on growth, bacteria might have another substantial effect on diatoms; specifically bacteria might affect the metabolism of diatoms. For instance, even though cultures of the diatom *Pseudo-nitzschia* produce domoic acid (Bates *et al.* 1989) there is a long debate if the diatom is the real producer of that toxin. One report attested that xenic diatom cultures a higher domoic acid production capability than axenic cultures (Bates *et al.* 1995) while others illustrated that free–living bacteria in xenic cultures do not produce the toxin without evaluating the effect of attached bacteria (Bates *et al.* 2004). Recently, other researchers tried to address this problem with a co-culturing approach but due to experimental limitations a final conclusion could not be drawn (Kobayashi *et al.* 2009; Sieg *et al.* 2011). However, clearly bacteria do play a crucial role for domoic acid biosynthesis, for example by the delivery of precursor molecules, even though this interaction is not completely understood.

A thrilling example of the complex interplay between bacteria and microalgae including growth promotion and metabolic switches was recently presented (**Figure 3**). Here, the bacterium *Phaeobacter gallaeciensis*, belonging to the clade of roseobacters, can support the growth of the coccolithophorid haptophyte *Emiliania huxleyi*. The alga

provides the bacteria with DMSP as sulfur source and with a surface for biofilm formation. In return, the bacteria deliver growth factors such as phenylacetic acid or the antibiotic tropodithietic acid resulting in a mutualistic partnership for both partners (**Figure 3** green). However, if the alga starts to excrete lignin breakdown products such as *p*-coumaric acid (typically during senescence) the bacteria alter their metabolism and start to produce algicides named roseobacticides (**Figure 3** red). After this metabolic switch the relationship turns parasitic (Seyedsayamdost *et al.* 2011b). Further elicitors such as sinapic acid and ferulic acid have been identified and expand the generality of this concept (Seyedsayamdost *et al.* 2011a). Still, an additional chemical signaling pathway might be involved in this interaction. It is well established that bacteria regulate their gene expression of various active compounds via a cell density dependent mechanism named quorum sensing (QS) (Bassler 1999). This process is governed by small molecules that are excreted and recognized by bacteria. Indeed, lately it has been demonstrated that the production of tropodithietic acid is regulated by N-acyl homoserine lactones (Berger *et al.* 2011), a class of compounds regularly involved in QS processes. Thus the bacteria might only deliver tropodithietic acid to the algae if they reach a certain threshold cell density which potentially helps to keep the metabolic costs for the biosynthetic machinery low.

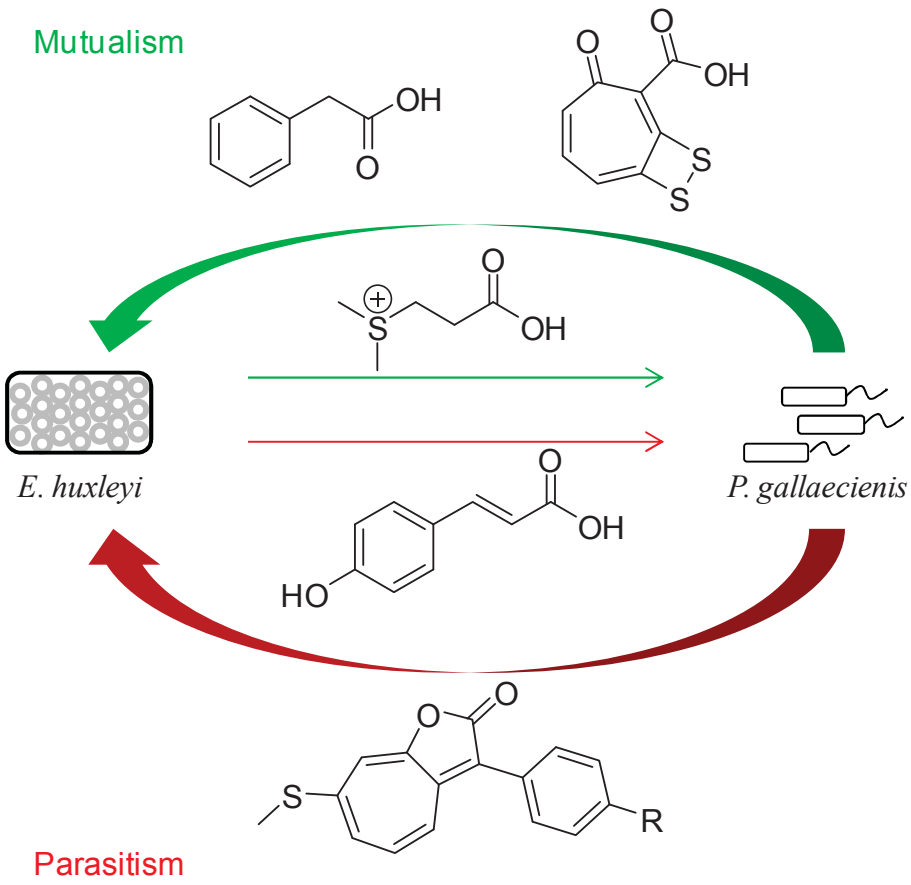

Figure 3: Interaction of E. huxleyi *with the bacteria* P. gallaeciensis. *The relationship can be either mutualistic (green) by* E. huxelyi *providing DMSP and receiving growth promoters and antibiotics. In case the alga releases* p-*coumaric acid the relation turns parasitic (red) with* P. gallaeciensis *starting to produce algicides (Seyedsayamdost* et al. *2011b).*

While keeping the multifaceted interactions, which include impacts on growth and metabolism, between bacteria and diatoms in mind, this thesis aims to focus on the interactions of bacteria with non-toxic diatoms which represent a major part of the phytoplankton community. I focused hereby on the discovery of active bacterial substances that affect diatom growth including its underlying regulation mechanisms as well as reasons for activity specificity. Furthermore, I endeavored to find compounds produced by diatoms by which diatoms regulate the bacterial community around themselves.

Polyunsaturated aldehydes, metabolites involved in multiple interactions?

One class of compounds which is highly debated to be involved in several interactions of diatoms is polyunsaturated aldehydes (PUA) which is highlighted in several reviews (Ianora and Miralto 2010; Leflaive and Ten-Hage 2009; Pohnert 2005). PUA are produced by diatoms upon wounding (Pohnert 2000) and are thought to be involved in the chemical defense against grazers such as copepods (Ianora *et al.* 2004; Miralto *et al.* 1999) even though this fact is still highly discussed since theoretical and field evidence suggest these compounds are not involved in chemical defense (Flynn and Irigoien 2009; Wichard *et al.* 2008). Other studies proposed different functions of these metabolites such as an involvement in diatom-diatom interactions (Vardi *et al.* 2006). This could be feasible since PUA are also released by intact diatoms during certain moments of their growth (Vidoudez *et al.* 2011b; Vidoudez and Pohnert 2008). PUA have also been suggested to have a function in the regulation of the bacterial community. One study used bacterial strains isolated from non-marine habitats and applied PUA in concentrations above what can be found in nature (Adolph *et al.* 2004) and others used bacteria isolated from a *Skeletonema* bloom combined with the application of still unrealistic high concentrations (Ribalet *et al.* 2007a), so the results of these studies are unlikely to reflect diatom bacterial interactions in nature. More ecologically relevant is a recent investigation which used PUA in concentrations that might occur in nature. The researchers found group specific effects of PUA on bacteria cell abundance and metabolic activity (Balestra *et al.* 2011). However, to this point there have been no experiments reported which tested the effect of PUA under ecological relevant conditions such as a field or mesocosm experiment. With this thesis I aim to tackle this problem and investigate the effects of PUA on bacterial abundance and bacterial community composition under ecological relevant conditions.

Model organisms

One diatom species frequently used in investigation dealing with PUA is the centric diatom *S. costatum* (**Figure 1B** and **Figure 1E**) (Ribalet *et al.* 2007b; Vidoudez *et al.* 2011a; Vidoudez *et al.* 2011b; Vidoudez and Pohnert 2008; Wichard *et al.* 2005). *Skeletonema* species are cosmopolitan phytoplankters that form dense blooms in coastal and marine environments (Castillo *et al.* 1995; Karentz and Smayda 1984; Kooistra *et*

al. 2008). A frequently used *Skeletonema* species is *S. costatum*. However, in 2005 it was demonstrated that strains reported to be *S. costatum* consist, in fact, of several different species. The most common species of the new identified species is *S. marinoi* (Sarno *et al.* 2005). Nevertheless, there is still certain confusion about the species affiliation of several *Skeletonema* strains since not all *Skeletonema* strains in laboratory use have been reclassified (Kooistra *et al.* 2008). For that reason the reader is encouraged to treat *S. costatum* and *S. marinoi* as the same species during this thesis.

S. costatum has been used in several investigations dealing with allelopathy. It was found to be inhibited by compounds produced by *H. akashiwo* (Yamasaki *et al.* 2009) but can also, depending on the growth stage, inhibit the growth *H. akashiwo* (Yamasaki *et al.* 2011). It was also shown to have unique abilities to undermine the allelopathic effect of *K. brevis* (Prince *et al.* 2008b) making it an interesting organism for allopathic research.

The bacterium *Kordia algicida* showing algicidal properties against *S. costatum* was isolated from a *S. costatum* bloom (Sohn *et al.* 2004). Interestingly, this bacterium is the first of its genus and had, at the time published, only a low 16S rRNA similarity of 93% with any other species (Sohn *et al.* 2004). Recently the genome of *K. algicida* was sequenced revealing interesting features such as the ability to degrade macromolecules as well as a gene cluster encoding for a polyketide synthase and a nonribosomal peptide synthase which were hypothesized to be involved in the algicidal action against *S. costatum* (Lee *et al.* 2011).

S. costatum was further used to develop a metabolomics based approach in plankton ecology. For instance, an ultra performance liquid chromatography coupled to a mass spectrometer (UPLC-MS) was used to profile *S. marinoi* exudates (Barofsky *et al.* 2009) while a gas chromatography-mass spectrometry (GC-MS) based approach was applied for the profiling of *S. marinoi* cellular metabolites during growth and day time revealing several metabolites differentially regulated during algal growth (Vidoudez and Pohnert 2011). Thus, there are already powerful methods optimized for *S. costatum* available which can be applied to investigate allelopathic interactions.

To investigate allelopathic interactions of benthic diatoms the diatom *Nitzschia* cf. *pellucida* was chosen. *Nitzschia* species, common in diatom biofilms (Patil and Anil 2005), are known to produce a variety of halogenated compounds (Hill and Manley

2009; Moore *et al.* 1996) with so far often unknown functions (Paul and Pohnert 2011). Further, *N.* cf *pellucida* showed strong allelopathic activity against competing species in preliminary experiments (Vanelslander, personal communications).

The approach: Bioassay guided fractionation *vs*. metabolomics

A classic approach to revealing active compounds in chemical ecology, specifically in allelopathic research, is a bioassay guided fractionation of the active compound(s) (Prince and Pohnert 2010). Here, the first step is the development of a bioassay which can reliable detect any active compound from the producing organism. Traditionally, the growth of a target organism, ideally ecological relevant, is monitored after the addition of nutrient enriched conditioned medium e.g. (Pratt 1966; Sharp *et al.* 1979). After a positive response the active filtrate or an active crude organic extract can be used for further partitioning according to a certain chemical feature such as polarity or molecular size. The obtained fractions can be further tested for its biological activity and a repetitive procedure of fractionation and biological testing should finally yield one active compound which chemical structure can be elucidated using various analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). This time-consuming procedure has, however, several limitations. For example compounds that act additively or synergistically might not be detected and activity might be lost due to a low stability of the active compound (Prince and Pohnert 2010). Additionally, it is not always possible to obtain enough substance to elucidate the structure of the active compound resulting only in preliminary descriptions of the active compounds e.g. (Prince *et al.* 2010; Yamasaki *et al.* 2012). Despite these limitations, the approach is still a valid method to spot active compounds in chemical ecology.

However, recently an approach called metabolomics has been applied to several research fields such as medical chemistry and plant science. Metabolomics is the endpoint of the "omics cascade" consisting additionally of genomics, transcriptomics and proteomics. This method gives information to the researcher which best represents an organism's phenotype (Dettmer *et al.* 2007). Metabolomics is a procedure which ultimately aims to identify and quantify all metabolites present at one particular moment (the metabolome) in an organism (Griffiths *et al.* 2007). Even though it is widely accepted that this ultimate goal is not achievable, different instrumental platforms

designed to detect the widest array of metabolites have been established. Mass spectrometers are often coupled with GC or LC and NMR based approaches have also been applied to metabolomics (Weckwerth and Morgenthal 2005). NMR based methods have advantages in that they cover a wide range of metabolites and deliver useful information for structure elucidation of compounds. However, these methods lack sensitivity in comparison to MS based metabolomics (Fernie *et al.* 2004). This makes NMR a less suitable method for applications dealing with only a limited amount of biomass. GC-MS based approaches offer great chromatographic resolution and have low limits of detection in the pmol or nmol concentrations (Dunn and Ellis 2005). However, a GC separation is limited to volatile, low molecular weight compounds. To overcome that problem a two-step derivatization consisting typically of oxime formation of carbonyl groups and silylation of exchangeable protons has been employed (Lisec *et al.* 2006). This strategy results in complex chromatograms of several hundreds of metabolites including multiple derivatization products e.g. of amino acids. One major advantage of GC-MS metabolomics methods is that electron impact ionization in combination with mass spectral libraries and analysis of retention time indices facilitates the identification of metabolites. This advantage can be most meaningfully used for the identification of intracellular metabolites in cellular metabolomics experiments, since most of the metabolites found here are well described metabolites of the primary metabolism. Metabolites released into the surrounding medium by diatoms might, however, span a broad range of polarity (Pohnert 2012). LC-MS covers a broader range of analyte polarity and is thus especially well suited to profile exuded compounds of diatoms. Furthermore, this technique requires only a limited sample preparation which accelerates sample processing significantly. Unfortunately, electrospray ionization does not offer as much structural information as electron impact ionization which makes the metabolite identification in LC-MS metabolomic experiments a challenging task. Recently, MS libraries for electrospray ionization based on high resolution MS and MS/MS data have been developed (Patti *et al.* 2012; Smith *et al.* 2005). However, so far these databases are not designed for plant or even diatom metabolomics experiments and are thus not applicable for the experiments presented within this thesis.

Metabolomics experiments can be used to compare organisms in two different states. For example, in systems biology a comparison of a mutant and the wild type can help to elucidate the function of specific genes. In other cases, researchers may want to know which metabolites are regulated in response to other stimuli, such as abiotic or biotic stresses. Bioinformatic tools are further used to pick out metabolites that are differentially regulated and are thus potentially important for this particular situation.

While metabolomics based methods are well established in plant science (Kueger *et al.* 2012; Roessner *et al.* 2001) for diatom research metabolomics is less widely applied. However, Nappo *et al*. used a GC-MS method to present a list of metabololites found in the diatom *Cocconeis scutellum* without giving these metabolites any function within an ecological and physiological context (Nappo *et al.* 2009). Using a GC-MS protocol specifically established for plant metabolomics (Lisec *et al.* 2006) diatom physiology was investigated by a combined non-targeted transcriptomic and metabolomic approach. For example, it was shown that under iron limiting conditions the diatom *Phaeodactylum tricornutum* changes its metabolism especially in processes employing iron rich components such as mitochondrial electron transport and photosynthesis (Allen *et al.* 2008). Even more recently, a similar approach was used to evaluate the importance of the urea cycle, a pathway that detoxifies ammonium by converting it to urea in vertebrates, in marine diatoms. By following the expression of genes involved in the urea cycle as well as a metabolomic survey of implicated metabolites it was revealed that the urea cycle is linked to other metabolic pathways such as the tricarboxylic acid cycle and contributes significantly to the metabolic response of diatoms to nitrogen availability (Allen *et al.* 2011). The diatom cell metabolism was further investigated employing a GC-MS protocol developed especially for diatom metabolomics, specifically *S. marinoi*, including crucial steps such as extracting, derivatization and data evaluation. This approach was subsequently used to describe metabolic changes of intracellular metabolites during different growth stages of a laboratory culture (Vidoudez and Pohnert 2011).

Besides intracellular metabolites produced by diatoms, extracellular metabolites exuded by these algae are of special interest because these compounds are likely to be involved in chemically mediated interactions. Diatom exudates can make up a significant portion of the photosynthetically fixed carbon, and in a previous study the

excretion of polysaccharides and amino acids was monitored for the diatom *Chaetoceros affinis* revealing a growth stage dependent release of amino acids (Myklestad *et al.* 1989). A global approach to detect a broad range of metabolites exuded by *S. marinoi* was recently performed utilizing solid phase extraction (SPE) in combination with a UPLC-MS method. Here, it was shown that various metabolites have different release pattern. For example, some metabolites, which could unfortunately not be identified, were predominantly excreted during exponential growth and not during stationary or the declining phase while others are not exuded during the exponential phase but later on during growth. The authors suggest that these complex patterns could have implications for the chemically mediated interactions of the diatom (Barofsky *et al.* 2009). Indeed, again an UPLC-MS approach was utilized to investigate the interaction of the diatom *S. marinoi* with the copepod *Calanus* sp. Here, copepods fed preferentially on *S. marinoi* in the post-bloom phase in mesocosm and laboratory assays. In parallel, the cellular profile of *S. marinoi* was monitored using UPLC-MS which showed changes in the chemical profile during culture development. Taken together, the authors conclude that chemicals other than nutrients are involved in the selective feeding behavior of copepods (Barofsky *et al.* 2010).

To investigate diatom-diatom as well as diatom-bacteria interactions one has not only consider effects that can be detected as an alteration of biomass parameter such as growth rate but also how co-existing organisms influence metabolite profiles inside and outside the diatom cell. Despite the importance of diatoms, for those interactions no metabolomics approach using either UPLC-MS or GC-MS has been applied. The reason for that might be due to methodological limitations. However, metabolomics has many advantages to techniques currently used. Traditionally, conditioned cell free medium of one donor-partner was applied to a receiving partner and the effect on growth monitored (Fistarol *et al.* 2005; Sharp *et al.* 1979). This method is limited because the nature and the amount of substances excreted by one organism might depend on the presence of another organism. For example, extracellular extracts obtained from one pot co-cultures consisting of *Karenia brevis* and *S. costatum* were significantly less allelopathic than extracts of *K. brevis* mono-cultures. Even though the reason for that phenomenon remains unknown it might be due to the interruption of biosynthesis of the active compound, metabolizing of the active compound or the excretion of counteracting

allelochemicals by *S. costatum*, emphasizing the dynamic interplay between these planktonic microalgae (Poulson *et al.* 2009; Prince *et al.* 2008b). Moreover, it has been recently shown that more complex microalgae assemblies react differently to allelopathic compounds exuded by *K. brevis* in comparison to mono-cultures. Again, these results highlight the importance and complexity of phytoplankton interactions for phytoplankton composition dynamics (Poulson *et al.* 2010).

The cultivation techniques: non-contact co-culturing and mesocosm

An experimental setup which would allow investigations of such interactions between diatoms and between diatoms and bacteria is an arrangement where chemically mediated communication is possible but simultaneously separates both interaction partners for example by a membrane. Such setups were recently used in studies dealing with interactions in marine (Yamasaki *et al.* 2007) and non-aquatic habitats (Schroeckh *et al.* 2009). In these studies only small volumes could be investigated due to limitations of commercially available setups. However, for the combination of such co-culturing technique with chemical analysis (e.g. UPLC-MS or GC-MS based metabolomics) these volumes are not sufficient. Thus, there is a need to develop a setup which enables a non-contact culturing of microalgae and bacteria. For this thesis I aimed to develop such an apparatus and apply it for studies on diatom-diatom and diatom-bacteria interactions.

However, no matter how elaborate such setups are in marine chemical ecology the results obtained in laboratory experiments should be verified in experiments which are closer to natural conditions. In this context, mesocosm experiments, which have an ecological relevance between laboratory experiments and field studies, offer great opportunities. Mesocosms are better controllable than field studies, for example by temperature and light manipulations, but are still closer to nature than laboratory experiments. For example, experiments dealing with the bacterial community's response to treatments will measure the response of the ecologically relevant bacterial community and not the response of single bacterial strains in the laboratory. For PUA, an effect on bacteria was described in laboratory based studies (Adolph *et al.* 2004; Balestra *et al.* 2011; Ribalet *et al.* 2008). During this thesis I further aimed to verify these results in a mesocosm study by the application of natural PUA concentration to a natural bacterial assembly.

2 Scope of the study

Diatoms live in a homogenous environment without an ecological niche as known from terrestrial habitats. However, these eukaryotic microalgae are surprisingly diverse with several species co-existing next to each other. Chemically mediated interactions of these diatoms are anticipated to be crucial for the conservation of biodiversity and essential for ecosystem functioning. This work aims to deepen the understanding of these interactions focusing on allelopathic interactions including both diatom-diatom and diatom-bacteria interactions.

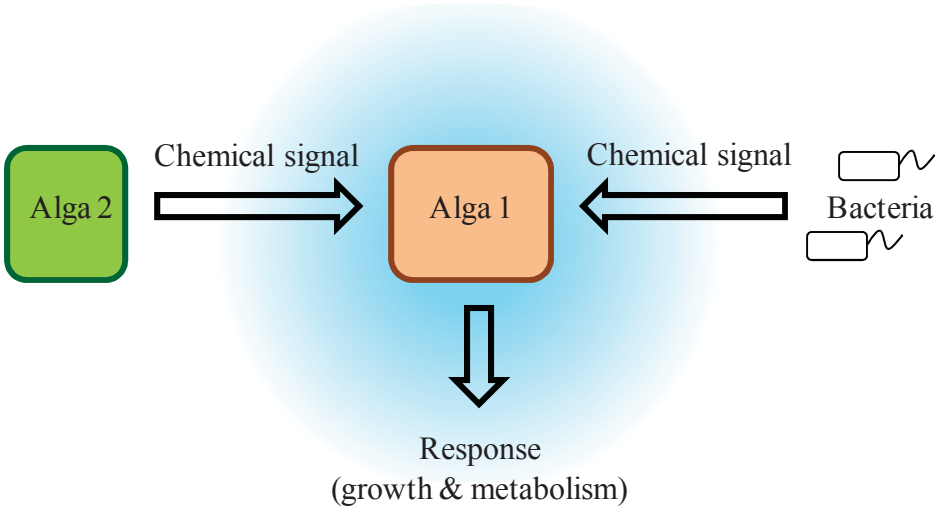

Figure 4: Summary of the aims of this thesis.

Therefore, I aimed to identify chemical signals excreted by diatoms and bacteria and investigate their effect on diatoms. Further, I intended to describe the general effect of bacteria and diatoms on the growth and metabolism of co-existing diatoms. In detail, this work comprises the following aspects:

Methodological development

I aimed to establish a setup which enables a non-contact co-culturing of diatoms or diatoms and bacteria. The setup should be easy to handle, have a high diffusion rate between both culturing chambers and provide sufficient volumes for metabolomics analysis (see chapter **4.4** and **4.5**).

Metabolic responses of diatoms to biotic stimuli using metabolomics

I aimed to use a co-culture approach in combination with metabolomics based on UPLC-MS. I specifically wanted to profile intra- and extracellular metabolites in order to evaluate the influence of co-existing diatoms on growth and metabolism of diatom cells (chapter **4.5**).

Additionally, I aimed to apply the co-culture setup for the investigation of the interaction of *T. pseudonana*, the first sequenced diatom, with the ubiquitary algal symbiont *Dinoroseobacter shibae*. Here, I tempted to apply a GC-MS based metabolomics approach to elucidate the effect of bacteria on the cell metabolism of diatoms (chapter **4.4**).

Chemical signaling between diatoms and bacteria

I used the marine bacterium *K. algicida*, originally isolated from a *S. costatum* bloom, to elucidate the active substance involved in this for *S. costatum* harmful interaction in a bioassay guided fractionation approach. Further, I attempted to investigate the underlying regulation mechanism of this interaction e.g. a regulation mechanism for the excretion of the active compound (chapter **4.1**). Moreover, the negative effect of *K. algicida* might well be species specific. I endeavored to find the reasons for that species specifity (chapter **4.2**). Finally, I wanted to verify the effect of PUA on bacteria and viruses in a mesocosm approach. Besides the effect on growth I aimed to study the effect of PUA on bacterial community composition in order to get a broader view of the effect of PUA on the bacterioplankton (chapter **4.3**).

Signals in diatom-diatom interactions

Here, I used the benthic diatom *N.* cf *pellucida* which showed a growth inhibition against co-occurring species in preliminary experiments. I co-worked on this topic and used different GC-MS methods to elucidate metabolites which were tested for their biological activity (chapter **4.6**). Since these metabolites were predominantly halogenated I synthesized the knowledge of halogenated metabolites produced by micro- and macroalgae in a review article focusing on biogeochemical importance, biosynthesis and the relevance for chemically mediated interactions of that class of compounds (chapter **4.7**).

3 Publication list

Manuscript A

Paul, C., & Pohnert, G. (2011). Interactions of the algicidal bacterium *Kordia algicida* with diatoms: regulated protease excretion for specific algal lysis. *PLoS One, 6*(6), e21032.

Marine bacteria can affect the performance of diatoms in contrasting ways, for example by promoting diatom growth by releasing vitamins or by inhibiting the growth of diatoms. The bioactive agent responsible for growth inhibition is so far often elusive. Here we show that the bacteria *K. algicida* release proteases which inhibit the growth of *Skeletonema costatum* but do not affect *Chaetoceros didymus*. The protease release was not influenced by co-occuring diatoms but was rather regulated by cell to cell signaling mechanism known as quorum sensing.

Author contribution

Carsten Paul	Designed research, performed research, analyzed the data, wrote the manuscript
Georg Pohnert	Designed research, wrote the manuscript

Manuscript B (unpublished)

Paul, C., & Pohnert, G. (2012). Induction of protease release by the diatom *Chaetoceros didymus* in a cryptic response to bacterial enzymes. In preparation

Diatoms react to chemical cues from bacteria in different ways. For example, the growth of some diatoms is strongly inhibited while others are not affected at all. Here we show that the growth of *C. didymus* is not inhibited by the pathogenic bacteria *K. algicida*. We found an induced physiological reaction in response to the presence of bacterial substances resulting in the release of algal proteases. Further bioassay guided fractionation revealed that bacterial proteins, responsible for detrimental effects *K. algicida* has against other diatoms, cause this release of algal protease. We suggest that such an induced reaction is involved in the resistance of that alga against *K. algicida*.

Author contribution

Carsten Paul	Designed research, performed research, analyzed the data, wrote the manuscript
Georg Pohnert	Designed research, wrote the manuscript

Manuscript C

Paul, C., Reunamo, A., Lindehoff, E., Bergkvist, J., Mausz, M. A., Larsson, H., *et al*. (2012). Diatom derived polyunsaturated aldehydes do not structure the planktonic microbial community in a mesocosm study. *Marine Drugs, 10*(4), 775-792.

Diatoms can produce fatty acid derived polyunsaturated aldehydes (PUA) in wound activated processes. These PUA have been suggested to play a role in predator-prey interactions and are frequently discussed as compounds which structure the planktonic bacterial community as well. While previous studies have used laboratory experiments with concentrations of PUA too high to be ecologically relevant, we applied PUA in ecological relevant concentrations in a mesocosm experiment, which is much closer to the field environment. Simulating a bloom of *S. marinoi* with two different diatom strains we could show that the bacterial community is not structured by realistic or elevated concentrations of PUA. Instead, the bacterial community composition was dependent on culture stage and at least partly dependent on the algal strain suggesting that factors other than PUA regulate the bacterial community.

Author contribution

Carsten Paul	Designed research, daily monitoring of mesocosm development, performed PUA sampling, extraction and addition of PUA, analyzed PUA data, combined different data sets, wrote the manuscript
Anna Reunamo	Sampling and analyzing T-RFLP data
Elin Lindehoff	Designed research, sampling, Analyzed phytoplankton as well as bacterial abundance data
Johanna Bergkvist	Analyzing of virus abundance data
Michaela A. Mausz	Performed PUA sampling and addition, daily monitoring of mesocosm development

Henrik Larsson	Technical attendance, chlorophyll and nutrients analysis, daily monitoring of mesocosm development
Hannes Richter	Extraction of PUA
Sten-Åke Wängberg	Sampling for virus abundance data, supervision of J.B.
Piia Leskinen	Supervision of A.R.
Ulf Båmstedt	Designed Research, supervision
Georg Pohnert	Designed research, wrote the manuscript, supervision of C.P., M.A.M. and H.R.

All co-authors wrote their technical part for the manuscript and commented on the manuscript.

Manuscript D (unpublished)

Paul, C., Mausz, M. A. & Pohnert, G., A co-culturing / metabolomics approach to investigate chemically mediated interactions of planktonic organisms reveals influence of bacteria on diatom metabolism, *Metabolomics*, submitted

Marine bacteria are extremely abundant, about 10^6 cells per mL, in the world's oceans and thus co-occur with marine diatoms. Although bacteria can have big influences on diatom growth, so far no metabolomics approaches have been conducted to explore how bacteria influence diatom cell metabolism. Therefore we developed and validated the functionality of a highly efficient co-culture device that facilitates the simultaneous cultivation of diatoms and bacteria. In this device the cells are physically separated by a membrane but are still able to exchange infochemicals.

The setup was used to co-culture the diatom *Thalassiosira pseudonana* with the bacterium *Dinoroseobacter shibae*. The results indicate that the amino acid metabolism of the diatom is especially stimulated by the presence of bacteria. Even though we cannot discriminate if this influence is due to an uptake of amino acids or a stimulation of amino acid production this example illustrates the complex interactions between organisms beyond a simple effect on growth.

Author contribution

Carsten Paul	Designed research, method development, cultivation, metabolomics experiment, wrote the manuscript
Michaela A. Mausz	Flow cytometry analysis and wrote the corresponding part of the manuscript
Georg Pohnert	Designed research, wrote the manuscript

Manuscript E

Paul, C., Barofsky, A., Vidoudez, C., & Pohnert, G. (2009). Diatom exudates influence metabolism and cell growth of co-cultured diatom species. *Marine Ecology-Progress Series, 389*, 61-70.

Marine diatoms can affect the growth of other diatoms by the release of biologically active yet unidentified compounds. However, the effect diatoms have on the metabolism of other diatoms remains largely unknown. Here we used a non-contact co-culturing approach combined with LC-MS based metabolomics to evaluate such effects. We found that the diatom *S. costatum* can influence the growth of the diatom *T. weissflogii* depending on the growth stage of both algae. The growth of *T. weissflogii* was not affected by *S. costatum* during the exponential and early stationary growth. In contrast, during late stationary growth *T. weissfloggii* cultures in the presence of *S. costatum* showed a stimulated growth in comparison to controls. Additionally, the metabolism of the algae was changed in co-cultures compared to mono-cultures emphasizing an interplay between both organisms.

Author contribution

Carsten Paul	Designed research, performed research, analyzed data, wrote the manuscript. Parts of the manuscript are based on my diploma thesis (Allelopathische Interaktionen zwischen Diatomeen, C. Paul, Friedrich Schiller University Jena 2008)
Alexandra Barofsky	Assisted during data analysis
Charles Vidoudez	Designed research
Georg Pohnert	Designed research, wrote the manuscript

All co-authors commented on a tentative version of the manuscript.

Manuscript F

Vanelslander, B., Paul, C., Grueneberg, J., Prince, E. K., Gillard, J., Sabbe, K., *et al*. (2012). Daily bursts of biogenic cyanogen bromide (BrCN) control biofilm formation around a marine benthic diatom. *Proceedings of the National Academy of Sciences of the United States of America, 109*(7), 2412-2417.

The active agent employed by diatoms in chemically mediated interactions with each other has remained so far mostly elusive. Here we show that the novel natural product BrCN is released by the benthic diatom *Nitschia* cf. *pellucida* to inhibit the growth of competing species. The release is triggered by the onset of light rather than dependent on a circadian clock. The biosynthesis is mostly likely accomplished utilizing haloperoxidases.

Author contribution

Bart Vanelslander	Designed research, involved in the performance of all aspects of the research, wrote the manuscript
Carsten Paul	Performed haloperoxidase and catalase assays, structure elucidation, wrote the manuscript
Jan Grueneberg	Structure elucidation
Emily K. Prince	Performed haloperoxidase assay
Jeroen Gillard	Performed research
Koen Sabbe	Designed research
Georg Pohnert	Designed research, wrote the manuscript, supervision of C.P., J. Grueneberg, E.K.P.
Wim Vyvermann	Designed research, wrote the manuscript, supervision of B.V., J. Gillard

Manuscript G

Paul, C., & Pohnert, G. (2011). Production and role of volatile halogenated compounds from marine algae. *Natural Product Reports, 28*(2), 186-195.

Both marine macro- and microalgae are well known to release a suite of volatile halogenated compounds. The review article combines the knowledge of biogeochemical and biosynthetic aspects of these algal products. Additionally the functions of these metabolites for the algal physiology as well as for algal interactions are discussed.

Author contribution

Carsten Paul	Wrote the manuscript
Georg Pohnert	Wrote the manuscript

4 Publications

4.1 Manuscript A

Interactions of the algicidal bacterium *Kordia algicida* with diatoms: regulated protease excretion for specific algal lysis

Carsten Paul and Georg Pohnert

PLoS One 2011, 6, 6, e21032

OPEN ACCESS Freely available online

PLoS one

Interactions of the Algicidal Bacterium *Kordia algicida* with Diatoms: Regulated Protease Excretion for Specific Algal Lysis

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Abstract

Interactions of planktonic bacteria with primary producers such as diatoms have great impact on plankton population dynamics. Several studies described the detrimental effect of certain bacteria on diatoms but the biochemical nature and the regulation mechanism involved in the production of the active compounds remained often elusive. Here, we investigated the interactions of the algicidal bacterium *Kordia algicida* with the marine diatoms *Skeletonema costatum*, *Thalassiosira weissflogii*, *Phaeodactylum tricornutum*, and *Chaetoceros didymus*. Algicidal activity was only observed towards the first three of the tested diatom species while *C. didymus* proved to be not susceptible. The cell free filtrate and the >30 kDa fraction of stationary *K. algicida* cultures is fully active, suggesting a secreted algicidal principle. The active supernatant from bacterial cultures exhibited high protease activity and inhibition experiments proved that these enzymes are involved in the observed algicidal action of the bacteria. Protease mediated interactions are not controlled by the presence of the alga but dependent on the cell density of the *K. algicida* culture. We show that protease release is triggered by cell free bacterial filtrates suggesting a quorum sensing dependent excretion mechanism of the algicidal protein. The *K. algicida* / algae interactions in the plankton are thus host specific and under the control of previously unidentified factors.

Citation: Paul C, Pohnert G (2011) Interactions of the Algicidal Bacterium *Kordia algicida* with Diatoms: Regulated Protease Excretion for Specific Algal Lysis. PLoS ONE 6(6): e21032. doi:10.1371/journal.pone.0021032

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Diatoms (Bacillariophyceae) are very abundant unicellular microalgae in marine and freshwater ecosystems and are highly ecologically relevant because of their position at the bottom of the marine food web [1]. Different diatom species can occur in dense blooms and dominate the phytoplankton community during short or prolonged periods. Because of their ecological importance, understanding the factors that limit diatom growth and proliferation is crucial. These can include abiotic factors such as extreme light or temperature conditions or nutrient limitation [2]. But also biotic factors such as grazing by zooplankton [3,4], allelopathic effects of other phytoplankton species [5], or viral infections can have a negative impact on diatoms [6,7]. It is also documented that bacteria can even control bloom termination processes [8,9].

In terms of cell numbers marine bacteria are even more abundant than diatoms and by utilization of organic matter they also play a key role in plankton communities [10]. Interactions between phytoplankton and bacteria have gained increasing attention as the relevance of the microbial loop for plankton communities becomes more evident [11,12,13]. Bacteria can act synergistically with diatoms and symbiotic interactions have been reported from several systems [11,14,15]. But bacteria can also control algal populations e.g. by inhibiting growth of diatoms and other phytoplankton members or by active lysis of algal cells [16,17,18]. Bacterial inhibition of algal growth either requires direct cell contact [19] or can be mediated by excreted extracellular substances [18,20]. Inhibitory interactions between bacteria and phytoplankton are mostly investigated with the goal of finding a biological control for harmful algal blooms [21,22]. In contrast, only few ecological studies on the bloom termination of non-harmful plankton species exist [12,18]. Besides few exceptions the identity of the compounds or enzymes responsible for the algicidal effect remains unknown. Lee *et al.* [20] demonstrated that *Pseudoalteromonas* sp. produces a high molecular weight extracellular protease which is able to inhibit the growth of the diatom *Skeletonema costatum*. But lower molecular weight algicidal compounds, such as rhamnolipid biosurfactants from *Pseudomonas aeruginosa* or the pigment prodigiosin from the bacterium *Hahella chejuensis* have also been identified [23,24].

The regulation of the production of such inhibitory compounds is mostly unknown. An exception is the report on genes potentially involved in prodigiosin biosynthesis [25]. Generally, bacterial production of inhibitory substances can be regulated by external factors which might also be a relevant mechanism for planktonic species. Examples from the terrestrial environment include mechanisms where secretion of active metabolites occurs only in the presence of the host or where the release of active compounds is dependent on the cell density of the bacteria [26]. The latter process is known as quorum sensing (QS). QS is a process governed by small molecules such as acyl homoserine lactones or peptides that are excreted from bacteria. Reception of such

metabolites allows bacteria to determine the local density of their population and to regulate gene expression. These changes in gene expression can result in a variety of physiological changes like the onset of bioluminescence, antibiotic synthesis or extracellular enzyme production [26].

In a screening of algicidal bacteria the aerobic, Gram-negative, non-motile *Kordia algicida* was isolated during a bloom of the cosmopolitan diatom *Skeletonema costatum*. The bacterium was able to kill *S. costatum* and also exhibited algicidal activity against other microalgae in co-culture experiments [27]. The genome sequencing of *K. algicida* is underway and interestingly, several genes coding for proteases have been identified and deposited in the databases. We decided to investigate *K. algicida*/diatom interactions in more detail. We reasoned that for any bacterium in the dilute matrix of the plankton, secretion of secondary metabolites or proteins that mediate lysis of diatoms is costly and thus we proposed the hypothesis that algicidal activity is controlled by biotic signals in the *K. algicida*/*S. costatum* system.

In this study we show that the algicidal bacterium *K. algicida* relies on diffusible enzymes >30 kDa to interfere with algal growth. We show that the activity is specific for certain diatoms, while others are not susceptible. Furthermore we show that the excretion of active proteases is not regulated by the presence of a co-cultured diatom species but is rather dependent on the bacteria cell density in a process that bears the hallmarks of quorum sensing.

Methods

Algal and bacteria culturing

The Gram-negative marine bacterium *Kordia algicida* strain OT-1 was originally isolated from a *Skeletonema costatum* bloom [27] and was obtained from the NITE Biological Resource Center (NBRC 100336). Cultures were grown at 15°C under constant shaking (90–100 rpm min^{-1}) in autoclaved ZoBell medium (5 g bacto peptone, 1 g yeast extract, 10 mg $FePO_4$, 34 g of Instant Ocean in 1 L bidistilled water) [28]. Dense cultures were used to prepare glycerol stock cultures (20 vol. %). Before each set of experiments a new culture was started from the glycerol stock.

Non-axenic *S. costatum* (RCC75) and *Thalassiosira weissflogii* (RCC76) were obtained from the Roscoff Culture Collection, France. *Phaeodactylum tricornutum* (UTEX 646) was obtained from the Culture Collection of Algae in Austin, TX, USA. *Chaetoceros didymus* (CH5) was isolated by S. Poulet, Station Biologique, Roscoff, France and is maintained in our culture collection. The strains were cultivated under a 14/10 hours light/dark cycle with 40–45 µmoles photons s^{-1} m^{-2} at 15°C in artificial seawater prepared according to Maier and Calenberg at a pH of 7.8 [29]. The nutrient concentrations were 620 µM nitrate, 14.5 µM phosphate and 320 µM silicate.

Estimating bacterial and algal growth

The optical density (OD) of *K. algicida* cultures was measured with a Specord M42 UV-vis spectrophotometer by Carl Zeiss (Jena Germany) at a wavelength of 550 nm. Bacterial growth rate was estimated graphically by plotting measured OD values on a logarithmic scale. Time points that showed a linear increase were used to perform an exponential regression with $OD_2 = OD_1\ e^{\mu t}$ wereμ represents the bacterial growth rate and OD_1 and OD_2 represent the optical densities at time point 1 and 2, respectively.

Algal growth was determined by measuring the *in vivo* chlorophyll a fluorescence using 300 µL of each culture in 96 well plates or 1.5 mL in 24 well plates. The fluorescence was measured with a Mithras LB 940 plate reader by Berthold Technologies (Bad Wildbad, Germany). Cell density was determined using a Fuchs-Rosenthal hematocytometer with an upright microscope (Leica DM 200, Leica, Germany).

Generation of cell free bacterial and co-culture filtrates

Exponentially growing *K. algicida* was inoculated into a 10:1 (vol. %) mixture of artificial seawater and ZoBell medium. After the culture reached an optical density (OD) >0.32 one mL was diluted into 50 mL of seawater. After 24 hours the cultures were gently filtered through a 0.22 µm sterile polyethersulfone (PES) filter (Carl Roth; Karlsruhe, Germany). To obtain a cell free filtrate of *S. costatum*/*K. algicida* co-cultures, 1 mL of bacterial culture in 10:1 seawater : ZoBell media (OD> 0.32) was inoculated with 50 mL of an exponentially growing *S. costatum* culture (ca. 1.5 10^6 cells mL^{-1}) and grown for 24 h before filtration as described above.

Monitoring algicidal activity

We inoculated 1.125 mL of the filtrate (see above) with 375 µL of the respective exponentially growing diatom culture in the wells of 24 well plates. For controls aliquots of 375 µL of the same starting cultures were diluted with 1.125 mL of artificial seawater. Plates were cultured under the previously mentioned conditions and measurements were performed over regular time intervals. The *in vivo* fluorescence of chlorophyll a of all cultures was measured as indicator for algal growth.

Size fractionation

Size fractionation experiments were performed with a filtrate of a co-culture of *S. costatum* and *K. algicida* as well as with filtrates of mono-cultures of these species (see above). A volume of 15 mL of the respective filtrates was fractionated using Amicon Ultra centrifugal filter units with a molecular weight cut off of 30 kDa (Millipore, Billerica, MA, USA) as described in the manufacturer's instructions. The high molecular weight fraction was diluted to 1.5 mL with artificial seawater. The biological activity of the filtrates was monitored in 96 well plates by inoculating 240 µL of raw or fractionated filtrates with 60 µL of exponentially growing *S. costatum*.

Heat inactivation of filtrates

Active cell free filtrates of *S. costatum*, *K. algicida*, and co-cultures were incubated at 80°C for 10 min. The algicidal activity was monitored after inoculating 375 µL of *S. costatum* culture in 1.125 mL of regular or heat treated filtrate in 24 well plates.

Conditioning of active filtrates

Replicates each containing 1.125 mL of active filtrate were inoculated with 375 µL of *S. costatum*, *C. didymus* or seawater in 24 well plates and incubated using the previously described culturing conditions. After 24 h each treatment was filtered through a 0.22 µm PES filter and the replicates within one treatment were combined. Aliquots of 1.125 mL of the combined filtrates were used to incubate 375 µL of exponentially growing *S. costatum* in 24 well plates. Other aliquots of the cell free filtrates were heat deactivated as described previously and inoculated with *S. costatum* in the same way to serve as controls.

Protease inhibition experiment

Cell free bacterial filtrates were harvested as described above and the irreversible serine-protease inhibitor phenylmethanesulphonylfluoride (PMSF; Sigma, Munich, Germany) was tested for its ability to reduce algicidal activity against *S. costatum*. A working stock solution (1 M in isopropanol) was used to add a final

concentration of 1 mM to active *K. algicida* filtrates and artificial seawater which was used as positive control. After incubation for 30 min in the dark at 15°C the filtrate was applied to *S. costatum* as described above and algal growth monitored as *in vivo* chlorophyll a fluorescence.

Protease activity

The measurement of protease activity in bacterial filtrates was based on the conversion of BODIPY FL (E 6638) to a fluorescent product [30]. The dye was purchased from Invitrogen (Carlsbad CA, USA) and the assay was performed following the manufacturer's instructions. Briefly, 10 µL of cell free filtrate of *K. algicida* cultures were diluted in 100 µL digestion buffer (Invitrogen) and 100 µL of the dye at a concentration of 10 µg mL^{-1} were added. After incubation at room temperature under exclusion of light for 1 h, the fluorescence was measured with a Mithras LB plate reader with an excitation filter of 470±5 nm and an emission filter of 510±20 nm. Linearity was ensured in independent calibrations.

Calculation of protease release rate

The protease release rate (PRR) was calculated according to PRR = Δ(protease fluorescence)/(OD(Av) * Δ(t) where Δ(protease fluorescence) represents the difference between the measured fluorescence at two time points, OD(Av) represents the average of the OD at these time points and Δ(t) the time in hours between these time points. PRR values not significantly different from 0 are not displayed in the figures.

Induction of protease release by conditioned bacterial filtrates

K. algicida was inoculated into 100 mL of a 10:1 mixture of artificial seawater and ZoBell medium in three replicates. The growth and the protease release rate were regularly monitored until the first significant protease release was measured. Afterwards the cultures were sterile filtered, the cell free filtrate was pooled and proteases as well as other high molecular weight constituents were removed using Amicon Ultra centrifugal filter units. A volume of 10 mL of filtrate was added to *i*) 10 mL of freshly inoculated *K. algicida* in 1:10 mixture medium and *ii*) to 10 mL *K. algicida* cultures inoculated 16 h before the addition of the conditioned filtrate. Protease activity in these inoculations was monitored as described above.

Extraction of homoserine lactones

The attempt to extract acyl-homoserine lactones was performed with cell free supernatant of dense bacterial cultures. The supernatant was extracted with CH_2Cl_2 according to an established protocol [31] and samples were run on an Perkin Elmer Auto System XL gas chromatograph (GC) equipped with a SPB-5 column (40 m, 0.32 mm internal diameter and 0.25 µm film thikness. He 5.0 was used as a carrier gas with a constant pressure of 160 kPa. The GC was coupled with a Perkin Elmer TurboMass mass spectrometer (Waltham, MA, USA).

Statistical analysis

The test for statistical significant differences at different time points over the course of an experiment was conducted using a two way repeated measures analysis of variance (RM-ANOVA) with Sigmaplot 11. Post hoc test of significance was performed using the Tukey method implemented in Sigmaplot 11. A student t-test was performed to exclude PRR values that were not significantly different from 0. Significance level was generally set for all analysis $P<0.05$.

Results

Effect of K. algicida and cell free filtrates on different diatom species

In an initial experiment we prepared a co-culture of *S. costatum* and *K. algicida* and monitored the cell growth of *S. costatum*. We observed a significant reduction of the diatoms cell density after 7 h ($P = 0.04$). After 25 h the cell density of the co-cultured diatoms was only 12.1% of the corresponding control (data not shown). All further experiments were performed with cultures of this active *K. algicida*. We tested the effect of a cell free filtrate of *K. algicida* on the diatoms *S. costatum*, *C. didymus*, *P. tricornutum* and *T. weissflogii* over the period of 64 h. Fig. 1 shows the *in vivo* chlorophyll a readings 39 h after inoculation. The cell growth of *S. costatum*, *P. tricornutum* and *T. weissflogii* were significantly inhibited ($P<0.001$ for all species). A two way repeated measures ANOVA revealed significant differences between treatment and the corresponding controls for all data points recorded 24 h after inoculation or later ($P<0.001$ for all species, data not shown). At the end of the experiment ($t = 64$ h) the *in vivo* chlorophyll a fluorescence in the treatments were only 8.7%, 8.7% and 19.4% of the respective control for *S. costatum*, *P. tricornutum*, and *T. weissflogii* respectively (data not shown). In contrast, *C. didymus* growth was not affected by *K. algicida* filtrate ($P \geq 0.553$ at all sampling points; RM-ANOVA). At the end of the experiment the *in vivo* chlorophyll a signal in the treatment was 99.7% of the corresponding control.

No regulation of algicidal activity in the presence of the host

The filtrate of active *K. algicida* cultures as well as the filtrate of *K. algicida*/*S. costatum* co-cultures both caused a significant decrease of cell growth of *S. costatum* in comparison to *S. costatum* filtrate as control ($P<0.001$ for both) (Fig. 2). No up-regulation of algicidal activity was observed in the presence of *S. costatum*, since the effect of the filtrate of *K. algicida* and of *K. algicida*/*S. costatum* co-cultures did not differ significantly ($P = 0.821$).

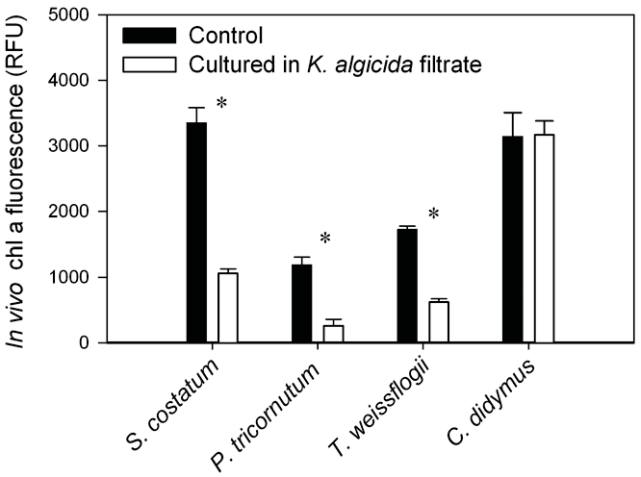

Figure 1. Screening for the susceptibility of four different diatom species to *K. algicida* filtrate. Mean values of *in vivo* fluorescence + SD (n = 4) displayed are measured after 39 h. Asterisks indicate significant differences between the respective control and treatment.
doi:10.1371/journal.pone.0021032.g001

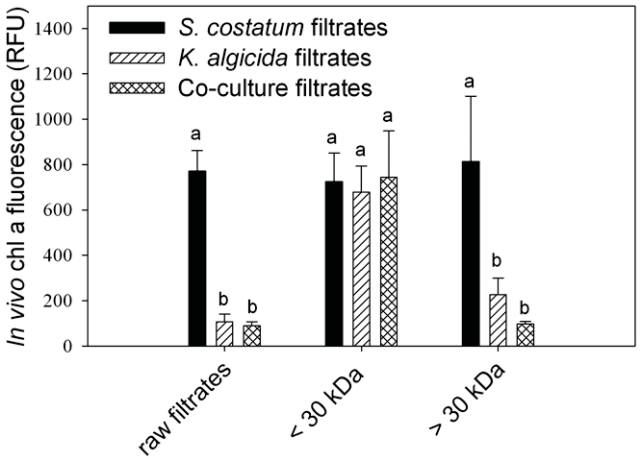

Figure 2. Effects of size fractionated *S. costatum, K. algicida* and co-culture cell free filtrates on the susceptible species *S. costatum* determined by *in vivo* fluorescence. Values displayed are measured after 47 h and are mean values + SD (n = 5 for all but *S. costatum* where n = 4). Different letters indicate statistically significant differences.
doi:10.1371/journal.pone.0021032.g002

Size fractionation of the bioactive filtrate

The filtrates of *K. algicida* and co-cultures of *K. algicida* and *S. costatum* containing only compounds with a molecular weight below 30 kDa had no inhibitory effect compared to the corresponding control using a <30 kDa fraction of medium from a *S. costatum* culture (P = 1 for both) (Fig. 2). None of these treatments were significantly different from a treatment with an unfractionated filtrate of a *S. costatum* culture ($P \geq 0.899$ in all cases). In contrast, treatments with the high molecular weight fraction >30 kDa of the *K. algicida* and co-culture filtrates resulted in a significant inhibition of algal growth in comparison to the control ($P < 0.001$ for both). The inhibition caused by the high molecular weight filtrate of the *K. algicida* culture was not significantly different from the inhibition by the high molecular weight filtrate of the co-culture (P = 0.832). The effects of high molecular weight filtrates of both *K. algicida* cultures and co-cultures were not significantly different as compared to the effect of the corresponding unfractionated filtrates (P> 0.321 for all comparisons) (Fig. 2).

Heat deactivated filtrates

The filtrates of *K. algicida* and co-cultures of *K. algicida* and *S. costatum* significantly inhibited the growth of *S. costatum* while aliquots of the same filtrates that were heated at 80°C for 10 min prior to the assay had no negative effect for any monitored time point (data not shown) ($P < 0.001$ for a comparison of the effect of filtrates versus heat treated filtrates at t = 38 h onwards). Filtrates of *K. algicida* and of the co-cultures again showed no significant difference in their activity over the complete time course of this experiment ($P > 0.982$).

Protease as the inhibiting enzyme

Aiming to identify the inhibiting activity of *K. algicida* we performed experiments adding commercially available protease from *Streptomyces griseus* to *S. costatum* and *C. didymus* in a concentration range of 1.7 U mg^{-1} to 0.2 U mg^{-1}. While *C. didymus* was not affected by any of these protease additions *S. costatum* was inhibited in growth by the external proteases (data not shown). Further evidence for the involvement of proteases in the interaction was gained by protease inhibition experiments. The addition of the serine-protease inhibitor phenylmethanesulphonylfluoride (PMSF) significantly decreased the inhibition of algal growth by *K. algicida* medium in comparison to controls without PMSF ($P < 0.038$). However, the protease inhibitor did not re-establish the complete algal growth and resulted in significant less *in vivo* chlorophyll a fluorescence compared to a seawater control (Fig. 3) ($P < 0.001$).

Test for detoxification of the K. algicida activity by *C. didymus*

An active filtrate of *K. algicida* was incubated for 24 h with a *C. didymus* culture to test whether *C. didymus* could deactivate algicidal activity. As controls aliquots of the same *K. algicida* filtrate were used without further treatment or after incubation with a *S. costatum* culture. After a second filtration to remove the diatoms, the respective filtrates were used in incubations with *S. costatum*. Neither incubation of the active filtrate with *C. didymus* nor with *S. costatum* resulted in decreased activity as compared to the control (Fig. 4) ($P \geq 0.956$ and $P \geq 0.585$, respectively over the entire time of the experiment). To test if this effect was due to a general loss of activity all three filtrates were heat inactivated, resulting in significantly reduced activity in all cases ($P < 0.005$ for all comparisons 54 h and onwards).

Protease release by *K. algicida*

Exponential bacterial growth started after a lag period of 29 h. During this period there was no detectable protease activity in the *K. algicida* medium (Fig. 5A). After 29 h the culture started to grow exponentially and reached a growth rate of $\mu = 0.142 \pm 0.004\ h^{-1}$. Exponential growth lasted from t = 29 until t = 48 h. In the beginning of the exponential growth phase there was no protease release. A significant release of proteases started after 44 h, in the late exponential phase. This release proceeded for 18 h and stopped after 62 h. In later stationary growth we could not observe any protease release. To exclude an underestimation of the protease release due to potential instability of the enzyme, we verified the protease stability in seawater over a period of 9 h. After this time period no detectable decrease of the protease activity could be observed (P = 0.866 in student t-test, data not shown).

Induction of protease release

In order to test if chemical communication regulates bacterial activity as known from quorum sensing we examined the effect of cell free bacterial filtrate on the excretion of protease from freshly inoculated *K. algicida* cultures and cultures that were incubated for 16 h (Fig. 5 B & C). The addition of *K. algicida* conditioned cell free filtrate to freshly inoculated bacteria cultures accelerated the protease release. These cultures exhibited already a significant protease release rate after 14 hours of cultivation (Fig. 5B) which was approximately 5 times higher than the release rate observed under standard growth conditions (Fig. 5A). Under standard cultivation conditions an optical density of >0.1 was needed before protease release occurred. In contrast cultures where conditioned cell free medium was applied started to excrete significant amounts of protease already at an optical density <0.01 (Fig. 5B). This protease release was stopped after 26 h and started again after 32 h when an optical density of >0.05 was reached.

If the same cell free filtrate was added to *K. algicida* cultures 16 h after inoculation we observed a significant release of bacterial proteases already after 8 h confirming an induction of enzyme release by a bacterial cell free filtrate (Fig. 5C).

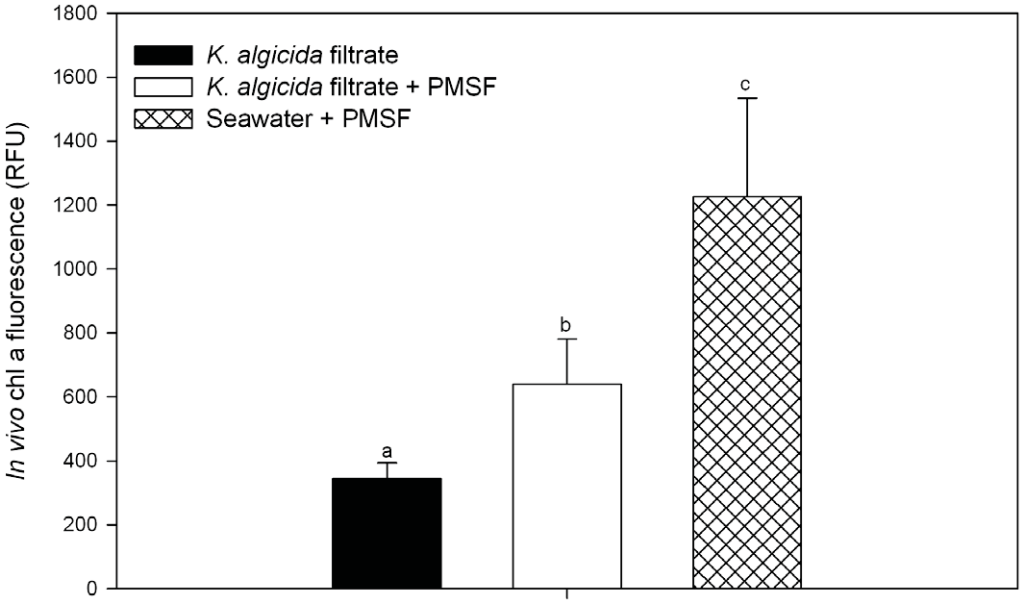

Figure 3. Effect of the protease inhibitor PMSF on the inhibiting effect of *K. algicida* filtrates. Values displayed are measured after 52 h and are mean values + SD (n = 5).
doi:10.1371/journal.pone.0021032.g003

Discussion

The marine bacterium *K. algicida* has a strong algicidal effect on the diatom *S. costatum*. In a direct contact situation a significant inhibition of diatom proliferation can be observed after 7 h if a dense bacteria culture is employed for incubation. This is consistent with previous findings of *S. costatum* cells that were killed quantitatively after 3 days in a co-culture with *K. algicida* [27]. The negative effect of the bacterium is not exclusively transmitted through contact with the diatom but can be also mediated via diffusible compounds. This is clearly demonstrated by the fact that activity of the *K. algicida* medium remains after removal of the cells by sterile filtration. Inhibition of growth relative to a control is observable within the first 24 hours of incubation, indicating rapid action of the algicidal compounds. Compared to reports from other systems, where a>24 h delay of effects on the algal cells was observed after algae were treated with algicidal bacteria, both the direct interaction as well as the action of the filtrate reported here are quick [8,32]. Diffusible substances mediating algicidal activity have been previously observed from bacteria and can include both, small molecular weight metabolites as well as proteins [33,34]. The use of dissolved substances to inhibit the growth of algae is common in bacteria belonging to the phylum of γ-proteobacteria which includes the genera *Alteromonas* [8], *Pseudoalteromonas* [22,35] and *Vibrio* [36]. However, *K. algicida* belongs to the *Cytophaga-Flavobacterium-Bacteroides* phylum (CFB). Genera within this group usually require direct cell contact to kill their prey [16,35], although there are exceptions reported [18]. *K. algicida* is thus a rare example of a CFB bacterium that does not require cell contact with its prey to inhibit the algal growth, but releases diffusible active enzymes.

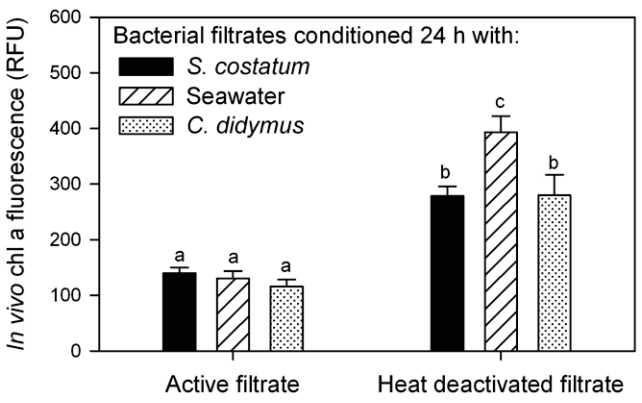

Figure 4. Left: Growth of *S. costatum* indicated as *in vivo* fluorescence in *K. algicida* filtrate conditioned for 24 h with *S. costatum, C. didymus* or seawater. Right: Incubations with the same filtrates that were heat inactivated before the start of the experiment. Displayed are mean values + SD (n = 6) taken 70 h after the start of the experiment. A statistically significant difference is indicates by different letters above the bar.
doi:10.1371/journal.pone.0021032.g004

The release of active substances by *K. algicida* allowed us to further explore the nature of the active principles. A first survey revealed that *K. algicida* filtrate is also active against other diatom species (Fig. 1). The activity against the pennate diatom *P. tricornutum*, as well as that against the centric diatom *T. weissflogii* was comparable to that observed against *S. costatum*. In contrast, another centric diatom, *C. didymus* was not susceptible against the diffusible factors released by *K. algicida*. This missing susceptibility is apparently not due to an active detoxification by *C. didymus* since medium from a *C. didymus*/*K. algicida* co-culture is still active against *S. costatum* (Fig. 4). The physiological properties which mediate *C. didymus* resistance cannot, however, be concluded from our experiments. Selectivity of algicidal activity is important to understand ecological interactions within the planktonic community. Additionally, proposals to apply bacteria in order to control red tides should seriously consider the selectivity of algicidal activity [35,37]. Different levels of specificity have been observed from algicidal bacteria. Selective activity against one algal species and universal activity against all tested species in a given taxon have been reported as well as all intermediate forms of specificity

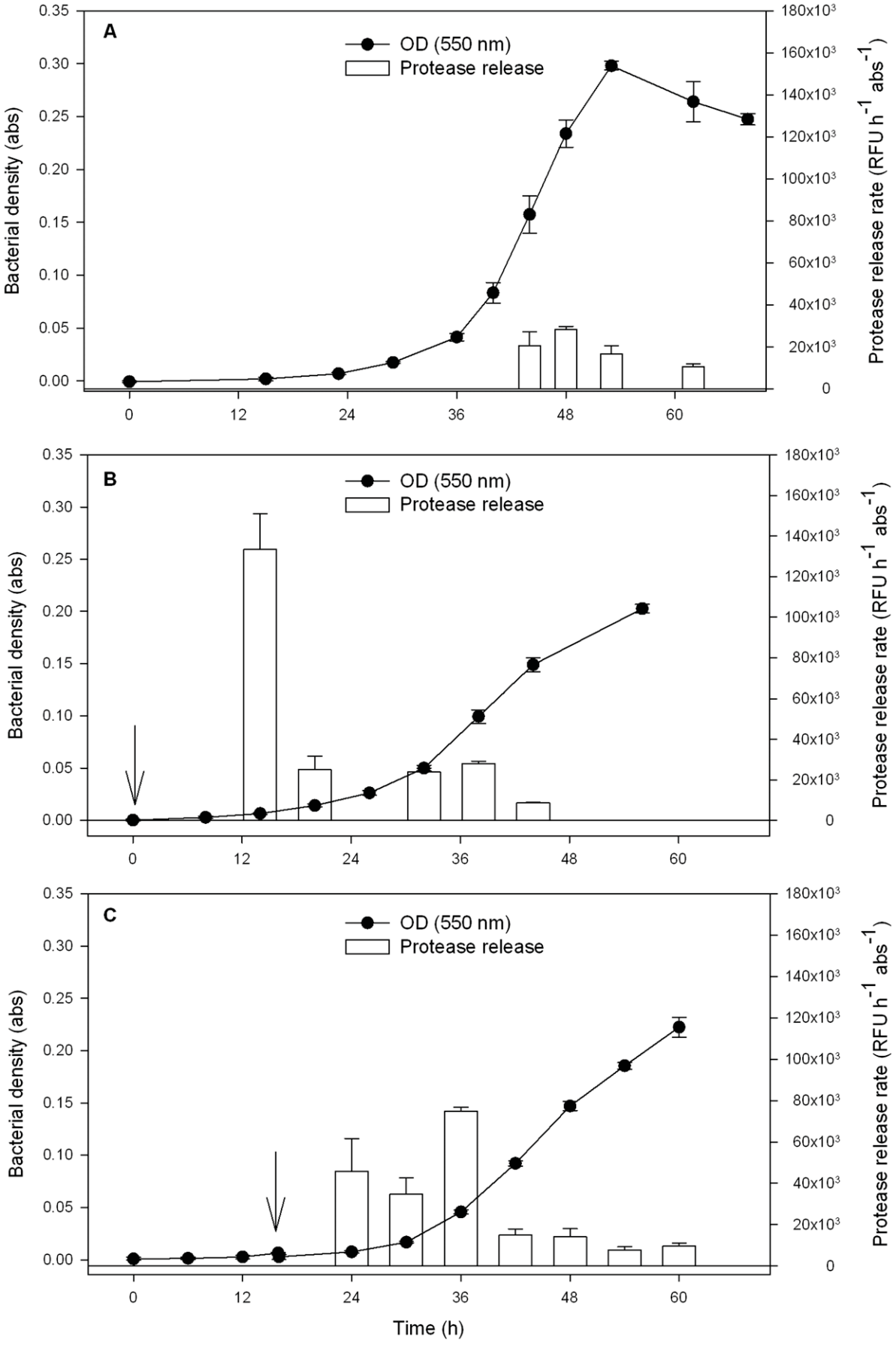
A
B
C
OD (550 nm)
Protease release
Bacterial density (abs)
Protease release rate (RFU h-1 abs-1)
Time (h)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
180x10³
160x10³
140x10³
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
0
12
24
36
48
60

Figure 5. Protease release by *K. algicida* during the growth of a culture. A) Protease release pattern of *K. algicida* under standard growth conditions. B) Protease release pattern of *K. algicida* with conditioned cell free filtrate added directly with the inoculation of the cultures. C) Protease release pattern of *K. algicida* with conditioned cell free filtrate added 16 h after the inoculation of the cultures. The line indicates bacterial growth measured as OD and the bars give the bacterial protease release rate. The arrows indicate the time of the addition of *K. algicida* conditioned cell free filtrate. Displayed are mean values + SD (n = 3).
doi:10.1371/journal.pone.0021032.g005

like they are shown here [16]. From an ecological perspective it is obvious that resistance mechanisms of algae have the potential to provide selective advantages. When other diatom species that are potential competitors for resources are inhibited, the unsusceptible alga can proliferate. Thereby the bacteria can directly influence plankton species successions.

Basic characterization of the released algicide showed that it bears all hallmarks of an enzyme. It has a molecular weight >30 kDa (Fig. 2) and the activity can be inactivated by heat treatment. A survey of the literature suggests that dissolved proteases are prime candidates for algicidal enzymes. Lee et al. were the first to demonstrate the activity of proteases in the interaction of the bacterium *Pseudoalteromonas* sp. and the diatom *Skeletonema costatum* [20]. After indirect evidence from bioassays they isolated a 50-kDa serine protease with algicidal activity. Several subsequent studies supported the role of enzymes from algicidal bacteria in the lysis of algae [17]. Using fluorescence based assays we were able to show that active medium from *K. algicida* and from *K. algicida*/*S. costatum* co-cultures exhibited substantial protease activity. Indeed, *S. costatum* was susceptible to protease treatment. If the protease from the bacterium *Streptomyces griseus* was applied the diatom growth was inhibited compared to a control. In agreement, application of the protease inhibitor PMSF to active *K. algicida* medium resulted in a significantly higher growth of *S. costatum* compared to uninhibited controls. The growth of *S. costatum* was, however, not fully restored after the application of the protease inhibitor. Similarly, PMSF did not fully neutralize the motility reduction of the dinoflagellate *Lingulodinium polyedrum* caused by bacterial proteases [38]. The inhibitor experiment demonstrates, however, the involvement of a protease in the interaction but it might well be that additional activities can be responsible for the observed interactions. Alternatively, the algicidal protease might not be very sensitive to the inhibitor PMSF and the applied concentration might not be sufficient for a quantitative inhibition.

It has been argued that the release of a freely diffusible algicide is unlikely to be energetically efficient for killing algal cells suspended in seawater [16]. Since ratio of the volume of bacterial cells to the volume of seawater they inhabit is ca. 10^{-7} in an average dilute situation in the plankton [39] an uncontrolled release of any active principle would most likely not result in concentrations sufficient for algicidal activity or result in high costs. However, a release of active metabolites could provide a selective advantage if it is under the control of a metabolic switch that is triggered only under environmental conditions where the production of algicides is beneficial. We tested the hypothesis that algicidal activity is only induced in the presence of susceptible algae or in the presence of signals of these algae. No evidence was found for such an induced mechanism since algicidal activity did not increase in the presence of diatoms (Fig. 2).

Another possibility to increase the success of released active compounds would be a metabolic switch dependent on the density of a bacterial population. Based on the findings that the algicidal activity observed in our study was caused by a protease, we monitored protease activity as a function of *K. algicida* culture density. We indeed observed a synchronized release of a protease, which could be explained by quorum sensing like mechanism in *K. algicida* (Fig. 5) [40]. We found support for such phenomenon using experiments with conditioned cell free supernatant of *K. algicida*. After adding such filtrates to freshly inoculated *K. algicida* cultures the protease release was remarkably accelerated (Fig. 5). These results fit to known quorum sensing dependent secretion mechanisms of other bacteria species such as the human pathogen *Pseudomonas aeruginosa* where the excretion of exoenzymes that determine virulence is controlled by bacterial density [41,42]. Quorum sensing in gram negative bacteria is often mediated by acyl homoserine lactones (AHL) as it can be for example observed in the *P. aeruginosa* pathogenicity [41,42]. We were, however, not able to detect any AHL in dichloromethane extracts of protease releasing cultures using sensitive GC/MS methods. Several other QS molecules that have been previously described for Gram-negative bacteria can be considered as alternative candidates and further tests will have to be performed in the search for the regulative principle in plankton assemblages [43,44]. Gram-negative bacteria found in all kinds of habitats often rely on quorum sensing signals to trigger metabolic events. In planktonic bacteria the alternative induction pathway (AI-2) for quorum sensing type regulation has been detected although it could not be directly linked to algicidal activity [18]. Evidence also exists for the QS-regulation of the production of the algicidal pigment PG-L-1 in a marine *γ-proteobacterium* [45]. While these studies give rather indirect evidence we can show here clearly that release rates of active principles are regulated. Comparable regulative mechanisms have also been suggested in a study of algicidal *Pseudoalteromonas* sp.. Mitsutani et al. [17] could show in gel electrophoretic experiments that the production of several enzymes was only observed during stationary phase and that bacteria only exhibited algicidal activity during this phase.

In the plankton such a density dependent release of proteases might provide an advantage if a sufficient bacterial density is required for efficient lysis of algae. Diffusible substances aiding algal lysis might provide a benefit for locally dense bacterial assemblages. Bacteria could jointly overcome defense systems of the alga in cases when active principles from single bacteria would not be effective. Lysis of algal cells could increase available nutrient concentrations in the vicinity of the bacterial assemblage and such a control could be an efficient means for a concerted mobilization of resources.

Our results on the specificity of the algicidal activity as well as on the density dependent regulation of the release of an active protease by an algicidal bacterium support the view that a multitude of chemical signals can regulate plankton interactions on all levels.

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Author Contributions

Conceived and designed the experiments: GP CP. Performed the experiments: CP. Analyzed the data: GP CP. Contributed reagents/materials/analysis tools: GP CP. Wrote the paper: GP CP.

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4.2 Manuscript B

Induction of protease release by the diatom *Chaetoceros didymus* in a cryptic response to bacterial enzymes

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In preparation

Induction of protease release by the diatom *Chaetoceros didymus* in a cryptic response to bacterial enzymes

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Introduction:

Diatoms (Bacillariophyceae) comprise an abundant group of unicellular microalgae distributed worldwide in marine and freshwater habitats. Diatoms play a crucial role in the marine ecosystems and are on the bottom of the marine food web [1] due to their massive carbon fixation. Thus, a detailed understanding of the diatom population dynamics is fundamental for a comprehensive view on plankton ecology. Factors influencing diatom bloom propagation are diverse, and range from abiotic factors such as temperature or nutrient conditions [2] to different biotic interactions. Biotic factors include defense reaction in response to grazers [3,4], allelopathic interactions with other phytoplankton species [5-7] and the effect of viruses on phytoplankton species [8]. Additionally bacteria can have a substantial effect on the performance of phytoplankton species including diatoms [9-11]. Some bacteria form mutualistic interactions with phytoplankton species. For example, the growth of the toxic dinoflagellate *Alexandrium fundyense* was dramatically increased by the presence of bacteria of the genus *Alteromonas* sp [12]. The growth promoting effect of bacteria on different algae can be due to a delivery of vitamins from the bacteria to the algae within a symbiosis [13,14]. However, the direction of interaction is labile. The bacterium *Phaeobacter gallaeciensis* can have a mutualistic interaction with the coccolithophore *Emiliania huxleyi* under certain circumstances by supplying the algae

with growth promoting factors and receiving dimethylsulfoniopropionate (DMSP) as a sulfur source. However, when the bacteria recognize *p*-coumaric acid released by the algae, the bacteria can switch their metabolism and produce algicides called roseobacticides which kill the algae and result in a surplus of nutrient for the bacteria [15,16]. In addition, bacteria have detrimental effects for the algae. For example, bacteria reduce the swimming motility of dinoflagellates [17] and the growth of diatoms [11,18] by the excretion of proteases. Even though there is evidence that non-enzymatic algicides are involved in the interaction between phytoplankton species and bacteria as well [19,20], enzymes, especially proteases, seem to be involved frequently. The effect of algicides is often species-specific. For instance, *Pseudoalteromonas* sp. releases a heat labile compound into the surrounding seawater which inhibits the growth of the dinoflagellates *Alexandrium catenella* but does not affect the growth of the diatom *Skeletonema* sp. or the cyanobacterium *Oscillatoria* sp. [21]. These inhibitory interactions of bacteria and phytoplankton, mostly dinoflagellates, have often been investigated with the aim of finding a biological control of harmful algal blooms (HABs) [22,23]. In comparison, the role of inhibitory bacteria on the bloom propagation of non-toxic diatoms has so far been mostly neglected. Diatoms occur in the ocean in complex mixtures [24] with several different species co-existing next to each other. Bacteria might promote this diversity by a species-specific effect of inhibitory substances.

Recently we showed that the bacteria *Kordia algicida* release proteases in a quorum sensing regulated manner into the surrounding seawater [11]. While several diatom species such as *Skeletonema costatum*, *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* showed a significantly reduced growth after exposure to proteases containing cell-free filtrates of *K. algicida* for approximately 40 h, the diatom *Chaetoceros didymus* was not inhibited. The reason why *C. didymus* is not susceptible to the enzymes released by *K. algicida* remained elusive. To our knowledge no defense mechanisms explaining the selective mode of action of algicides are currently known, even though cell surface associated polysaccharides were suggested to play a role by protecting the cell against proteolytic stress [10].

Here we show that *C. didymus* is indeed resistant to proteolytic attack by *K. algicida*. This resistance may be explained by the fact that *C. didymus* releases additional proteases induced by excreted proteins of *K. algicida* which might serve as a chemical defense.

Materials and Methods:

Algal and bacteria culturing

The bacterium *Kordia algicida* strain OT-1 was obtained from the NITE Biological Resource Center (NBRC 100336). Culture were grown at room temperature under constant shaking (80 to 100 rpm) in ZoBell medium (5 g bacto peptone, 1 g yeast extract, 10 mg $FePO_4$, 34 g Instant Ocean (Aquarium Systems, Sarrebourg, France) in 1 L Milli Q water). An exponentially growing culture was used to prepare a glycerol stock of this culture which was subsequently used to prepare a starting culture in ZoBell media before each set of experiments.

Skeletonema costatum (RCC 75) was obtained from the Roscoff culture collection, France. *Chaetoceros didymus* was originally isolated by S. Poulet at the Station Biologique in Roscoff, France and maintained in our culture collection. The diatoms were cultivated in artificial seawater [25] buffered at a pH of 7.8 at 15°C under a light/dark rhythm of 14/10 hours and an illumination of 40-45 µmoles photons s^{-1} m^{-2}. The final nutrient concentrations in the medium were 620 µM nitrate, 14.5 µM phosphate, and 320 µM silicate.

Estimating bacterial and algal growth

Bacterial growth was monitored using the optical density (OD) measured with a Specord M42 UV-vis spectrophotometer by Carl Zeiss (Jena, Germany) at 550 nm.

The diatom growth was monitored to investigate the harmful effect of bacterial exudates measuring the chlorophyll a fluorescence with a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany). The fluorescence of 1.5 mL *C. didymus* cultures in 24 well plates (Greiner Bio-One, Frickenhausen, Germany) was measured *in vivo*.

Harvesting cell free bacterial filtrates

The harvesting of cell free bacterial filtrate was performed as described [11]. Briefly, exponentially growing *K. algicida* was inoculated into a mixture of artificial seawater and ZoBell medium in a ratio of 10:1 until the culture reached an OD > 0.3. Afterwards, 1 mL of the culture was inoculated into 50 mL of seawater. After 24 h the cell free filtrate was harvested by sterile filtration with polyethersulfone 0.2 µm filters (Carl Roth, Karlsruhe, Germany).

Monitoring effect on algal growth

To check for effect of bacterial filtrates on diatom growth 1.125 mL of bacterial filtrate was inoculated with 0.375 mL of exponentially growing *C. didymus* culture in 24 well plates. Controls were run in parallel using seawater instead of bacterial filtrates. The *in vivo* chlorophyll a fluorescence was regularly measured as indicator for algal growth.

Measuring protease activity

The protease activity of cultures filtrates was measured applying the conversion of BIODIPY FL casein (E 6638) (Invitrogen, Carlsbad, CA, USA) to fluorescent cleavage products [26]. First, 10 µL of *C. didymus* or *S. costatum* culture was mixed with 100 µL of digestion buffer (Invitrogen, 200 mM Tris, pH = 7.8, 2 mM azide) and 100 µL of the dye at a concentration of 10 µg ml^{-1}. After incubation for 1 h at room temperature in the dark the fluorescence of the cleavage products was measured using a Mithras LB plate reader with an excitation filter of 470 ± 5 nm and an emission filter of 510 ± 20 nm.

Protein concentration

Protein concentrations were analyzed from *K. algicida* and *C. didymus* cultures as well as from cultures of *C. didymus* grown in *K. algicida* conditioned medium. The cultures were sterile filtered using 0.2 µm filters before adding 30 mL of the cell free medium to Amicon centrifugal filter units (Millipore, Billerica, MA, USA) with a molecular weight cut off of 30 kDa as described in the manufacturer's instructions. The resulting filtrate was concentrated to a final volume of approximately 300 µL representing a 100 fold concentrated protein fraction.

SDS-polyacrylamide gel electrophoresis and zymograms

The concentrated protein fraction of samples was mixed 1:1 (vol %) with loading buffer (3 g SDS, 3 mg bromophenol blue, 3 mL glycerol, 3.75 mL 500 mM Tris, pH = 6.8, volume adjusted to 10 mL with ultra pure water). From each sample, 20 or 30 µL were loaded onto a standard SDS gel consisting of a 5% polyacrylamide stacking gel and a 12% polyacryamide separating gel [27]. The molecular weight of the proteins was estimated based on the comparison to molecular weight standards (Fermentas, St. Leon-Rot, Germany). The gels were run first at a voltage of 80 V until the samples reach the separating gel. Afterwards the voltage was increased to 120 V until the loading dye was close to the end of the gel.

After electrophoresis a protease activity staining was performed according to a modified protocol of Garcia-Carreno [28]. Briefly, the gels were washed in deionized water before incubating in a 0.75% casein Hammerstein (VWR, Dresden, Germany) solution at 4°C for 30 min and at room temperature for 60 min. Afterwards the gels were washed 3 times with deionized water and subsequently incubated in PBS buffer (pH = 7.4) for 60 min. After washing the gels again 3 times the proteins were fixed in 12% trichloroacetic acid (VWR) for 30 min. The gels were then stained using standard Coomassie Brilliant Blue solution for 60 min and destained in methanol/acetic acid/water (415 mL/83 mL/502 mL) for 30 min.

Statistical Analysis

The test for statistical significant differences in growth experiment and for protease activity was performed using a two way repeated measures analysis of variance (RM-ANOVA) with Sigmaplot 11 (San Jose, CA, USA). The test of significance was performed using the Tukey post-hoc test implemented in Sigmaplot 11. Differences were accepted as significant for every comparison when $P<0.05$.

Results:

C. didymus is not susceptible to proteases of *K. algicida*

After addition of the bacterial filtrate we followed the growth of *C. didymus* indicated by its chlorophyll a (chl a) fluorescence over a period of 64 h (Fig 1). During this period we could not observe any significant difference in the chl a fluorescence at any time point ($P>0.553$ at each time point compared to controls). Recently it has been shown that *K. algicida* releases proteases which inhibit the growth of several diatom species such as *S. costatum* [11]. To verify that the applied filtrate was harmful for such organisms, the filtrate was also added to *S. costatum* and a significant growth reduction was observed ($P<0.01$ after 24 h and onwards, data not shown).

Increased protease activity in *C. didymus* cultures

Because *C. didymus* was not affected by proteases of *K. algicida* we aimed to investigate if *C. didymus* has developed a special mechanism to deal with such proteases stress, possibly by inhibiting or degrading the bacterial enzymes. Thus, we measured the protease activity in a *C. didymus* culture over for a period of 3 days after stressing it with cell free bacterial filtrate. We compared this protease activity with a culture of *S. costatum* shown to be susceptible to proteases of *K. algicida* (Fig. 2) [11]. During the first two days of the experiment we did not observe any difference between *S. costatum* and the *C. didymus* cultures ($P>0.855$). Interestingly, after 2 and 3 days the protease activity was significantly increased in *C. didymus* cultures in comparison to *S. costatum* cultures ($P<0.001$) indicating a release of additional protease(s) by *C. didymus*.

Induced protease release

After we monitored an increased protease activity in *C. didymus* cultures conditioned with the cell free *K. algicida* filtrate we hypothesized that *C. didymus* releases its own protease as a mechanism to counteract lytic enzymes of *K. algicida*. We performed a SDS polyacrylamide gel electrophoresis of concentrated cell free supernatants of a i) *C. didymus* standard culture, ii) *C. didymus* culture conditioned with cell free bacterial filtrate for 3 days and iii) *K. algicida* culture. In order to stain specifically proteases we performed a zymogram. In this method a clear zone on a blue background indicates the presence of a protease. We observed no presence of protease released by *C. didymus* under standard growth conditions (Fig. 3, lanes 1 and 2). In contrast, when *C. didymus* is grown in cell free filtrates of *K. algicida* we observed several additional

proteases in the extracellular protease profile (Fig. 3, lanes 3 and 4). The molecular weights of these induced proteases were approximately 85, 70 and 35 kDa. The band with the molecular mass of approximately 35 kDa seemed to be the most prominent band indicated by the broad and bright character of the band. The extracellular protease profile of *K. algicida* (Fig. 3, lanes 5 and 6) showed only bands with very low intensities at molecular masses of approximately 110 and 50 kDa.

Protease induction by proteinaceous bacterial compounds

After we verified the protease release from *C. didymus* in response to bacterial metabolites we aimed to characterize the eliciting bacterial substances in more detail. Therefore, we compared the extracellular protease profile of *C. didymus* grown in bacterial filtrate (Fig. 4, lanes 1 and 2) with the profile of released proteases of *C. didymus* cultures grown in modified *K. algicida* filtrate. As a modification we used filtrates that were heat treated prior to the addition to *C. didymus* (Fig. 4, lanes 3 and 4), a high molecular weight fraction (> 30 kDa) of the bacterial filtrate (Fig. 4, lanes 5 and 6) and low molecular weight fraction (< 30 kDa) of the *K. algicida* filtrate (Fig. 4 lanes 7 and 8). *C. didymus* showed the induction of proteases of the molecular weight of 85, 70 and 35 kDa, similar to our previous results (Fig. 3). Neither the heat treated filtrate nor the low molecular weight fraction of the filtrate induced the release of proteases (Fig. 4, lanes 3 and 4, and 7 and 8 respectively). In contrast, after application of the high molecular size fraction of the bacterial filtrate to *C. didymus* we observed a release of proteases with the molecular mass of approximately 85, 70 and 35 kDa (Fig. 4, lanes 5 and 6) similar to the induction initiated by unfractionated bacterial filtrate.

Discussion

The bacteria *Kordia algicida*, the first of its genus and originally isolated from a diatom bloom, is capable of inhibiting the growth of several diatom species. In a previous study the diatoms *Skeletonema costatum*, *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* showed a reduced chl a fluorescence after a two day exposure to *K. algicida* spent medium while *C. didymus* was not affected by the active proteases [11]. To further elucidate the bacteria-algae interactions we tested the effect of *K. algicida* on the growth of *C. didymus*, a diatom genus that is widespread in several marine waters such as the Baltic Sea or Dabob Bay, USA [29,30], and found no effect at any of the time points tested which verifies initial findings. Subsequently we hypothesized that *C. didymus* may actively deactivate bacterial proteases for example by protease inhibitors. Therefore we followed the protease activity in the culture medium of both the susceptible diatom *S. costatum* and the non-susceptible diatom *C. didymus* after the application of the *K. algicida* cell free filtrate. We used a fluorescence based method which allows the quantitative evaluation of protease activity including several types of proteases such as serine or metalloproteases [26]. At the beginning of the experiment concentration of *K. algicida* proteases in *S. costatum* and *C. didymus* cultures was the same. In cultures of *S. costatum* the protease activity remained constant over time. This can be completely attributed to the stability of the bacterial proteases since measurements of proteolytic activity in seawater incubated with *K. algicida* proteases were also constant over the time of the experiment (data not shown).

Interestingly, the *C. didymus* cultures showed a completely different picture neglecting the idea of active protease inhibitors. Here, we found that the protease activity increased during the experiment suggesting an induced protease release by the alga due to diffusible bacterial compounds. In order to verify that the increased protease activity was due to a release of diatom proteases and not an artifact of the fluorescence assay caused e.g. by pH changes during culture growth [31] we profiled the exuded proteases of *C. didymus*. We used zymograms, a technique which allows the specific detection of proteases in polyacrylamide gels, and were able to detect proteases in a highly sensitive manner. Although *C. didymus* did not excrete any detectable proteases under standard growth conditions, we observed a dramatic induction of several proteases with a molecular weight range of 30 to 200 kDa after the addition of bacterial cell free conditioned medium. This induction can be clearly attributed to the application of bacterial

substances because neither *C. didymus* under standard growth condition nor *K. algicida* release a similar protease profile.

The protease profile of *K. algicida* cultures did show only minor amounts of proteases. Such low amount of protease detected in the protease profile of *K. algicida* might be surprising. However, two points need to be considered here. First, the activities of algal and bacterial proteases in the fluorescence assay and in the zymograms are most likely not the same and thus the actual amount of protease cannot be deduced from this experiment. Further, the protease activity displayed in Figure 2 includes also normal background fluorescence of the assay which was normally in the range of 8000 to 10000 RFU. Thus, the relative increase of the induction is higher than the protease activity caused by the bacterial protease.

The induction observed for *C. didymus* can be completely attributed to the algae and not minor amounts of contaminating bacteria. Even though we tried to keep bacterial contamination in *C. didymus* cultures as low as possible e.g. by single cell isolation and constant work under sterile conditions, the cultures were not axenic. However, in a comparable study it was calculated that with a maximal bacterial contamination of nearly 10^6 cells mL^{-1} and an average bacterial volume of 1-2 μm^3 that bacterial proteins could not contribute more than 0.4% of the total protein content [32]. Thus, it is very unlikely that the proteins detected in our zymograms are of bacterial origin unless these enzymes have a particularly high activity.

The influence of bacteria on the exudation of organic matter by diatoms has recently been appreciated. For instance, researchers have shown that the diatom *T. weissflogii* requires co-existing attached bacteria to form transparent exopolymer particles (TEP) and thus the diatom is only able to aggregate in the presence of bacteria [33]. Furthermore it has been demonstrated that diatoms release dissolved organic matter (DOC) in the presence of co-occurring bacteria [34]. However, that effect of bacteria on diatom DOC release was highly variable and changed over time with the available nutrient concentration. Interpretation of these experiments is complicated because they were performed by the addition of living bacterial cells which are able to metabolize the DOC pool [35]. Moreover, lately the induction of a diatom protease by a bacterium could be demonstrated. In a co-culture of the diatom *P. tricornutum* with the bacterium *Escherichia coli*, an extracellular algal protease was only detected in the presence of *E. coli*. Interestingly, the induction of that particular protease could be triggered by the addition of cell free bacterial spent medium. Although the use of *E. coli* as co-cultured bacteria offers several advantages e.g. the easy handling and the huge amount of data available in genome and proteome databases, *E. coli*

is not a marine bacterium and thus the ecological relevance of these experiments might be limited. Further, the eliciting substance was not characterized in this study [36]. We performed a size fractionation of the bacterial filtrate and tested the effect of two fractions, one smaller than 30 kDa and one fraction containing substances larger than 30 kDa, on *C. didymus*. *C. didymus* released proteases in response to the fraction containing high molecular mass substances but not the fraction containing low molecular mass compounds, indicating that the release of the diatom proteases is a direct response to bacterial proteins which are responsible for the growth inhibiting effect against several diatom species [11].

Overall, it is a compelling hypothesis that the release of diatom proteases by *C. didymus* is involved in the resistance against *K. algicida*. Supported by the fact that susceptible diatoms such as *S. costatum* do not release proteases in response to *K. algicida*, we tested the hypothesis that diatom proteases degrade bacterial proteins and thus potentially detoxify the bacterial exudates. We hypothesized that if *C. didymus* was able to release compounds that broke down the bacterial proteases, bacterial exudates exposed to *C. didymus* would be harmless to *S. costatum.* In fact, *S. costatum* grew less when exposed to the bacterial filtrate exposed to *C. didymus* than when exposed to the bacterial control filtrate, indicating that the induced *C. didymus* proteases additionally inhibit the growth of *S. costatum*. Thus, two hypotheses occur that are not distinguishable with our experiments. First, the algal proteases break down the bacterial proteins that cause themselves an inhibiting effect against *S. costatum* but are also inhibiting *S. costatum* growth. Secondly, algal proteases do not degrade bacterial proteins and the enhanced growth reduction is an additive effect of bacterial and algal proteases.

The role of diatom proteases remains unclear, but may be part of a general stress response. A 19-fold increase in protease transcription was also found in the diatom *Chaetoceros compressum* immediately after heat treatment at 30°C in comparison to cells grown at standard condition of 20°C [37] suggesting that proteases are a way to cope with temperature stress. An induction of specific intracellular proteases was also found for *T. weissflogii* upon nutrient or light limitation and has been suggested to be a sort of autocatalyzed cell death [32]. Accordingly, intracellular cysteinyl aspartate-specific caspase orthologues called metacaspases are suggested to play a major role in the programmed cell death of unicellular phytoplankton [38,39]. Thus, the induction of intracellular protease in response to abiotic stresses such as nutrients and temperature is well documented.

Even though our data suggests an involvement of induced proteases in the chemical defense of diatoms against pathogenic bacteria final evidence is lacking due to methodological limitations. Here we demonstrated the release of extracellular protease upon biotic stress. We suggest that these finding might have implications on growth dynamics of both bacteria and co-existing diatoms.

Figure 1:

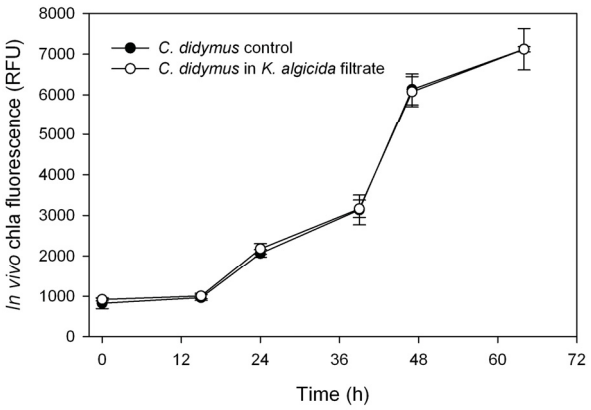
8000
7000
6000
5000
4000
3000
2000
1000
0
In vivo chla fluorescence (RFU)
C. didymus control
C. didymus in K. algicida filtrate
0
12
24
36
48
60
72
Time (h)

Figure 2:

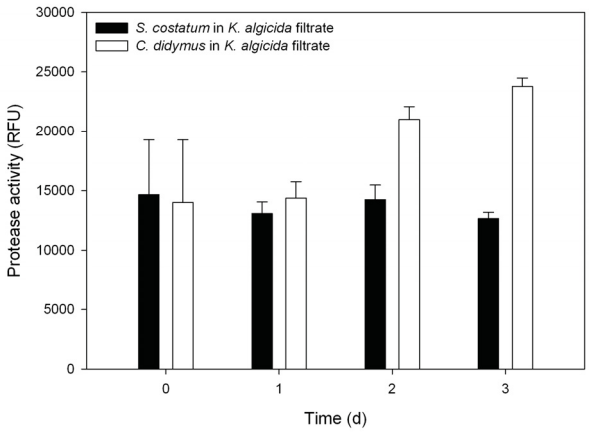
S. costatum in K. algicida filtrate
C. didymus in K. algicida filtrate
Protease activity (RFU)
30000
25000
20000
15000
10000
5000
0
0
1
2
3
Time (d)

Figure 3

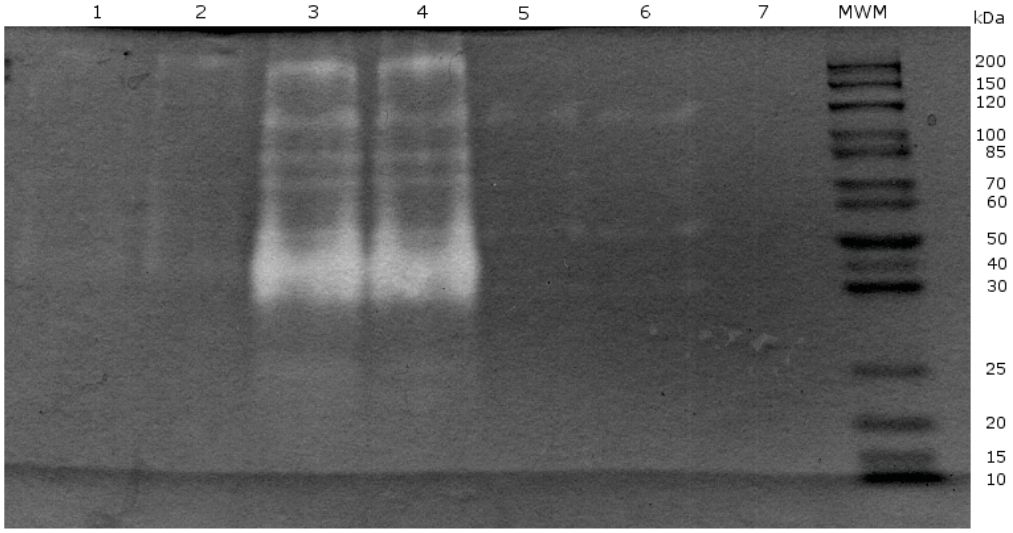

Figure 4

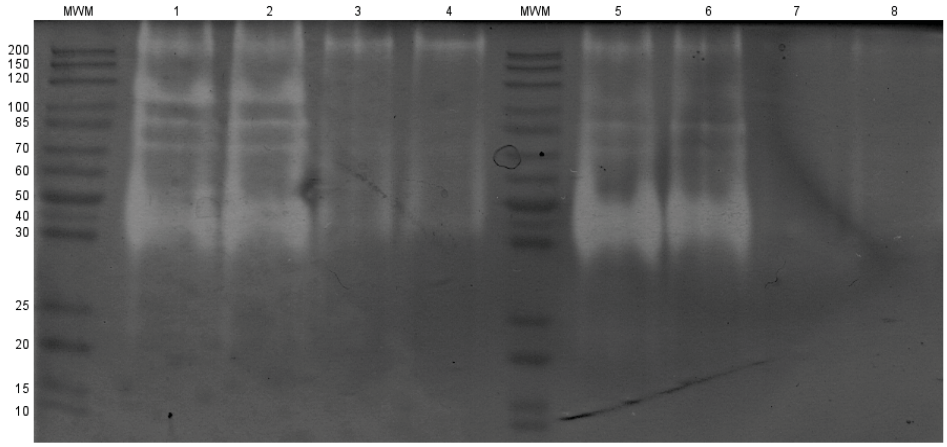

Figure legends

Figure 1: Growth of *C. didymus* indicated by in vivo chlorophyll a fluorescence in *C. didymus* standard cultures and in *C. didymus* cultures supplemented with cell free conditioned *K. algicida* filtrate. Displayed are the mean values + SD, n = 5).

Figure 2: Protease activity within cultures of the susceptible *S. costatum* and the non-susceptible diatom *C. didymus* after the application of conditioned cell free *K. algicida* filtrate (n=4).

Figure 3: Profiles of released proteases determined by zymography. Lane 1 and 2 represent the release of *C. didymus* under standard growth conditions; lane 3 and 4 shows the profile of induced proteases after the application of conditioned cell free *K. algicida* medium; and lane 5 and 6 gives the profile of the added *K. algicida* medium. MWM gives the molecular weight marker in kDa.

Figure 4: Characterization of the inducing compounds within the bacterial filtrate. Lane 1 and 2 was obtained after the induction with unfractionated filtrate serving as positive control, lane 3 and 4 indicate the release profile after the addition of heat treated bacterial filtrate, lane 5 and 6 represent the induction after the compounds > 30 kDa with in the bacterial filtrate while in lane 7 and 8 *C. didymus* was induced with bacterial filtrates containing only metabolites > 30 kDa. MWM indicates the molecular weight marker

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4.3 Manuscript C

Diatom derived polyunsaturated aldehydes do not structure the planktonic microbial community in a mesocosm study

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Diatom Derived Polyunsaturated Aldehydes Do Not Structure the Planktonic Microbial Community in a Mesocosm Study

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Abstract: Several marine and freshwater diatoms produce polyunsaturated aldehydes (PUA) in wound-activated processes. These metabolites are also released by intact diatom cells during algal blooms. Due to their activity in laboratory experiments, PUA are considered as potential mediators of diatom-bacteria interactions. Here, we tested the hypothesis that PUA mediate such processes in a close-to-field mesocosm experiment. Natural plankton communities enriched with *Skeletonema marinoi* strains that differ in their PUA production, a plankton control, and a plankton control supplemented with PUA

at natural and elevated concentrations were observed. We monitored bacterial and viral abundance as well as bacterial community composition and did not observe any influence of PUA on these parameters even at elevated concentrations. We rather detected an alternation of the bacterial diversity over time and differences between the two *S. marinoi* strains, indicating unique dynamic bacterial communities in these algal blooms. These results suggest that factors other than PUA are of significance for interactions between diatoms and bacteria.

Keywords: mesocosm; plankton interactions; aldehydes; oxylipins

1. Introduction

Diatoms comprise a group of phototrophic microalgae with worldwide dominance in higher latitudes. Some diatom species and isolates are known to produce a multitude of fatty acid-derived secondary metabolites such as polyunsaturated aldehydes (PUA) [1]. A negative effect of PUA on the reproduction success of herbivorous copepods was proposed by several authors based on evidence from feeding experiments [2], application of pure PUA [3] or PUA encapsulating liposomes [4] (reviewed in [5,6]). While some evidence for the action of PUA was found in the field [7], several investigations failed to correlate diatom abundance with reproduction failure of copepods [8,9] as well as PUA production with lower egg hatching success [10–13]. Additionally, modeling studies suggest that PUA-based chemical defense against herbivory is not advantageous for a diatom population, since it cannot be considered as an evolutionary driving force [14]. Several studies point to other potential functions of this class of metabolites such as infochemicals involved in diatom-diatom interactions [15,16] or mediators of the microbial community around diatom cells [17]. For such interactions PUA excretion by intact diatoms would be required and indeed, PUA release (hepta- and octadienal) was recently shown for the species *Skeletonema marinoi* in culture [16] as well as in mesocosm experiments [18]. PUA were also detected in subnanomolar concentrations during a *S. marinoi* bloom in the Adriatic Sea [19] indicating that e.g., co-existing bacteria are exposed to these metabolites during a diatom bloom. Several adverse effects of PUA on microorganisms have been reported and it has been suggested that diatom-derived PUA can regulate the bacterioplankton community [3,17,20]. This assumption is supported by the observation that PUA are generally strongly bioactive, and capable of disturbing normal cell functions in several organisms [21]. However, the results obtained from laboratory studies so far lacked experimental verification under ecologically relevant conditions. While agar diffusion assays were used to assess the cell toxicity of PUA on bacteria isolated from habitats unrelated to diatom distribution [3], Ribalet *et al.* tested, among others, bacterial strains isolated from a *S. marinoi* bloom in liquid nutrient enriched bacterial growth medium. Concentrations used in these experiments were in the high micromolar range [17] exceeding average natural conditions in the water by several orders of magnitude [18,19]. Most ecologically relevant is a recent study that applied 7.5 nM PUA, which is in the range of natural concentrations, to bacterial strains isolated from the Mediterranean Sea, and observed group specific effects [20]. However, all these experiments are laboratory tests based on application of pure PUA on cultivable bacterial strains.

Given the evidence of an activity of PUA on bacteria and the notion that these metabolites are indeed found in the seawater, we aimed to investigate their role in a set-up that is close to a field situation. We therefore designed a mesocosm experiment to test the hypotheses that (1) PUA influence the abundance of the microbial community, including bacteria and viruses, and (2) PUA produced by diatoms influence the biodiversity of the bacterial plankton communities. As a test organism, we used the diatom *S. marinoi*, since its PUA content and the release of these metabolites into seawater has been documented in laboratory, mesocosm and field experiments [16,18,19]. Despite significant quantitative variability between isolates, it can be stated that this species generally produces the shorter chain length PUA, 2,4-heptadienal, 2,4-octadienal, and sometimes to a minor extent 2,4,7-octatrienal, while it lacks C10 PUA. *Skeletonema* spp. are regularly found at the study site even if they contribute often only little to the phytoplankton community and are thus ideally suited to manipulate existing communities. The experimental set-up was rigorously replicated and included mesocosms where cultivated PUA-producing *S. marinoi* strains were used to trigger a diatom bloom and mesocosms where synthetic PUA were introduced in different concentrations as well as controls. We undertook a comprehensive monitoring of phytoplankton development, chemical exudates, bacterial abundance and community composition as well as viral abundance and observed no effects by PUA-producing diatoms. Additions of synthetic PUA in natural and above natural (*ca.* 1000×) concentrations did not result in major changes of the bacterial and viral abundance.

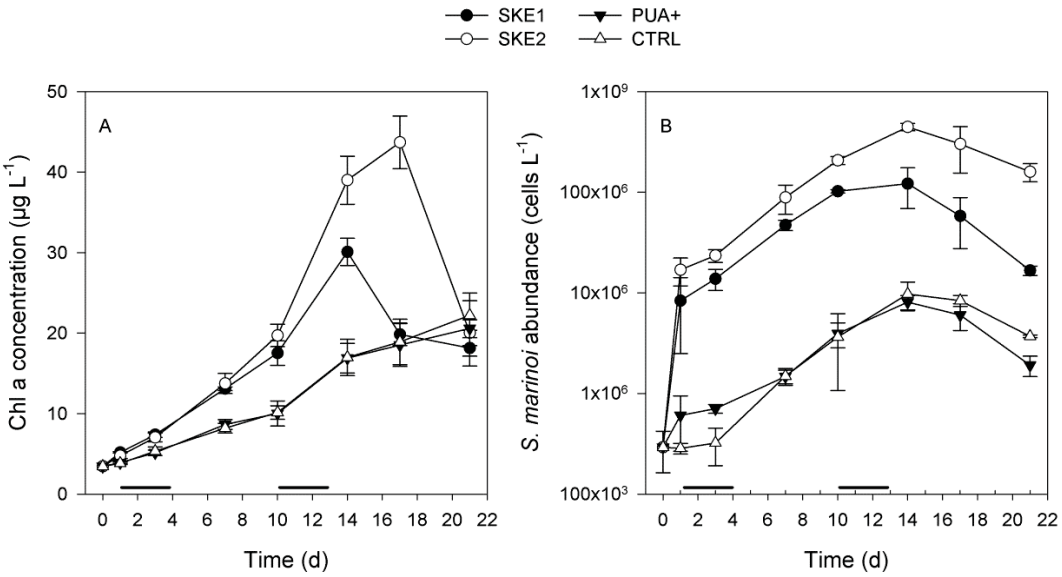
2. Results

2.1. Phytoplankton Development

In the following, the treatments are abbreviated with SKE1 and SKE2 for the mesocosms inoculated with the two *S. marinoi* strains, PUA+ for the mesocosms with additions of PUA and CTRL for the untreated control. All treatments started with a total chlorophyll a concentration of 3.5 $\mu g \cdot L^{-1}$. A phytoplankton bloom developed in SKE1 and SKE2 as indicated by an increasing chlorophyll a concentration. SKE2 reached the highest chlorophyll a concentration of more than 40 $\mu g \cdot L^{-1}$, peaking at day 17. In SKE1, chlorophyll a was peaking at day 14 with a maximal concentration of 30 $\mu g \cdot L^{-1}$ (Figure 1A). These two treatments showed significant differences to each other at day 14 and day 17 ($P < 0.001$).

PUA+ and CTRL showed very similar progressions in their chlorophyll a concentration and no significant differences were recorded at any time point ($P > 0.66$ for all comparisons). In these treatments, chlorophyll a concentration steadily increased and from day 7 to day 14; chlorophyll a values were significantly lower compared to SKE1 and SKE2 ($P < 0.02$ for all comparisons). *S. marinoi* cell counts revealed that in CTRL and PUA+ treatments a population of *S. marinoi* developed, which was consistently lower in abundance compared to the SKE1 and SKE2 treatments (Figure 1B). *S. marinoi* cell counts in PUA+ showed no significant difference to CTRL over the course of the experiment ($P = 1$ for all comparisons). SKE2 showed a significantly increased *S. marinoi* cell abundance in comparison to SKE1 at day 10 and day 17 ($P = 0.001$ and $P < 0.001$ for day 10 and day 17, respectively).

Figure 1. Development of (**A**) total chlorophyll a concentration and (**B**) the cell abundance of *Skeletonema marinoi* during the time course of the experiment. SKE 1 and SKE2 were inoculated with two different *S. marinoi* strains at day 0. The black lines on top of the time axis indicate the first (low PUA concentration) and second (high PUA concentration) PUA addition to the PUA+ treatment.



The phytoplankton composition was dominated in every treatment by diatoms reaching maximum 94% and minimum 70% of total biomass at the end of the experiment (day 21) (Figure 2). *S. marinoi* accounted for up to 20% of the biomass in SKE1 and up to 40% of the biomass in SKE2 (data not shown). The second most abundant class of algae was dinoflagellates. These accounted for approximately 4% of the total biomass at day 21 in PUA+ and CTRL and 14% and 11% in SKE1 and SKE2, respectively. Euglenophyceae and Chrysophyceae contributed less to the biomass and were most abundant in SKE1 with respectively 6% and 9% at the end of the experiment. In all other treatments, these classes were only present in very minor amounts.

2.2. Bacterial and Viral Abundance

The initial bacterial abundance was very similar in all treatments with roughly 10^6 cells·mL^{-1} at day 1, and showed no significant difference between the four treatments ($P > 0.829$). Irrespective of the first PUA addition or the developing *S. marinoi* blooms, the bacterial abundance developed uniformly in all treatments until day 10 when densities of ca. 1.8×10^6 cells·mL^{-1} were reached, again not showing any significant differences between any treatments ($P > 0.954$ for all comparisons) (Figure 3A). At day 17 PUA+ and CTRL showed significantly higher bacterial abundances compared to SKE1 and SKE2 ($P < 0.001$ for all 4 comparisons). Bacterial abundance in SKE1 and SKE2 did not differ significantly during the entire experiment ($P > 0.088$ at any time point, Figure 3A).

Figure 2. Development of phytoplankton composition in SKE1 and SKE2, PUA+ and CTRL. Only phytoplankton classes that contribute more than 0.4% to the total biomass are displayed. PUA additions to the PUA+ were between days 1 and 4 (low concentration) and between days 10 and 13 (high concentration).

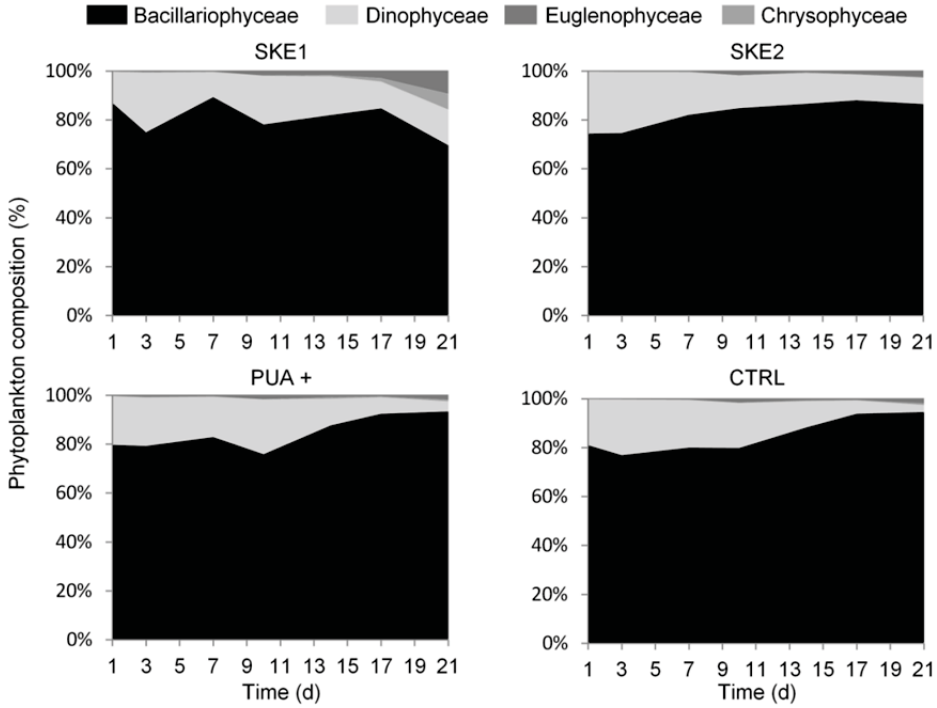
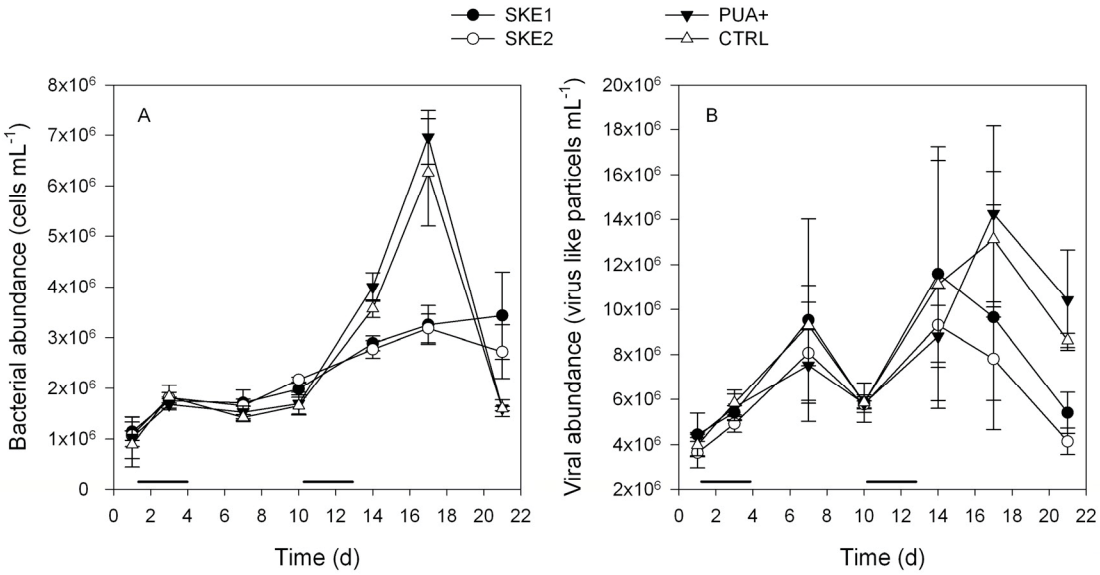


Figure 3. Bacterial (**A**) and viral (**B**) abundance during the experiment. The black lines on top of the time axis indicate the two periods of PUA addition.

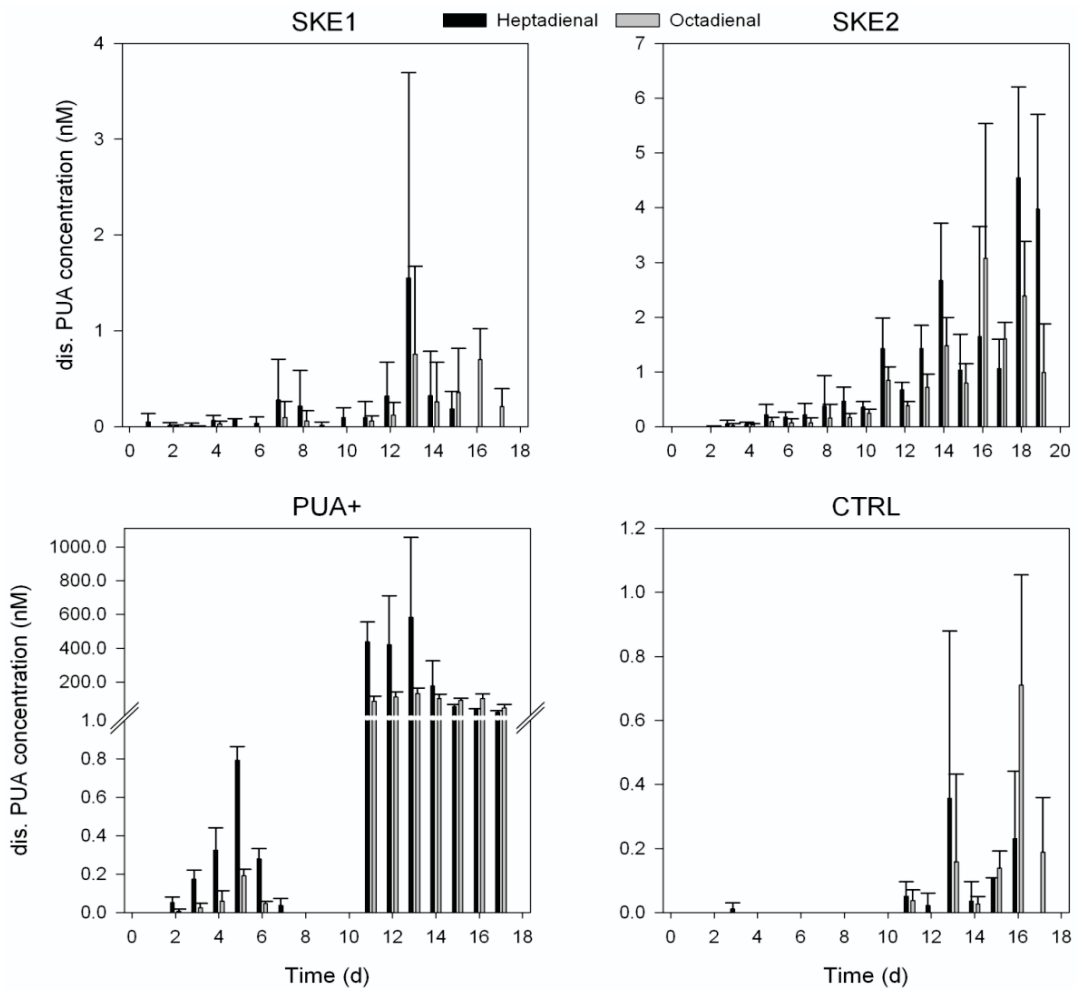


Initial viral abundance in all treatments was at 4×10^6 particles·mL^{-1} and increased to a maximum of 14×10^6 particles·mL^{-1} in PUA+ by day 17. No significant differences were observed until day 14 ($P > 0.416$). After day 14 PUA+ contained significantly more virus like particles than SKE1 and SKE2 ($P < 0.05$ for all comparisons). CTRL and PUA+ as well as SKE1 and SKE2 did not differ in their virus abundance at any time point ($P > 0.572$ at each time point, Figure 3B).

2.3. Dissolved PUA Concentration

To mimic natural concentrations occurring during diatom blooms, the first PUA addition was adjusted to result in subnanomolar concentrations of hepta- and octadienal in the mesocosms [19]. PUA were added after the sampling at days 1, 2, 3 and 4. A second repeated addition with 1000fold more PUA was conducted at days 10, 11, 12 and 13. The concentration of dissolved PUA found in the PUA+ treatment reflected the PUA addition. From day 2 to day 7, we detected PUA starting from 0.05 nM for heptadienal and very minor amounts of octadienal. At day 2 the concentrations were increasing to a maximum of 0.8 nM heptadienal and 0.2 nM octadienal at day 5. From day 5 to day 7 the measured PUA concentration decreased again until it was not detectable from day 8 to day 10, which marks the start of the second PUA addition period (Figure 4). After the repeated addition of high PUA concentrations up to 570 nM heptadienal and 125 nM octadienal were reached at days 11 to 13. Later, the heptadienal concentration decreased. In the CTRL treatment, neither heptadienal nor octadienal could be detected during the first 10 days of the experiment, excluding a very low amount of heptadienal detected at day 3. After day 10, heptadienal as well as octadienal in variable but low amounts could be detected in the CTRL mesocosm but not exceeding concentrations of 0.8 nM (Figure 4).

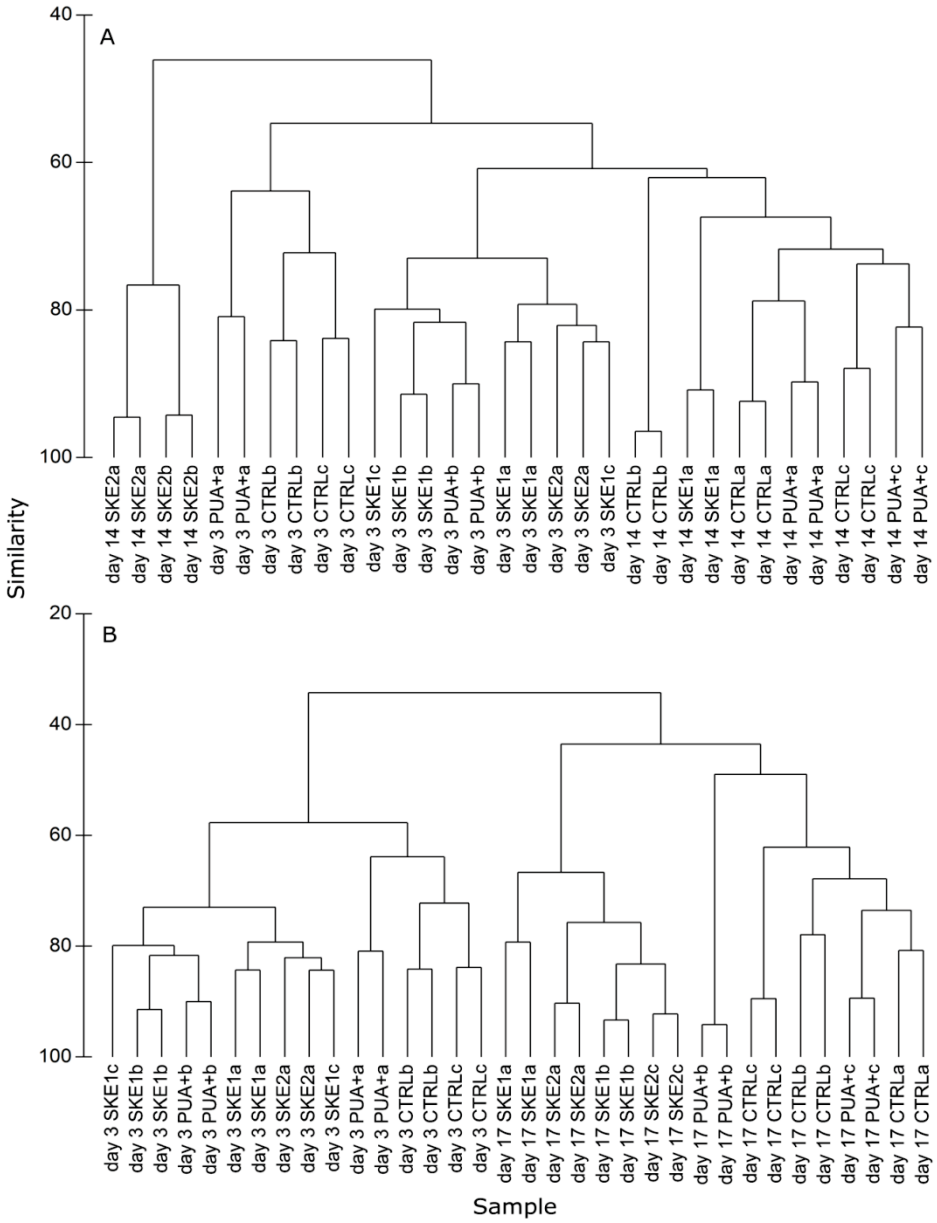
Figure 4. Daily concentration of dissolved PUA in all treatments. Please note the different scales on y-axes in each graph. SKE2 has additionally a different time axis due to a prolonged algal growth in this treatment.



In SKE1 subnanomolar concentrations of dissolved PUA were detected during the beginning of the experiment and only towards the end (day 13) were levels around 1 nM or higher observed. In general, PUA levels were higher in SKE2. Similar to SKE1 only subnanomolar concentrations of hepta- and octadienal were detected during the first 10 days. Towards the end of the experiment PUA levels in SKE2 reached up to 4 nM heptadienal (day 18) and up to 3 nM octadienal (day 16, Figure 4).

2.4. Bacterial Community Composition

Figure 5. Cluster analysis of T-RFLP samples taken during low (day 3), directly after high (day 14) PUA addition period, and towards the end of the experiment (day 17). Cluster analysis of the day 3 and day 14 samples (**A**), in comparison to a cluster analysis of day 3 and day 17 (**B**). Replicates that did not fulfill our reproducibility criteria were discarded from the analysis. Each sample was measured twice and both technical replicates were used for the analysis. Technical replicates obtained from the same biological sample are indicated with the same letter.



The cluster analysis (Figure 5) included samples taken during the first PUA addition (day 3), samples taken directly after the second PUA addition period (day 14) and samples taken towards the end of the experiment. On day 3, CTRL and PUA+ did not group differently from each other, indicating a homogeneous bacterial community in both treatments. Additionally, neither SKE treatments differed from the control and neither differed from each other. In addition, at day 14 we could not discriminate between CTRL and PUA+. All samples, with the exception of SKE2, grouped together (Figure 5A). This unique bacterial community in SKE2 was not observable at day 17 (Figure 5B). In contrast to day 14, at day 17 SKE1 and SKE2 grouped closely together, but clustered apart from CTRL and PUA+. In total, all samples of day 3 grouped separately from samples of day 17, indicating a change of the bacterial community over time (Figure 5B).

3. Discussion

Addition of nutrients and inoculation of the diatom *S. marinoi* allowed us to generate diatom dominated phytoplankton blooms in the mesocosms SKE1 and SKE2. We reached a total chlorophyll a concentration of up to 40 $\mu g \cdot L^{-1}$ for SKE2 and 30 $\mu g\ L^{-1}$ for SKE1. The chlorophyll a concentration was thus 6 to 8 fold higher than typically observed during a spring bloom in the Bothnian Sea which reaches around 5 $\mu g \cdot L^{-1}$ [22]. Chlorophyll a concentrations were comparable to those typically obtained in fertilized diatom mesocosm experiments using *Skeletonema* strains [13] and other diatom species [23]. Irrespective of the inoculation, the phytoplankton composition was dominated by diatoms in every mesocosm. Depending on time and treatment, diatoms accounted for 70 to 94% of the total biomass in our experiments. In the Bothnian Sea diatoms dominate the spring bloom and were estimated to account for 50 to 70% of the total phytoplankton biomass [22]. The major diatom species found during a recent field survey were *Achnanthes taenicata*, *Chaetoceros* spp. and *Thalassiosira* spp. while *Skeletonema* spp. were present only in minor amounts [22]. This is also reflected in the uninoculated mesocosms CTRL and PUA+, where *Skeletonema* spp. reached only low cell counts. The second most abundant group of algae in all mesocosms was Dinophyceae, which is again in line with the phytoplankton composition of the Bothnian Sea [22]. Further, cell counts and chlorophyll a determinations confirmed that the mesocosms CTRL and PUA+ were suitable to test the effect of PUA on the plankton community in a close-to-field situation. SKE1 and SKE2 were ideally suited to test the potential effect of diatom blooms of very similar isolates from one species that have a different PUA production potential.

Recent work detected heptadienal and octadienal in concentrations of approximately 0.1 nM during a *S. marinoi* bloom in the Adriatic Sea [19]. In mesocosm studies, concentrations of approximately 1 nM PUA were detected during an induced *S. marinoi* bloom [18]. We thus selected initially an addition of PUA that was calculated to result in a final concentration of 1 nM. Nevertheless, only a fraction of this concentration was detected 23 h after PUA addition. This lower concentration was anticipated due to potential adsorption of PUA on the mesocosm containers, instability of the metabolites, microbial uptake and transformation, as well as potential loss due to volatility. However, after repeated PUA additions, concentrations up to 1 nM were reached. We can thus assume that up to day 6 the PUA addition resulted in concentrations that match roughly those found in nature. In our experiments, we added PUA as dilute aqueous solution and immediately mixed the mesocosms to

reach a homogeneous concentration of these aldehydes. Simple hydrodynamic considerations suggest that PUA-producing diatoms will build up a concentration gradient of these metabolites around the cells, resulting in elevated local concentrations on a microscopic scale. We therefore also added 1000 nM heptadienal and 250 nM octadienal to overcompensate such potential local effects. Again, these concentrations were not detected after the first addition, but up to *ca.* 600 nM PUA were found after the repeated additions. Since we inoculated the SKE1 and SKE2 mesocosms with *S. marinoi* strains that were different in their PUA production, we could also test the effect of PUA release by otherwise very similar algae from one species. Indeed, PUA concentrations in SKE1 and SKE2 developed differently with SKE2 reaching higher PUA concentrations over the entire experiment.

The bacterial abundance in all treatments was 1×10^6 cells·mL^{-1} at the beginning of the experiment. Due to the filling procedure of the mesocosms, we conclude that microorganisms at day 0 consisted of the local bacterial community in the Bothnian Sea. During the first 10 days, no difference in the bacterial cell number between the 4 treatments were detected, indicating no effect of the addition of low amounts of PUA or the onset of *S. marinoi* blooms. Afterwards, both CTRL and PUA+ exhibited a significantly enhanced bacterial growth compared to SKE1 and SKE2. The suppression of bacterial growth in the two SKE treatments might result from a competition for essential nutrients [24], or from weak antibacterial activity of the algae. Such antimicrobial activity of *Skeletonema costatum*, recently renamed to *S. marinoi* [25], was lately observed. This alga produced antimicrobial substances particularly during the steady-state growth phase [26] that could explain the observed trend in bacterial abundance (Figure 3A). We can conclude, however, that this effect is not due to the PUA of *S. marinoi*, since comparable concentrations in the first days of the PUA+ treatment had no effect on the bacterial abundance, and actual measurements of bacterial production showed highest values in PUA+ (not shown). Since CTRL and PUA+ did not differ throughout the experiment, the addition of PUA has obviously no effect on bacterial abundance, even if concentrations way above those reached in nature were applied. Apparently, the antibacterial activity of PUA detected in laboratory studies [3,17] is not sufficient to inhibit bacterial growth in the plankton. This might be explained by the fact that in laboratory studies concentrations higher than 3 μM PUA are required to trigger activity, but the highest PUA concentration reached in the PUA+ mesocosms was only around 700 nM (Figure 4). It might, however, not be excluded that elevated concentrations of dissolved organic matter (DOC), resulting from the PUA addition, support bacterial growth. Such a positive effect might be counterbalanced by antibacterial activity of PUA. The virus abundance was also not affected by PUA. Similar to the trend observed for bacterial abundance, PUA+ exhibited significantly higher viral particle counts than SKE treatments, while not differing from the control at the end of the experiment. Thus, neither the added PUA nor the PUA released by *S. marinoi* negatively affects virus abundance in natural plankton assemblages or artificially induced diatom blooms. Since we did not see any increase of viral abundance during the decline of the *S. marinoi* bloom, we conclude that the diatom succession was not controlled by viruses. However, viral termination of phytoplankton blooms has been reported for the Prymnesiophyceae *Emiliania huxleyi* (e.g., [27–29]); also, diatom lysis by viruses has been observed [30]. However, since we did not separate between viruses infecting bacteria or phytoplankton, the more abundant bacteria viruses could mask changes in phytovirus abundances.

Bacterial community composition is as important as bacterial abundance for the structure and succession of plankton communities. The bacterial community composition in all mesocosms was

dependent on the growth stage indicated by a distinct grouping between samples taken during the beginning and towards the end of the experiment. Since PUA induce different effects on marine bacteria from different taxonomical groups in cultures [31], we also addressed the question of whether PUA or PUA-producing algae influenced the bacterial community composition in a close-to-field mesocosm environment. Using T-RFLP, we could not detect an effect of PUA addition on bacterial diversity. During the first PUA addition (day 3), in low concentrations equivalent to those detected in nature, we did not see any grouping within the cluster analysis. The CTRL, which was the only treatment without any detectable PUA at this time, was not separated from the two SKE treatments or from PUA+ in which subnanomolar concentrations of dissolved PUA were detected. Even the second PUA addition resulting in above-natural conditions did not cause detectable changes. Recent investigations have evaluated the effect of PUA addition on bacteria representing a natural community from the coastal area of the Mediterranean Sea [20]. Using nanomolar additions of PUA, no strong effect of PUA on the total abundance or the actively respiring cells was observed in short-term incubations. However, a decreased metabolic activity in response to PUA addition was detected, indicating that PUA may have the potential to shape the bacterial community composition. In our experiments, we compensated for potential limitations of short term incubations and tested the effect of different PUA concentrations over 16 days and found no detectable effect on bacterial diversity. This suggests that the postulated effects might be very subtle and cannot be observed even during an extended mesocosm experiment. Nevertheless, our methods are clearly suitable to pick up changes in the bacterial community, which is supported by the observation that bacterial communities differ in all mesocosms between early and late stages of the experiment (Figure 5B). The primers used in our PCR reaction can potentially amplify 16S rRNA of diatom chloroplasts. However, bacteria are in higher abundance than diatoms and the sequence similarity of the primers in comparison to *Skeletonema* chloroplasts is not high enough to ensure sufficient amplification. Thus, the influence of amplified eukaryotic rRNA can be neglected. This is additionally supported by the fact that we do not observe a differentiation between inoculated and non-inoculated treatments in the T-RFLP profiles which would be expected for amplified rRNA from *Skeletonema* chloroplasts. We saw a very distinct separation of the bacterial community in SKE2 after day 14. This change can most likely not be attributed to PUA since SKE1 was also releasing PUA at the same time, although in lower concentrations. Comparable specific shifts in bacterial communities were reported by several authors for diatom cultures. Laboratory studies revealed that unique bacterial communities develop if axenic cultures of the diatoms *Thalassiosira rotula* and *S. costatum* are inoculated with natural bacterial assemblages [32]. Other mesocosm studies with the diatom *S. costatum* demonstrated a change of the bacterial composition over time, similar to the results presented here [23]. The observed exclusive bacterial community in SKE2 at day 14 was indicated by a low similarity in the cluster analysis. However, three days later, the bacterial community of SKE2 grouped together with SKE1 indicating no long-lasting effect. Such dynamic temporal shifts in bacterial composition were shown already in mesocosm experiments [23] as well as in field studies [33]. Interestingly, complex patterns of metabolite release were shown recently for diatom species [34], which turned out to be crucial for the growth of competing phytoplankton species [35]. Thus, exudates of diatoms might influence the plankton community, but according to our study, the metabolites influencing bacterial communities are not PUA.

4. Experimental Section

4.1. Experimental Design

The experiment was performed at the mesocosm facility of the Umeå Marine Science Center (UMSC, Umeå, Sweden), between 3 May (day 0) to 22 May (day 19) 2010. The experiment was run in 12 indoor polyethylene mesocosm towers of 5 m depth and a volume of approximately 2000 L each. Thermal advection, achieved by heating the lowest section of the tanks, resulted in a slow mixing of the water column with a turnover time of approximately 24 h. All tanks were filled with unfiltered seawater (29 April) with its natural microbial community from the Bothnian Sea and were kept at 8 °C to 10 °C, corresponding to ambient field conditions outside the mesocosm [36]. Nutrients were added to all tanks directly after filling to a concentration of 13 µM $NaNO_3$-N, 4 µM NH_4Cl-N and 4 µM NaH_2PO_4. The nutrient levels mimicked the unlimited conditions at the time of the spring bloom in the Bothnian Sea [36]. Nutrient levels were regularly monitored on days 8 and 14 and adjusted if necessary with nitrate and phosphate and additionally, on day 17, with silicate, to initial conditions. Four days after filling (3 May), we started 4 different treatments, each in triplicates. Two treatments were supplemented with two different *S. marinoi* strains (*S. marinoi* abbreviated SKE1 and *S. marinoi* abbreviated SKE2) having different growth and PUA-releasing characteristics. The addition of *Skeletonema* was adjusted to roughly double the concentration of chlorophyll *a* in the tanks after 4 days of growth and thereby leading to a *Skeletonema* dominated phytoplankton community throughout the experiment. Additionally, one treatment consisted of unfiltered natural seawater only (CTRL) and the final treatment consisted of unfiltered natural seawater that was supplemented with different concentrations of PUA at two different time points (PUA+). During days 1 to 4 we added daily hepta- and octadienal, corresponding to a final concentration of 1 nM, representing similar concentrations as found in *S. marinoi* dominated mesocosms [18]. Therefore, heptadienal (22 µL) and octadienal (24 µL) stock solutions (10 $mg \cdot mL^{-1}$ in methanol) were dissolved in 5 L 0.2 µm filtered seawater and thoroughly mixed. This 5 L seawater solution was afterwards added to each tank of PUA treatment and 46 µL of methanol in 5 L filtered seawater were added to the control tanks. All 12 mesocosms were subsequently mixed 3 times with a Secchi disk. To evaluate the effect of PUA on the natural microbial community under artificially elevated PUA concentration, we daily added heptadienal to a calculated concentration of 1000 nM and octadienal to a concentration of 250 nM during day 10 to day 13 of the experiment. Here 2200 µL of a heptadienal and 620 µL of an octadienal stock solution (100 $mg \cdot mL^{-1}$ in methanol) were added daily to 5 L of filtered seawater and which were handled identically to the low PUA supplement (addition of 2820 µL of methanol in 5 L seawater to the control). The relative PUA concentrations were adjusted to match the heptadienal/octadienal ratios produced by *S. marinoi* laboratory batch cultures [16]. All treatments were run for 21 days.

4.2. General Sampling

Samples for bacterial diversity, bacterial abundance, viral abundance, chlorophyll a, phytoplankton cell abundance and inorganic nutrient determinations were taken 7 to 8 times during the experiment depending on the analysis. In order to accomplish this, 10 L were sampled from each tank at a depth of approximately 1 m into a plastic container. All samples for the corresponding analysis were taken from

this container. Samples (1 L) for dissolved PUA analysis were additionally taken daily at days when no regular sampling was scheduled to ensure complete PUA monitoring during the experiment. After each major sampling, all tanks we refilled with 0.2 µm filtered seawater to keep the water volume constant.

4.3. Chlorophyll a Concentration

All samples were treated in duplicate under reduced light. Depending on cell abundance 100 or 50 mL portions of the sample were filtered through a 25 mm GF/F filter (Whatman, Kent, UK), using a vacuum of about 20 kPa. The filters were then transferred into 15 mL plastic Falcon tubes containing 4 steel balls of 5 mm diameter and blanks were prepared using Milli-Q water. A 10 mL portion of 95% ethanol was added to each tube, the stopper tightened firmly and a rack positioned with the tubes horizontally on a large amplitude linear shaker at about 200 rpm for 5 min, thereby crushing the filters thoroughly. Next, the tubes were kept overnight in the dark and the next day each tube was briefly shaken by hand prior to centrifugation at 3500 rpm for 10 min using a Heraeus Instruments Labofuge 400 (Hanau, Germany). The fluorescence of each solution was recorded on a Perkin Elmer LS 30 spectrofluorometer (Waltham, MA, USA) operating at excitation and emission wavelengths of 433 and 673 nm, respectively. The chlorophyll a content of each sample was calculated using a calibration constant obtained in a previous calibration.

4.4. Phytoplankton Abundance

Samples for phytoplankton identification and monitoring of phytoplankton abundance were fixed with 0.5% acid Lugol's solution. Volumes of 10 or 3 mL of fixed samples were settled in a sedimentation chamber for 12 to 24 h and counted according to the Utermöhl technique [37] in an inverted microscope (NIKON Eclipse TE 300). Phytoplankton, identified to species or class level, were counted and measured for calculations of biomass using the equations and size classes described for the Baltic Sea [38].

4.5. Bacterial Abundance

Samples for total bacterial abundance including attached and free living bacteria (50 mL) were preserved with 0.2 µm filtered formaldehyde (1.5% final concentration). For analysis, 4 mL sample was filtered onto black 0.22 µm 25 mm polycarbonate filters (Osmonics Inc., Minnetonka, MN, USA) and stained with acridine orange dissolved in MilliQ water (0.01% final concentration). Samples were analyzed in an epifluorescence microscope (Zeiss Axiovert 100) connected to an image analysis system using blue excitation light and a 450 to 490 nm filter [39].

4.6. Viral Abundance

Virus samples (1 mL) were filtered subsequently through 0.8 and 0.2 µm Supor membrane syringe filters (Pall Corporation, Newquay, UK), fixed in glutaraldehyde (0.5% final concentration) for 30 min at 4 °C, frozen in liquid nitrogen, and stored at −70 °C [40]. The staining procedure with SYBR Green I (Molecular Probes Inc. Eugene, OR, USA) was modified from Marie *et al.* [40]. In short, virus samples were thawed at 35 °C for a few min and then diluted 100 times in 0.99 mL TE buffer (10 mM

Tris-HCl and 1 mM EDTA, pH 8.0) to avoid coincidence (two or more particles being present at the same time in the sensor zone) [40,41]. Diluted virus samples were stained with 10 µL SYBR Green I or 10 min in the dark at 80 °C. Final concentration of SYBR Green I was a 10^{-4} dilution of the commercial stock. Virus samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW 488 nm air-cooled argon-ion laser and standard filters. The trigger was set to green fluorescence. Readings were collected in logarithmic mode and analyzed with the software CellQuest (version 6.0; Becton Dickinson, San Jose, CA, USA, 2007). A total of at least 5000 events were recorded for each sample. Green fluorescence (GFL), side scatter (SSC) and total events were recorded. The data were normalized to fluorescent beads (yellow beads of 1.5 µm; Polysciences Inc., Warrington, PA, USA) which were added as an external standard.

4.7. PUA Analysis

Sampling and analysis of dissolved PUA measurements were based on a protocol published previously [16]. Briefly, a 1 L sample was transferred to a 1 L glass bottle and 5 µL of a benzaldehyde solution (1 mM in methanol) were added as internal standard. First, this sample was transferred by vacuum through a sand cartridge to gently remove diatom cells. The filtrate was immediately loaded on a 3 mL EASY Chromabond cartridge (200 mg, Macherey-Nagel; Düren, Germany) previously treated with 1 mL of a 25 mM *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) solution in 100 mM TRIS-HCL at pH 7.2. The EASY cartridge was rinsed with water and dried by applying a gentle vacuum. PUA were eluted with 4 mL of a 5 mM PFBHA solution in methanol under gravity and stored at −80 °C until further extraction. Extraction was performed in 25 mL glass flasks filled with 8 mL hexane and 8 mL water. After addition of 1.5 mL of concentrated sulfuric acid (95%), the hexane phase was separated and dried with sodium sulfate. The hexane phase was then evaporated to dryness under reduced pressure and the remaining derivatized PUA were re-dissolved in 100 µL hexane. GC-MS measurements were performed on an Agilent 6890N gas chromatograph equipped with a DB-5ms 30 m column (0.25 mm internal diameter, 0.25 µm film thickness) coupled to a Waters GCT Premier mass spectrometer (Manchester, UK). Helium was used as carrier gas at a constant flow rate of 1 mL·min^{-1}. The calibration was performed using synthetic standards of heptadienal and octadienal normalized to benzaldehyde as internal standard with R^2 = 0.9794 and R^2 = 0.9764 for heptadienal and octadienal, respectively.

4.8. Bacterial Community Composition

For DNA extraction, 100 mL of sample was filtered on 47 mm diameter, 0.22 µm pore size polycarbonate filters (GE Water & Process Technologies, Fairfield, CT, USA). Filters were stored at −80 °C until DNA extraction which was carried out as described previously [42]. For T-RFLP analysis the 16S rRNA-gene was partially amplified by PCR using bacterial 16S primers 27F_FAM (AGA GTT TGA TCC TGG CTC AG) [43,44] and 926R_HEX (CCG TCA ATT CCT TTG AGT) [43]. The reaction mixture consisted of 1 µL of non-diluted, half-diluted or 10^{-1} diluted template DNA, 25 mM dNTPs, 10 µM of each primer, 10× buffer and 0.5 units of KAPA Taq polymerase (KAPA Biosystems, Woburn, MA, USA) for 25 µL reaction. The amount of PCR products were estimated in a 1% agarose gel and then purified with a PCR purification kit (Macherey-Nagel, Düren, Germany).

Restriction of purified PCR products was done with HhaI and RsaI restriction enzymes separately. After a restriction time of 6 h, products were precipitated in ethanol solution (the final mixture containing 97% ethanol with 5 v% 3 M NaOAc) for 1 h at −70 °C. Then, samples were centrifuged in a microcentrifuge for 30 min at 15,000 rpm and supernatants were carefully removed. Pellets were washed with 500 µL 70% ethanol and centrifuged for 10 min at 15,000 rpm. Finally, samples were left to air-dry in a laminar hood and re-suspended in 10 µL of sterile water. For the T-RFLP run with an ABI analyzer, 2–6 µL of sample (depending on the amount of PCR product) was mixed with 6–10 µL of Hi-Di solution (Applied Biosystems, Carlsbad, CA, USA) and 0.01 µL of size standard 600LIZ (Applied Biosystems). Each sample was measured twice and both technical replicates were used for the analysis. The fluorescent peak profiles were analyzed with Peak Scanner software (Applied Biosystems, Carlsbad, CA, USA); the peak data was processed and the peak profiles constructed using T-REX [45], a web-based open access program. The T-REX uses the approach of Abdo *et al.* [46] for noise filtering and the method of Smith *et al.* [44] for peak alignment. The program default values were used for noise filtering, peak alignment was done using a clustering threshold of 0.6 and relative peak heights were used for constructing the peak profiles. The community profiles were clustered based on the Bray-Curtis similarity matrix of log(x + 1) transformed data using PRIMER-6 (version 6.1.12; Primer-E Ltd., Plymouth, UK). Because of the high number of peaks in each profile (due to two fluorescent primers) and because we used peak heights for calculation of results, the maximum similarities between any samples were restricted to approximately 97%. However, based on 75% similarity observed between some of the replicates from the same tanks, we considered that sample profiles that had more than 70% similarity represented similar communities. Samples with replicates less similar than this were removed from the analysis. The evaluated peak heights reflect the relative amounts of different species in the sample and thus give more information on the bacterial community structure than presence/absence data. However, they add to the total complexity of the community profiles, thus reducing the similarity between samples.

4.9. Statistical Analysis

Difference in cell abundance and chlorophyll a data were assessed using a Repeated Measures Analysis of Variance (RM-ANOVA) with the Tukey *post-hoc* test. The analyses were performed with the software package Sigmaplot 11 (Systat Software Inc., San Jose, CA, USA, 2008).

5. Conclusion

In summary, we show that PUA are released by *S. marinoi* and that these aldehydes did not affect the abundance of both bacteria and viruses. Additionally, PUA did not affect the natural bacterial community composition under environmentally realistic and also not at elevated concentrations (1000 fold). Moreover, we observed that different *S. marinoi* strains have unique bacterial communities which change in a dynamic way. Taken together, these results suggest that PUA play no significant role in diatom-bacteria interactions but do not exclude an influence on interactions with other co-existing organisms.

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Samples Availability: Available from the authors.

4.4 Manuscript D

A co-culturing / metabolomics approach to investigate chemically mediated interactions of planktonic organisms reveals influence of bacteria on diatom metabolism

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ORIGINAL ARTICLE

A co-culturing/metabolomics approach to investigate chemically mediated interactions of planktonic organisms reveals influence of bacteria on diatom metabolism

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Abstract Chemically mediated interactions are hypothesized to be essential for ecosystem functioning as co-occurring organisms can influence the performance of each other by metabolic means. A metabolomics approach can support a better understanding of such processes but many problems cannot be addressed due to a lack of appropriate co-culturing and sampling strategies. This is particularly true for planktonic organisms that live in complex but very dilute communities in the open water. Here we present a co-culturing device that allows culturing of microalgae and bacteria that are physically separated but can exchange dissolved or colloidal chemical signals. Identical growth conditions for both partners as well as high metabolite diffusion rates between the culturing chambers are ensured. This setup allowed us to perform a metabolomic survey of the effect of the bacterium *Dinoroseobacter shibae* on the diatom *Thalassiosira pseudonana.* GC–MS measurements revealed a pronounced influence of the bacterium on the metabolic profile of *T. pseudonana* cells with especially intracellular amino acids being up-regulated in co-cultures. Despite the influence on diatom metabolism, the bacterium has little influence on the growth of the algae. This might indicate that the observed metabolic changes represent an adaptive response of the diatoms. Such interactions might be crucial for metabolic fluxes within plankton communities.

Keywords Diatom · Metabolomics · Plankton interactions · Co-culture · *Thalassiosira pseudonana*

1 Introduction

Planktonic ecosystems harbor a variety of different microscopic organisms sharing the same habitat. Among these, unicellular algae contribute significantly to the global carbon cycle by performing up to 50 % of the world wide carbon fixation (Field et al. 1998). Nowadays, there is an increasing recognition that chemically mediated interactions between planktonic organisms are essential for the structure and function of the ecosystem (Ianora et al. 2011; Pohnert et al. 2007; Sieg et al. 2011; Van Donk et al. 2011). Planktonic microalgae, such as diatoms, dinoflagellates and prymnesiophytes can detect chemical signals that trigger chemical defense (Ianora et al. 2004; Pohnert 2000), intraspecific cell to cell communication (Vardi et al. 2006; Vidoudez and Pohnert 2008) and allelopathic interactions (Legrand et al. 2003; Paul et al. 2009; Yamasaki et al. 2009). Metabolites regulating these interactions are very diverse in their chemical nature. Indeed gaseous metabolites such as dimethylsulfide (DMS) (Wolfe et al. 1997) and cyanogen bromide (Vanelslander et al. 2012), complex secondary metabolites such as saxitoxins (Selander et al. 2006) and even high molecular mass protein complexes (Yamasaki et al. 2009) can cause responses in phytoplankton. This diversity makes the elucidation of chemical communication highly challenging. Chemically mediated interactions in pelagic organisms can be stimulated by direct cell contact or feeding activity. In these cases mechanical contact of interacting partners is required to trigger physiological or chemical responses (see e.g. Ianora et al. 2004; Wolfe et al. 1997; Pohnert 2000). Such interactions are local but can influence e.g. predator prey dynamics. In

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contrast, interactions that are mediated by diffusible signals have the potential to influence plankton communities by triggering responses in entire populations or in patches in the open ocean. These interactions require the release and perception of chemical signals that are active within the open water of the ocean (Pohnert et al. 2007). Investigation of plankton interactions have thus to distinguish between these fundamentally different chemically mediated processes but available methods are limited.

Several studies dealing with the influence of co-existing organisms on the metabolism of phytoplankton focused on the effect of grazers on the production of specific metabolites (Selander et al. 2011). However, from an ecological perspective the interactions of diatoms with bacteria also have a substantial influence on algal abundance and performance (Mayali and Azam 2004; Paul and Pohnert 2011; Teeling et al. 2012) even though only few studies document the influence of bacteria on diatom metabolism. For example, the production of the toxic domoic acid by the diatom *Pseudo-nitzschia multiseries* could be stimulated by the addition of bacteria (Bates et al. 1995). In a non-contact co-culturing experiment where axenic *P. multiseries* was separated by a cellophane tubing from non-axenic cultures, domoic acid concentrations in axenic cells were lower than in non-axenic ones suggesting that a direct cell contact between diatom and bacteria supports domoic acid production (Kobayashi et al. 2009). The importance of chemical communication was recently also emphasized for the interaction between the coccolithophore *Emiliania huxleyi* and the bacterium *Phaeobacter gallaecienis*. Bacteria either promoted or inhibited growth of *E. huxleyi* depending on the nature of released algal metabolites (Seyedsayamdost et al. 2011).

A major bottleneck in the identification of chemically mediated plankton interaction is the availability of suitable co-culture setups. Ideally, such devices should separate interaction partners but still allow diffusion of potential infochemicals. Previous setups using dialysis bags or cellophane tubing placed in a glass pot could not ensure identical growth conditions for the interacting partners or a sufficient diffusion between both culturing chambers (Jensen et al. 1972; Kobayashi et al. 2009; McVeigh and Brown 1954; Sieg et al. 2011). Commercial setups where membrane coated inserts are used to separate interaction partners have been employed in studies using marine organisms (Yamasaki et al. 2007). These setups allow only the investigation of small volumes of up to 5 mL. Since phytoplankton grows often only in low cell abundance such setups do not allow chemical analysis due to lack of sufficient extractable biomass.

Here we introduce a co-culture setup that overcomes these limitations. A fully replicated co-culturing experiment of the diatom *Thalassiosira pseudonana* and the bacterium *Dinoroseobacter shibae* was carried out and sufficient biomass for GC–MS based metabolomics according to Vidoudez and Pohnert (2012) was obtained. Since genome data for *T. pseudonana* as well as for the universal algal symbiont *D. shibae* are available, this study gives substantial insight into the interaction of well established model species (Armbrust et al. 2004; Wagner-Döbler et al. 2010). We observed that bacteria substantially influence specific pathways in the alga and we propose that such metabolically mediated interactions might play a fundamental role in ecosystem functioning.

2 Materials and methods

2.1 Co-culture setup

The co-culture setup consists of two modified glass vessels each holding ca. 500 mL with a 100 mm flat edge opening (Fig. 1). Modification of the commercially available Duran® flask (VWR, Dresden, Germany) included the generation of an opening and the addition of a 29 mm neck by a glass blower. Both vessels can be fitted together by a holding clamp and a 0.22 µm hydrophilic polyvinylidene fluoride (PVDF) membrane filter (Durapore, Millipore, Billerica, MA, USA) can serve to separate two culturing chambers. An O-ring made out of silicone between both vessels ensures a leak proof sealing. Each culture vessel has one 29 mm opening for filling and sampling purposes that can be covered with aluminum foil during culturing. Culture vessel, O-ring, aluminum foil, and membrane filter can be autoclaved separately and assembled under a sterile hood.

2.2 Diffusion assay

Each partition of the co-culture setup was filled with 300 mL artificial seawater (Maier and Calenberg 1994) and one chamber was supplemented with 15 µL of heptadienal (Sigma-Aldrich, Munich, Germany, 100 mM in methanol), 300 µL dimethylsulfoniopropionat (DMSP) (100 mM in water) and 177 µL of a 1.05 M sodium nitrate solution. The setup was kept at 15 °C and shaken at approximately 90 rpm during the entire experiment. To follow the kinetics of diffusion through the membrane, samples were taken from both compartments and analyzed as described below. Results are based on three independent replicates.

2.3 Instrumentation

LC–MS measurements were performed on an Acquity ultra performance liquid chromatography (UPLC) equipped with an Acquity BEH HILIC column (1.7 µm, 2.1 mm × 50 mm) coupled to a Q-ToF Micro mass spectrometer

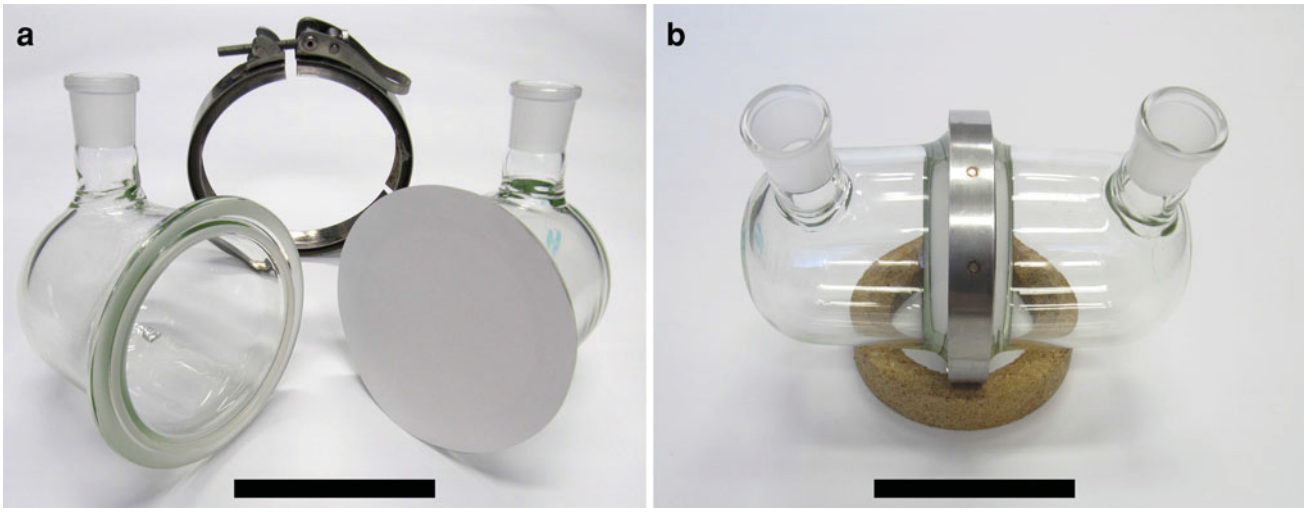

Fig. 1 Co-culture setup to grow organisms without direct contact but ensuring the exchange of metabolites. **a** Disassembled setup with all parts required for the setup and **b** assembled co-culture device. A 0.22 µm membrane separates both chambers that can each be filled with up to 500 mL medium. The black bar represents 10 cm

(Waters, Manchester, UK). Heptadienal and metabolomics samples were measured in random order with an Agilent 6890N gas chromatograph (Waldbronn, Germany) equipped with a 30 m DB-5 ms column (internal diameter 0.25 mm, film thickness 0.25 µm) protected by a 10 m Duraguard pre-column (Agilent, Waldbronn, Germany). The GC was coupled to a GCT premier mass spectrometer (Waters, Manchester, UK). He 5.0 was used as carrier gas with a constant flow rate of 1 mL min^{-1}. The MS scan rate was set to 2 and 5 scans s^{-1} for heptadienal and metabolomics samples respectively in dynamic range extension mode with the electron impact source at 70 eV. The GC oven temperature for the heptadienal analysis was held initially at 60 °C for 2 min followed by an increase of 8 °C min^{-1} to 240 °C and of 15 °C min^{-1} to 280 °C. For metabolomic samples the initial temperature of 60 °C was held for 1 min followed by an increase with a rate of 15 °C min^{-1} to 310 °C which was held for 10 min.

2.4 Solvents for extraction and derivatization

The following solvents were used for the extraction and derivatization: methanol (ChromasolvPlus, Sigma-Aldrich, Munich, Germany), chloroform (HiPerSolv, VWR, Dresden, Germany), ethanol (LiChrosolv, Merck, Darmstadt, Germany) and pyridine (ChromasolvPlus, Sigma-Aldrich, Munich, Germany).

2.5 Heptadienal analysis

For the analysis of dissolved heptadienal 10 mL water samples were derivatized with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (Fluka, Basel, Switzland) and measured using GC–MS as previously described (Vidoudez and Pohnert 2008). The quantification was based on the ratio of the peak area of heptadienal and benzaldehyde that was added as internal standard.

2.6 DMSP analysis

The quantification is based on a published protocol (Spielmeyer and Pohnert 2010). Briefly, 1 mL water samples were supplemented with 100 µL of a 200 µM [$^2H_6$]-DMSP solution as internal standard and kept at −80 °C until further analysis. The sample was diluted 1:10 (v:v) with acetonitrile before measuring with UPLC–MS.

2.7 Nitrate analysis

Nitrate concentrations were determined spectrophotometrically using a Specord M42 UV–Vis spectrophotometer (Carl Zeiss, Jena, Germany) according to a protocol developed by Zhang and Fisher (Zhang and Fischer 2006) that is based on the conversion of resorcinol.

2.8 Diatom-bacteria co-cultures

An axenic *T. pseudonana* culture was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (CCMP 1335) and grown subsequently in autoclaved artificial seawater (Maier and Calenberg 1994) at a constant temperature of 15 °C under a 14/10 h light/dark regime with 40 µmol photons m^{-2} s^{-1} illumination. The axenity of the culture was regularly checked by plating 100 µL of the culture on marine broth agar plates. *D. shibae* DFL 12 was obtained from the German

Collection of Microorganism and Cell Cultures (Strain No. 16493) and grown in artificial seawater supplemented with 10 mM succinic acid (Roth, Karlsruhe, Germany) (SW+) as sole carbon source at 15 °C on an orbital shaker (90–100 rpm). To prepare the co-culture experiments both *T. pseudonana* and *D. shibae* were inoculated in SW+ medium and kept in exponential growth phase by repeated dilution with fresh culture medium. Co-culturing (Tp/Ds) was started by inoculation of one chamber with exponentially growing *T. pseudonana* to reach 50×10^3 cells mL^{-1} in a final volume of 350 mL. The other culturing chamber was inoculated with *D. shibae* resulting in 1.8×10^6 cells mL^{-1} in 350 mL. As controls cultures of *T. pseudonana* were inoculated into both chambers with identical medium and concentrations as above (Tp/Tp). All setups were placed on an orbital shaker (90 rpm) at 15 °C with a light/dark regime of 14/10 h using fluorescent tube Osram T8 36 W 840 with approximately 40 µmol photons s^{-1} m^{-2}. Samples (1 mL) were taken daily during the course of the experiment under sterile conditions to estimate cell growth, chlorophyll a fluorescence and photosystem II (PSII) efficiency. Tp/Tp and Tp/Ds were both replicated 5 times. All data including cell growth, PSII efficiency and metabolic profiling in *T. pseudonana* control cultures are based on the culture in only one culture chamber. One replicate of the mono-cultures was discarded from the metabolic profiling evaluation due to high bacterial contamination.

2.9 Monitoring algal and bacterial growth parameters

Algal growth was estimated by counting approximately 300 cells using a Fuchs-Rosenthal hematocytometer with an upright microscope (Leica DM 2000, Heerbrugg, Switzerland). Bacterial cell growth was determined by flow cytometry. Therefore, 990 µL of the sample were fixed with 10 µL of a 25 % glutaraldehyde solution (electron microscopy grade, Sigma Aldrich, Munich, Germany) reaching a final concentration of 0.25 %. The sample was then vortexed for approximately 1 min before keeping it in the dark at 4 °C for 15 min. After freezing with liquid nitrogen, samples were stored at −80 °C before further analysis. Samples were thawed at room temperature and, to avoid coincidence (two or more particles being simultaneously present in the sensor zone), diluted in ultra-pure water one, five or tenfold depending on the growth stage. Aliquots of 100 µL diluted samples were diluted with 250 µL ultra-pure water and stained for 10 min in the dark with 5 µL of SYBR Gold (Invitrogen, Carlsbad, CA, USA) in 45 µL of PCR buffer (Fermentas, St. Leon-Rot, Germany). After addition of 100 µL of a calibration standard (see below) the final concentration of SYBR Gold for the measurement was 10^{-4} lower compared to the commercial stock. Samples were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with CXP-software, a 20 mW 488 nm air-cooled argon-ion laser and standard filters. The discriminator was set to green fluorescence and the samples were analyzed for 1 min at a flow rate of 30 µL min^{-1}. Data were calibrated to polystyrene fluorospheres (3.6 µm in diameter; Beckman Coulter) measured at 620 nm using CXP analysis software and the mean of three repetitive measurements per sample was used.

For PSII efficiency measurements 200 µL of the diatom culture were added to black 96 well plates. After storing the samples in the dark at 15 °C for 30 min the initial fluorescence (F0) was measured using a Mithras LB 940 plate reader with an excitation filter of 430 nm and an emission filter of 665 nm (Berthold Technologies, Bad Wildbad, Germany). After addition of 15 µL of 3-(3,4-dichlorphenyl)-1,1-dimethylurea (71.7 µM in water) (Sigma-Aldrich, Munich, Germany) to a final concentration of 5 µM the fluorescence was measured again resulting in the maximal fluorescence (Fm). The PSII efficiency was calculated according to (Fm − F0)/Fm (Roy and Legendre 1979).

2.10 Sampling, extraction and derivatization for metabolomics

At the end of the experiment (day 5) 250 mL out of the *T. pseudonana* containing chambers (only one chamber in the Tp/Tp experiments) were concentrated on 47 mm GF/C filters (Whatman, Kent, UK) under reduced pressure (~500 mbar). The wet filters containing the cells were transferred to 25 mL beakers and immediately extracted with 2 mL of cold (−20 °C) extraction solvent mix (methanol:ethanol:chloroform, 2:6:2, v:v). Extracts were transferred to 2 mL microcentrifuge tubes. The extracts were kept at −80 °C until further work up, approximately 1 month after extraction. Immediately before measurement all samples were derivatized simultaneously. Therefore, samples were adjusted to room temperature, treated in an ultrasound bath for 10 min and centrifuged for 15 min at 4 °C with 17,000 rpm. A volume corresponding to the extract of 10^8 extracted cells of the supernatant was transferred into 1.5 mL glass vials and evaporated to dryness under reduced pressure. For derivatization (Vidoudez and Pohnert 2012) 50 µL of a methoxyamine hydrochloride (Sigma-Aldrich, Munich, Germany) solution (20 mg mL^{-1}) in pyridine were added and incubated at 60 °C for 1 h and at room temperature for additional 9 h. Subsequently, 50 µL of N-methyl-N-trifluoroacetamide (MSTFA) (Macherey–Nagel, Düren, Germany) supplemented with decane, pentadecane, nonadecane, octacosane, dotriacontane (final concentration for all 40 µM) and hexatriacontane (final concentration 20 µM) (all Sigma-Aldrich, Munich, Germany) as retention time index were

added to each sample and incubated at 40 °C for 1 h. The samples were transferred into 100 µL glass inserts of 1.5 mL vials and directly measured with GC–MS.

2.11 Data processing

Data processing was adapted from a previously published protocol (Vidoudez and Pohnert 2012). Briefly, MassLynx (version 4.1, Waters) was used to correct the chromatograms for background noise applying the Component Detection Algorithm. The chromatograms were converted to netCDF files using MassLynx DataBridge prior to running them as one batch job in AMDIS (version 2.65, NIST, http://www.nist.gov). The parameters were: Minimum match factor: 30; type of analysis: simple; component width: 12; excluded *m/z*: 147, 176, 193, 207, 219; adjacent peak subtraction: 2; resolution: low; sensitivity: medium; shape requirement: low. The AMDIS output files were used to quantify the identified metabolites using MET-IDEA (version 2.05, http://bioinfo.noble.org) with the following parameters: average peak width: 0.08; AMDIS transfer: 0.5; maximum peak width: 2; peak start/stop slope: 1.5; adjusted retention time accuracy: 0.25; peak overload factor: 0.9; MS type: TOF; mass accuracy: 0.1; mass range: 0.3; lower mass limit: 100; ion per component: 1. The resulting data matrix consisting of metabolite identity and corresponding peak areas was copied into excel 2007 (Microsoft, Redmont, USA). Signals resulting of the retention time index were deleted prior to statistical evaluation.

2.12 Statistical analysis

Differences in growth and PSII efficiency were evaluated using two way repeated measure analysis of variance (RM-ANOVA) implemented in SigmaPlot 11 (Systat Software, San Jose, CA, USA). To evaluate differences between Tp/Ds and Tp/Tp metabolic profiles unconstrained (principal coordinate analysis, PCO) and constrained methods (canonical analysis of principle coordinates, CAP) were used. Therefore, the data matrix was converted to a *.txt file and fed to the software CAP 12 (http://www.stat.auckland.ac.nz/~mja/Programs.htm). The following parameters were used: transformation: none; standardization: none; similarity measure: Bray–Curtis. The data of the resulting output file was used to graph the PCO and CAP plot using SigmaPlot 11. Correlation of metabolites with the CAP axis was considered if the correlation coefficient was above or below 0.75 or −0.75, respectively.

2.13 Identification of metabolites

The identity of all metabolites recognized as significant for the separation of both treatments in CAP analysis was manually examined based on their mass spectra and retention time. Therefore, the chemical structure suggested by AMDIS was reviewed of one exemplary chromatogram using the following mass spectral libraries: NIST library version 2005, T_MSRI_ID 2004-03-01 Golm metabolome database (Wagner et al. 2003) and an in-house library implemented in the software MS search (version 2.0 d, NIST).

The structure was accepted if the following criteria were met. First, the structure as suggested by AMDIS had to be confirmed by the used libraries. Second, the reverse match factor had to be >800, structures with a reverse match between 800 and 700 are tagged with a "?" and structures with a reverse match below 700 are marked with "??". Reverse match factors below 600 were not accepted. Structures that fulfilled these criteria needed additionally to have a retention index within the range of the retention index provided by the mass spectral libraries. To verify the identity of amino acids, retention times and mass spectra of authentic standards had to be identical with respective signal.

3 Results and discussion

3.1 Development of the co-culture setup

We developed a setup enabling non-contact co-culturing of planktonic organisms in which two species grow in separated compartments under identical conditions. This set-up allows diffusion of potential infochemicals between the separated compartments and thus a chemical communication between the interaction partners. The vessels were constructed so that regular sampling for standard growth parameters as well as metabolomic investigation of the cells is possible under sterile conditions. We constructed a custom made setup with two glass chambers separated by a membrane (Fig. 1) in which each chamber can be sterilized, filled and manipulated independently. As culturing chamber we used 500 mL flat edged glass ware that was modified with an opening for filling and sampling purposes. After autoclaving membrane, sealing, and glassware the setup was assembled under sterile conditions, which took about 3 min per co-culturing device. As biocompatible membrane we used a 0.22 µm PVDF filter which proved to be suitable to separate microalgae as well as bacteria (data not shown). In contrast to co-culture setups used previously where separation went ahead with unequal growth conditions for both partners (Paul et al. 2009), both compartments in our setup supported equal growth. This was verified by inoculation of both chambers with 50×10^3 cells mL^{-1} *T. pseudonana*. The algae started to grow exponentially and their cell counts did not differ significantly in the compartments ($P > 0.3$ for comparisons

at day 1, 2 and 3). An advantage over commercially available setups (Yamasaki et al. 2007) is the large culture volume of up to 500 mL that allows sampling for metabolomic investigations even in dilute growth situations that match cell counts in the sea (Vidoudez et al. 2011).

3.2 Diffusion of relevant metabolites

To allow planktonic organisms to interact via chemical signals the diffusion within such a co-culturing setup has to be quick and ideally result in equilibrium conditions. The nature of signal molecules that have hitherto been identified in the plankton is very heterogeneous and ranges from small ionic to larger more lipophilic compounds (Pohnert 2012). We therefore monitored the diffusion of DMSP, a zwitterionic low molecular weight metabolite with multiple functions including osmolytic regulation, antioxidant properties as well as a potential involvement in chemical defense (Groene 1995; Pohnert et al. 2007; Steinke et al. 2002). If added to one compartment diffusion readily occurred and within 24 h the concentrations in both compartments were nearly at equilibrium. After 48 h no differences were detected (Fig. 2b). Additionally, we tested the distribution of the less polar heptadienal after inoculation in one of both chambers. Heptadienal plays a role in the chemical defense of diatoms (Ianora et al. 2004) and might also be involved in the cell to cell communication (Vidoudez and Pohnert 2008) of diatoms. The diffusion of ca. 3.5 µM heptadienal resulted in nearly identical concentrations in both chambers after 24 h (Fig. 2c). In contrast to DMSP, the final concentration was only around one-third of the initial concentration, which can be explained by low stability and volatility of the compound. Further, heptadienal might be adsorbed by the glass or the membrane reducing the concentration of dissolved heptadienal. We also tested the diffusion of nitrate, an essential macronutrient supporting algal growth, added to one growth chamber (Fig. 2a). Nitrate diffusion leads also to equal concentrations in both chambers within 24 h. The same diffusion assays were performed with a 10 kDa dialysis membrane separating the culturing chambers and the results revealed very similar diffusion kinetics of the relevant metabolites (data not shown). Due to their ease of handling we decided to use 0.22 µm membranes in further experiments. We thus demonstrated that the set-up allows the testing of the influence of nutrients as well as common infochemicals. In certain cases of high molecular weight or highly lipophilic metabolites diffusion would have to be evaluated in a preliminary experiment.

In all cases the diffusion processes observed lead already to a significant exchange of metabolites between the chambers within the first hours after addition. This is definitely sufficient for the observation of most chemically mediated interactions, that often require prolonged times for an interaction to manifest. For example, the inhibitory effect of polyunsaturated aldehydes such as 2,4-heptadienal is known to take several days (Ianora et al. 2004). If however faster diffusion would be required the experimental set-up could be optimized by introducing a larger membrane in modified vessels.

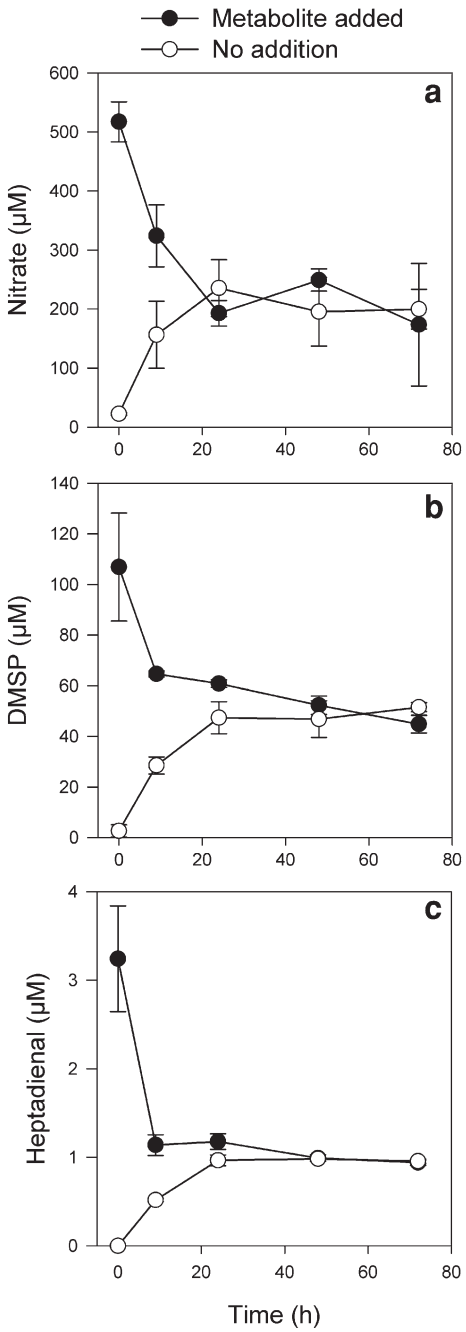

Fig. 2 Diffusion of **a** nitrate, **b** DMSP and **c** heptadienal trough a 0.22 µm membrane in co-culture devices. The respective compounds were added to one compartment and their concentrations were monitored in both compartments over time

3.3 Growth in co-cultures

Growth of *T. pseudonana* developed similarly in both treatments within the first days of the co-culturing experiments. From day 4 onwards the cell abundance in Tp/Ds was higher compared to Tp/Tp ($P = 0.002$ and $P < 0.001$ for day 4 and 5, respectively, $n = 5$). While *T. pseudonana* in Tp/Ds reached a final cell abundance of 3.1×10^6 cells mL^{-1}, Tp/Tp cultures had a cell abundance of 2.3×10^6 cells mL^{-1}. *D. shibae* was inoculated to an initial abundance of ca. 2×10^6 cells mL^{-1}. During the course of the experiment the cell abundance increased reaching a final bacterial abundance of 70×10^6 cells mL^{-1} (Fig. 3a). By plating out 100 µL culture of the Tp compartment axcenity could be verified on day 1. During the further course of the experiment bacteria with different morphology and in significantly lower amounts compared to *D. shibae* could be detected. These traces of contaminant bacteria might affect the overall metabolism of *T. pseudonana* but since they were found in both co-culture setups (Tp/Tp and Tp/Ds) in comparable amounts we conclude that they did probably not influence the outcome of the comparative metabolic profiling. This will rather reflect the effect of additional *D. shibae* bacteria in high abundance. Medium effects can be excluded since conditions were identical in both setups. The stimulated growth in Tp/Ds can be caused by several factors. Certain bacteria are known to increase algal growth by supplying vitamins to algae (Cole 1982; Croft et al. 2005; Seyedsayamdost et al. 2011). Especially vitamin B1 and B12 from *D. shibae* can support algal growth as demonstrated in co-cultures of the bacterium with *Prorocentrum minimum* (Wagner-Döbler et al. 2010). Alternatively, essential nutrients might be used up quicker in Tp/Tp since twice the cell counts with respect to the total volume were reached. However, this possibility is less likely since the experiment was conducted only during the first days of the exponential growth phase where sufficient nutrients should be available.

We estimated the health and nutrient status of the cultures by monitoring the PSII efficiency for Tp/Tp and Tp/Ds. The PSII efficiency was similar in both treatments during the entire experiment ($P > 0.088$ for all comparisons, Fig. 3b) suggesting an equal performance of the diatoms. The photosystem II efficiency allows drawing conclusions on the nutrient and health status of a culture. PSII efficiency measurements are a valid diagnostic tool for nitrogen limitation in *T. pseudonana* batch cultures (Kolber et al. 1988). This suggests no nitrate limitation, which was also supported by the fact that nitrate concentrations in both treatments did not differ (average 380 µM, $P = 0.455$). A limitation in available phosphorous leads also to a decreased quantum yield efficiency in algae (Beardall et al. 2001; Liu et al. 2011). Furthermore silica

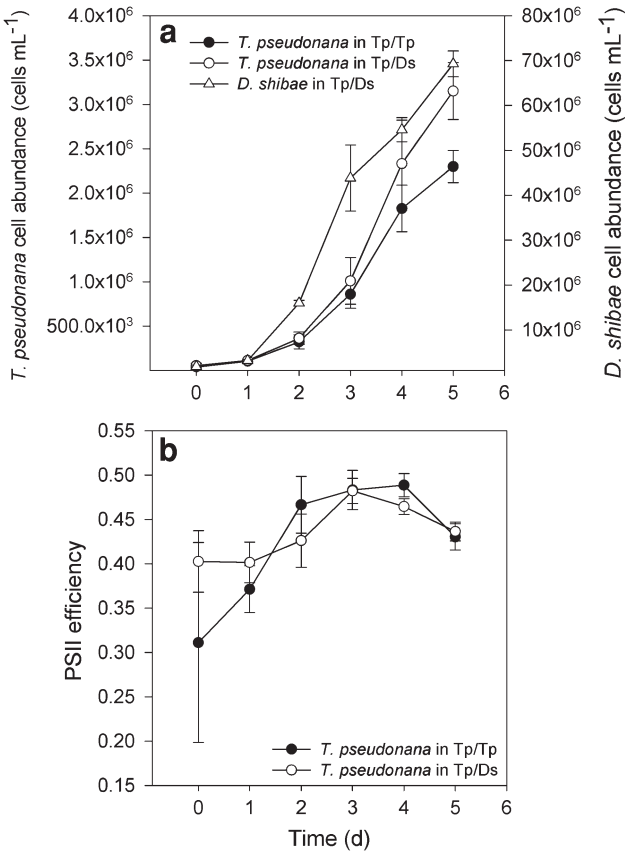

Fig. 3 Growth parameters of *T. pseudonana* and *D. shibae*. **a** Cell abundance of *T. pseudonana* in mono- and co-cultures and the cell abundance of co-cultured *D. shibae*. **b** Photosystem II (PSII) efficiency of *T. pseudonana* in mono- and co-culture

limited cells of *Thalassiosira weissflogii* had a significant reduced PSII efficiency which could be recovered after the addition of silicate (Lippemeier et al. 1999). Since the PSII efficiency did not differ between *T. pseudonana* in Tp/Tp and in Tp/Ds we conclude that *T. pseudonana* cells were not nutrient limited or did at least not have a significant different health status that could influence the metabolic profile.

3.4 Metabolic profiling

At the end of the experiment (day 5) cellular metabolites of *T. pseudonana* were extracted to evaluate the effect of exudates from bacteria on the metabolism of these diatoms. After extraction, the equivalent of 10^8 diatom cells was derivatized using methoxyamine hydrochloride followed by a silylation employing MSTFA. Analysis with GC–MS and AMDIS evaluation of the data allowed the identification of 510 ± 22 and 544 ± 30 compounds for Tp/Tp and Tp/Ds cells respectively being not significantly different from each other ($P = 0.1$). The peak areas were integrated using the MET-IDEA software and the data set consisting

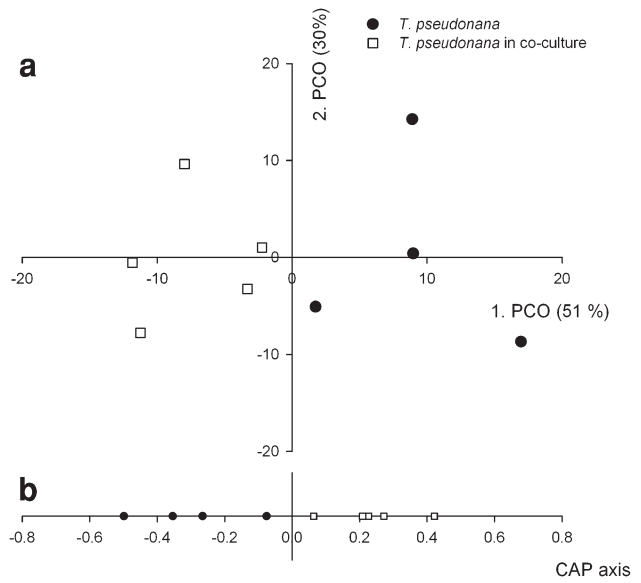

Fig. 4 Illustration of the multivariate separation of cellular metabolites of *T. pseudonana*. Separation using **a** the unconstrained method of analysis of principal coordinates (PCO) and **b** the constrained method of canonical analysis of principle coordinates (CAP). Diagnostic correlation coefficients were obtained for the correlation of each metabolite with the canonical axes

of tentatively identified substances with their corresponding peak areas was used to perform principal coordinate analysis (PCO) and canonical analysis of principal coordinates (CAP). Both protocols were previously successfully applied to discover patterns in large ecological data sets (Anderson and Willis 2003; Nylund et al. 2010) and in metabolomics experiments (Nylund et al. 2011; Vidoudez and Pohnert 2012). The unconstrained PCO as well as the constrained CAP gave a visual separation of both treatments (Fig. 4). Statistical evaluation gave a high eigenvalue of 0.9 and a P value of 0.046 for the permutation test. The misclassification was 11.1 % representing one out of nine samples to be misclassified. The correlation of each variable with the canonical axis allowed identifying variables that contribute to the separation of the two groups and that are thus potential candidates for metabolites regulated in bacteria-phytoplankton interaction. Metabolites were tentatively identified using MS libraries and the retention index, and the peak areas were plotted resulting in the heat map shown in Table 1.

The metabolic profiling procedure applied here is adapted from a protocol optimized for the diatom *Skeletonema marinoi* (Vidoudez and Pohnert 2012). As with *S. marinoi* we were able to obtain complex chromatograms with hundreds of metabolites (unedited list including substance identification according to AMDIS and peak area obtained with MET-IDEA can be found in a supplementary excel file). Interestingly, the metabolites exuded from bacteria generally stimulate the metabolism of *T. pseudonana* since nearly all observed differences are due to an increased abundance of metabolites in Tp/Ds (Table 1). We identified no substance that was negatively correlated with the canonical axis and thus down regulated in the presence of bacteria ($R < -0.75$). Since extracts were derived from a normalized amount of cells and since the cell volume did not differ between both treatments, this observed effect of the co-culturing is not simply caused by a higher amount of biomass extracted. Moreover, the activation of metabolism is not uniformly found for all metabolites, but rather specific pathways are up-regulated in the presence of bacteria. Especially amino acids including serine, proline, phenylalanine and the glutamic acid derivatives acetylglutamic acid and pyroglutamic acid are found in higher concentration in Tp/Ds compared to Tp/Tp. Pyroglutamic acid, which is not an algal metabolite, can be formed from glutamate during the analytic procedure (Gehrke and Leimer 1971; Leimer et al. 1977). The importance of amino acids for diatom-bacteria interactions was recently also recognized in biofilms. In the benthic freshwater diatom *Fragilaria pinnata* the pool of extracellular dissolved free amino acids is significantly altered if diatom-bacteria co-cultures were compared with axenic diatoms. For example, the concentration of dissolved isoleucine was significantly higher in co-cultures while the concentration of histidine was significantly reduced (Bruckner et al. 2011). However, the reason for that observation could not be elucidated in detail. Besides amino acids we detected several short and medium length acids such as glutaric acid and C16 and C18 fatty acids that were found in higher concentrations in Tp/Ds cells. Additionally, several sugars of which the identity was not elucidated in detail were strongly up-regulated in the presence of bacteria.

We cannot conclude if any of these metabolites is up regulated in response to signals from co-cultured bacteria or if diatoms can take up metabolites released by bacteria. It is known that benthic diatoms such as *Cylindrotheca closterium* (Nilsson and Sundback 1996) and planktonic diatoms such as *Thalassiosira* sp. and *Phaeodactylum* sp. (Admiraal et al. 1984; Admiraal et al. 1986; Flynn and Wright 1986) can take up dissolved free amino acids from the culture medium. Interestingly, it was also demonstrated that free amino acids can enhance primary production in pelagic and benthic diatoms (Flynn and Syrett 1986; Linares 2006), which might explain the stimulated algal growth in our co-culturing. However, cellular concentrations of free amino acids depend on a multitude of additional factors. For example, it was found that amino acid concentration varies depending on the culture growth stage in targeted (Myklestad et al. 1989) and non targeted analysis (Vidoudez and Pohnert 2012). The observed effect could also be explained with an uptake of vitamins released from the bacteria that could influence specific biosynthetic pathways of the algae (Cole 1982; Croft et al. 2005).

Table 1 Heatmap of intensities of cellular metabolites positively correlated with the CAP axis ($R > 0.75$) from *T. pseudonana* in mono- and co-cultures

Metabolite	RT (min)	Mono-cultures				Co-cultures					R
Monomethylphosphate ?	6.58	151	385	1332	468	1745	1334	1070	2446	1651	0.77
Glyceric acid	8.01	1500	2638	9483	1910	30180	12455	13380	30094	18717	0.85
Picolinic acid ?	8.05	5010	6452	11680	6101	24703	12189	14292	25658	13067	0.84
Serine *	8.29	15784	25122	23085	15992	32164	29821	40887	34010	24809	0.83
Threonic acid-1,4-lactone	8.42	2046	2881	2469	1652	3555	2431	3465	3306	2552	0.78
Glutaric acid ??	8.73	519	425	386	366	1200	849	753	588	460	0.79
Maleic acid ??	9.03	1542	1209	1180	781	2602	1725	2443	3449	2177	0.81
Pyroglutamic acid *	9.75	177925	322289	620549	289735	709794	745946	635337	930276	536875	0.87
N-acetylglutam acid ?	9.82	16235	46012	79388	22005	253266	242190	343970	422207	233094	0.86
Phenylalanine *	10.03	1092	4364	5390	1883	10400	5958	17097	12335	13194	0.77
Proline *	10.24	154271	299905	335246	180343	422092	355007	428996	373481	286540	0.86
Pentonic acid-1,4-lactone	10.76	452	515	610	324	928	592	1329	1078	801	0.8
Pentonic acid-1,4-lactone	11.41	3551	3729	3496	1449	3875	4183	5229	4633	3847	0.76
C4 sugar ?	12.11	4943	5631	6853	4919	13452	8078	8200	11694	6901	0.86
C4 sugar	12.34	13072	15747	19597	13398	23516	19251	26090	31259	15933	0.77
C4 sugar	12.42	9612	17911	19902	14319	29140	20610	18576	28631	19817	0.76
Hexadecenoic acid	13.51	311648	19096	352998	16322	312906	261116	403752	413417	342359	0.75
Octadecenoic acid ?	14.68	21914	16361	26675	14033	29900	26950	37148	34691	19123	0.83
Octadecatrienoic acid ?	15.56	49658	42673	45053	23870	64219	49337	75074	74334	54889	0.84
Monohexadecanoylglycerol	16.65	10075	19666	28349	10033	28171	20502	36361	38430	23327	0.76

The color reflects the intensities of the metabolites with bright coloring for low intensities and dark coloring for high intensities. The identity of metabolites indicated with an "*" was verified with authentic standards. Metabolites marked with a "?" had reverse match between 800 and 700 and marked with "??" a reverse match of below 700

The metabolite picolinic acid, a tryptophan catabolite, was also found in higher concentration in Tp/Ds diatom cells compared to Tp/Tp. Picolinic acid can potentially form metal complexes with limiting trace elements such as iron. Assuming a synthesis of this metabolite by the co-cultured bacteria and an uptake of iron complexes by the aglae this would be a way to establish a mutualistic interaction. Notably, a derivative of picolinic acid named thallusin with a 2,6-dicarboxy pyridine moiety was isolated from marine bacteria in symbiosis with the macroalga *Monostroma oxyspermum*. In this symbiosis the substance is essential since it induces cell differentiation in the alga (Matsuo et al. 2005). Detailed further investigations using e.g. the co-culture set-up introduced here would however be required to support such considerations.

4 Concluding remarks

We describe a co-culturing device, which enables a contact free co-culturing of both planktonic microalgae and bacteria. This setup facilitates high diffusion rates for several plankton relevant metabolites and secures identical growth conditions for both co-cultured partners. It was successfully utilized to co-culture *T. pseudonana* with *D. shibae*. While control and co-cultured *T. pseudonana* had an identical health status during the experiment as indicated by similar PSII efficiencies, the cell abundance in diatom/bacteria co-cultures was higher at the end of the experiment in comparison to mono-culture controls. Metabolic profiling of diatom cellular metabolites revealed a higher metabolic activity of co-cultured *T. pseudonana* with especially amino acids and amino acid derivatives being up-regulated. These findings might have significant ecological significance since, for example the food quality of algae might change in the presence of bacteria thereby influencing energy transfer between different trophic levels of the plankton. Further, bacteria might also influence the excretion of algal metabolites, which can in turn influence chemically mediated interactions and element cycling. Even though not addressed in our study, the presented co-culture device and the metabolomic routine will facilitate the investigation of a multitude of other plankton interactions in the future.

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Diatom exudates influence metabolism and cell growth of co-cultured diatom species

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Diatom exudates influence metabolism and cell growth of co-cultured diatom species

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ABSTRACT: We surveyed the role of chemically mediated interactions between the 2 diatom species *Skeletonema costatum* and *Thalassiosira weissflogii*. *S. costatum* promoted the growth of *T. weissflogii* in non-contact co-cultures, allowing an exchange of exuded metabolites. In contrast, cell counts of *S. costatum* were not affected by *T. weissflogii*. Metabolic profiling of the co-culture medium revealed a significant change in diatom-derived metabolites in comparison with those of monoculture controls. Several compounds detected in monocultures were not present in the co-culturing set-up, indicating either a transformation or uptake of released metabolites by the competing species. In addition, metabolic profiling of intracellular metabolites revealed that the biochemical processes of both diatoms changed in the presence of the co-cultured species. The present study illustrates that chemical cross-talk between diatom species is possible and that these types of chemical interactions lead to physiological responses and might even result in changed cell abundances.

KEY WORDS: Allelopathy · Metabolomics · Plankton interactions · Chemical ecology · Phytoplankton

INTRODUCTION

Diatoms (Bacillariophyceae) are highly abundant unicellular microalgae in ocean waters and play a crucial role in the marine food web (Fenchel 1988). In the natural environment diatoms occur in complex species assemblages and encounter stress from a variety of sources, including nutrient limitation and predation, as well as competing phytoplankton species. Stress from other phytoplankton often results from competitor-induced light or nutrient limitation (Tilman 1982). However, in some cases, phytoplankton species may also directly regulate the density of competitors via the exudation of chemical compounds. Molisch (1937) introduced the term allelopathy to apply to both inhibitory and stimulatory biochemical interactions between either primary producers or primary producers and microorganisms.

Allelopathic interactions between such phytoplankton are believed to influence plankton community composition (Maestrini & Bonin 1981, Cembella 2003, Gross 2003, Legrand et al. 2003, Pohnert et al. 2007). The effects of toxic dinoflagellates such as *Alexandrium* sp. and *Karenia brevis* have been particularly intensely studied. Both *Alexandrium* sp. (Fistarol et al. 2005) and *K. brevis* (Kubanek et al. 2005, Prince et al. 2008) have been reported to exude allelopathic compounds that inhibit the growth of competitors. Although in some cases allelopathic interactions between diatoms and other members of the phytoplankton community have been reported (Pratt 1966, Fistarol et al. 2004, Prince et al. 2008), little is known about the chemically mediated interactions between non-toxic diatom species (but see Sharp et al. 1979, Pouvreau et al. 2007).

In most studies of planktonic allelopathic interactions, experiments only monitor the effect of extracellular extracts or nutrient re-enriched media from a mono-culture of one species on a culture of the interacting organism. Other studies maintain mixed cultures in single culture vessels, thereby observing an overlapping effect of chemically mediated and cell contact mediated interactions (Talling 1957, Wang & Tang 2008). Yamasaki et al. (2007) separated cultures of the diatom *Skeletonema costatum* and the flagellate *Heterosigma akashiwo* by a dialysis membrane,

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thereby physically separating the cultures but allowing chemical interaction. This type of a spatially separated co-culturing is a useful method to investigate chemical communications between phytoplankton because it allows observation of chemical cross-talk without interference from direct cell contact (e.g. McVeigh & Brown 1954, Jensen et al. 1972, Vardi et al. 2002).

Current approaches for detecting chemically mediated interactions between plankton often consider only a small set of parameters, such as growth rate (Fistarol et al. 2005), morphology and/or the abundance of a specific class of compounds (Myklestad 1995). While useful, these approaches are often unable to detect previously unknown chemical cues and responses outside of the narrow range of established parameters. Metabolic profiling, a 'global' analysis of biological samples for low molecular mass organic metabolites, is a survey of released (exo-metabolome) and intracellular compounds (endo-metabolome). This non-targeted method allows the study of the physiological basis of signal production and reception by monitoring metabolic responses to external factors (Wilson et al. 2005).

In the present study, we combined a non-contact co-culturing approach with metabolic profiling to monitor the interactions between the diatoms *Skeletonema costatum* and *Thalassiosira weissflogii*. *S. costatum* is an abundant diatom in ocean waters (Kooistra et al. 2008) and has been intensely studied both in terms of ecology and physiology (e.g. Granum et al. 2002, Dutz et al. 2008, Zhao et al. 2009). *T. weissflogii* was selected as a competitor because it is known to co-occur with *S. costatum* (e.g. in English Channel waters). The metabolic profiling included analysis of both the metabolites released into the media and the metabolites retained within the cells. We observed changes in both the metabolic profiles and in cell growth in co-cultures relative to controls, indicating a direct physiological response of one species on the presence of another. We discuss these results and how this interaction might be described in light of the concept of allelopathy.

MATERIALS AND METHODS

Cell culturing. *Skeletonema costatum* (Strain RCC 75) and *Thalassiosira weissflogii* (Strain RCC 76; can also be found in the CCMP collection strain, CCPM 1336) were obtained from the Roscoff Culture Collection (Roscoff, France). Cells were cultivated under a 14:10 h light:dark cycle at 15°C, under illumination at approximately 80 µmol photons s^{-1} m^{-2}. The culture medium was sterilized, artificial, buffered seawater prepared as described by Maier & Calenberg (1994).

Cells were cultivated in standing cultures of 300 ml; transfer of cultures was performed using autoclaved tools and culture vessels under a clean bench. Repeated culturing to the exponential phase and subsequent dilution was performed to guarantee similar starting conditions for both algal species despite different absolute cell counts. For larger-scale inoculations, 300 ml of exponentially growing cultures were diluted with 1200 ml of medium to reach cell densities of 27×10^3 and 1×10^3 cells ml^{-1} for *Skeletonema costatum* and *Thalassiosira weissflogii*, respectively. Growth rates were similar to those found in earlier studies (Montagnes & Franklin 2001, Grossart & Simon 2007, Vidoudez & Pohnert 2008). Throughout the experiment samples from the outer chamber were monitored by microscopy for contamination and discarded if any increased levels of microorganisms were observed. At the end of the experiment the inner chambers were monitored for bacterial contamination, and results were not considered if contaminations were observed.

Co-culturing experiment. Dialysis tubes (dialysis tubing cellulose membrane, pore size 12 kDa, 76 mm × 49 mm; Sigma Aldrich) were used to separate the cultures during co-culturing experiments. Dialysis tubes were cut into sections of approximately 32 cm and washed according to the recommendations of the manufacturer. After washing, one end was closed by knotting the tube. A short piece of Teflon tubing (5 cm long and 3 mm in diameter) was attached to the other end of the dialysis tube using a nylon line. These dialysis bags were sterilized in boiling water for 15 min and were kept in a sterile container until use. The resulting sterile dialysis bags were inoculated with diatom cultures via the Teflon tubing. After filling, a silicon stopper was inserted into the Teflon tubing to ensure tight closure.

Co-cultures were made by transferring 100 ml of a freshly inoculated *Skeletonema costatum* culture (27×10^3 cells ml^{-1}) to a sterilized 500 ml glass flask with a wide mouth (Weck GmbH). Using a sterile syringe, a dialysis bag was then filled with 100 ml of a freshly inoculated *Thalassiosira weissflogii* culture (1×10^3 cells ml^{-1}) and tightly closed. The dialysis bag was subsequently placed into the *S. costatum* culture, ensuring that the whole dialysis bag was submerged. The reverse co-cultures, with *S. costatum* inside the dialysis bag (referred to here as the inner culture) and *T. weissflogii* in the glass flask (referred to as the outer culture), were also prepared. This second co-culture prevented differences in growth conditions within and outside of the dialysis bag from confounding experimental results. As a control, cultures were prepared identically to the treatments except that the same species was in both the dialysis bag and in the glass flask. All treatments were performed in triplicate. A blank was prepared by filling a glass flask and the dialysis bag with 100 ml of medium each.

The co-cultures were agitated on a laboratory shaker at 90 rotations min^{-1} and cultured under the conditions described above.

Sampling and cell concentrations. All cultures were sampled under sterile conditions. To follow cell growth, samples of approximately 75 µl were taken from the outer culture every 2 to 3 d. Sampling out of the inner culture was not possible before the last day of the experiment, since dialysis bags needed to remain sealed to avoid contamination. The cell density was determined by counting at least 400 cells on a Fuchs-Rosenthal hematocytometer with an upright microscope (Leica DM 2000, Leica).

Growth rate calculations. Growth rates (µ) were calculated as $\mu = (\ln N_t - \ln N_0) / \Delta t$, where N_t and N_0 are cell densities at time t and time 0, respectively, and Δt is the time difference between the measurements in days.

Extraction of extracellular and intracellular metabolites. On Day 38, the media and the cells of the outer culture and of the inner culture were independently extracted. The cells were separated from the medium by filtration on approximately 4 g of sand under gentle vacuum. The sand (VWR) was washed 5 times with deionized water beforehand. Sand filtration was selected since it allows separation of cells from the medium without induction of the release of metabolites due to stress, as has been observed when using other filtration techniques (Vidoudez & Pohnert 2008). The resulting cell-free medium was extracted on solid phase extraction (SPE) cartridges (Chromabond EASY, 200 mg, Macherey-Nagel). The loaded cartridges were washed with 4 ml of water (Chromasolv Plus, Sigma Aldrich) and eluted with a 1:1 (v:v) mixture of methanol (Chromasolv, Sigma Aldrich) and tetrahydrofurane (HiPerSolv, Chromanorm, VWR) into 4 ml glass vials. To obtain the cell extract, the sand used for the filtration containing the cells was immediately transferred to 15 ml tubes (Falcon) and covered with 4 ml of methanol. After vortexing for 1 min, the tubes were centrifuged and the methanol extract was transferred to 4 ml glass vials. The samples were stored at −80°C until analysis.

Ultra-performance liquid chromatography electrospray mass spectrometry (UPLC-ESI/MS) analysis. Metabolic profiling was performed using a Waters Acquity ultra-performance liquid chromatography (UPLC) coupled to a time of flight Q-ToF micro-mass spectrometer (Waters) equipped with electrospray ionization (ESI). Mass spectra were recorded in negative ion mode. A BEH C_{18} UPLC column (2.1 mm, 1.7 µm; Waters) at 30°C was used for separation. The injection volume of each sample was 2 µl and the flow rate was 0.6 ml min^{-1}. Every sample was injected 3 times. The mobile phases were 0.1% formic acid and 2% acetonitrile in water (=A) and 0.1% formic acid in acetonitrile (=B). The solvent gradient changed from 100% A to 100% B in 7 min, was held at 100% B for 2 min, was returned to 0% B by 9.5 min and was held at 0% B for another 30 s (all solvents ULC/MS, Biosolve). Mass spectra were recorded at a scan rate of 1 scan s^{-1} with an inter-scan delay of 0.1 s and a scan range of 100 to 1000 *m/z*. The collision energy was 5 V, the sample cone was 34 V, the cone gas flow was 150 l N_2 h^{-1} and the desolvation gas flow was 650 l N_2 h^{-1}.

Statistical analysis and quantification. Unless otherwise stated, values are given as mean ± SD. Differences in cell density were detected with a 2-tailed *t*-test assuming equal variances with Microsoft Excel 2007. For concentrations of cellular metabolites of *Thalassiosira weissflogii*, unequal variances were calculated and a *t*-test was performed assuming unequal variances (see Table 3). Controls and the corresponding co-cultures were compared with a 2-way repeated-measures analysis of variance (RM-ANOVA). In order to statistically analyze differences between *T. weissflogii* cultures, 1 culture was excluded due to missing data, in all other cases n = 3. The analysis was conducted with the software Graphpad Prism 5.0. In all cases, differences were accepted as significant when $p < 0.05$.

Raw MS data were used to automatically generate lists of intensities of mass-retention time pairs using the normalized and mean centered peak area, calculated with Apex Track peak detection (implemented in the MarkerLynx V4.1 software supplied by Waters). These data were used for principal component analysis (PCA) employing the same software (for experimental details see Barofsky et al. 2009).

In order to compare concentration differences of certain metabolites between different treatments, the peak area was determined with the software QuanLynx V4.1 (Waters) using standard program peak detection. The area of peaks in the cell extracts were normalized to the cell density and filtered volume, medium extracts were normalized to the filtered volume. Significance of differences between peak areas were evaluated with 2-tailed *t*-tests as described above.

RESULTS

Cell densities of co-cultured diatoms

We did not observe an effect on *Skeletonema costatum* cell density when it was co-cultured with *Thalassiosira weissflogii* (Fig. 1A). Both co-cultures and control cultures of *S. costatum* had similar growth rates (0.71 ± 0.03 d^{-1} for the co-culture, 0.70 ± 0.03 d^{-1} for the control, calculated between Days 0 and 6). The growth rates are not significantly different ($p = 0.41$). Logarithmic plotting of cell densities confirmed that growth

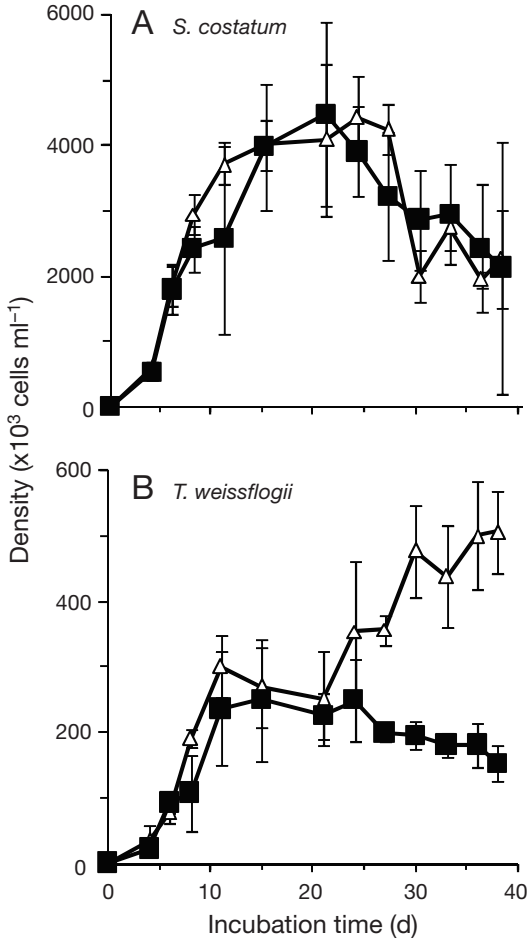

Fig. 1. *Skeletonema costatum* and *Thalassiosira weissflogii*. Growth during co-culturing. (A) Cell density of *S. costatum* in the presence of *T. weissflogii* (Δ) compared to a control in the presence of *S. costatum* (■) and (B) cell density of *T. weissflogii* in the presence of *S. costatum* (Δ) compared to a control in the presence of *T. weissflogii* (■). Values are mean ± SD (n = 3), except for the co-culture (Δ) in (B) where the values from Day 24 are given as the average and range (n = 2). The outer cultures of the co-culturing set-up were sampled

was exponential from Day 0 to 6 (data not shown). The maximum cell densities reached by *S. costatum* in the presence of *T. weissflogii* and in the control were nearly identical (4480 ± 1400 × 10^3 cells ml^{-1} [Day 21] and 4450 ± 630 × 10^3 cells ml^{-1} [Day 24], respectively). *S. costatum* reached a maximum cell concentration 3 d later when cultured with *T. weissflogii* than when cultured alone. However, this is not significant ($p = 0.49$). The *S. costatum* cell concentration decreased after a period of stationary growth (Day 27). Control cell concentrations decreased after Day 21. Furthermore, a repeated-measures ANOVA showed no significant difference for the cell density between co-cultures and controls at any time point ($p > 0.05$).

Significant effects on *Thalassiosira weissflogii* were observed during prolonged co-culturing with *Skeletonema costatum*. During the first 30 d of the experiment, the presence of *S. costatum* had no significant effect on the cell concentrations of *T. weissflogii* (Fig. 1B). *T. weissflogii* had a growth rate of 0.73 ± 0.04 and 0.76 ± 0.03 d^{-1} for the co-culture and the control, respectively, between Days 0 and 6, which is not significantly different ($p = 0.16$). Neither co-cultures nor control cultures reached significantly different maximum cell concentrations ($p = 0.44$), with 300 ± 48 × 10^3 cells ml^{-1} (Day 11) and 249 ± 93 × 10^3 cells ml^{-1} (Day 15), respectively. From Day 30 on, the cell concentrations between controls and co-cultures differed. While cell concentrations of *T. weissflogii* in the control cultures decreased ($\mu = -0.03$ d^{-1} between Days 30 and 38), an increase in cell density ($\mu = 0.01$ d^{-1} between Days 30 and 38) in the presence of *S. costatum* was observed. That increase resulted in a final cell density of *T. weissflogii* in the co-cultures that was more than 3 times greater than the final cell density of control cultures. A repeated-measures ANOVA calculation revealed a significant difference between co-cultures and controls from Day 30 on ($p < 0.001$ for all days).

Since inner cultures were not accessible to daily sampling, we determined their cell density only on the last day of the experiments (Fig. 2). Here we observed cell concentrations of *Skeletonema costatum* of 1890 ± 570 × 10^3 cells ml^{-1} for the co-cultures (n = 2) and of 890 ± 780 × 10^3 (n = 3) cells ml^{-1} for the control cultures (Fig. 2A). However, we were unable to test for significance because of the low number of replicates.

We found a significant difference in the cell densities of *Thalassiosira weissflogii* when comparing the inner cultures of the co-cultures and the control (Fig. 2B). While the cell concentration in the control was 152 ± 20 × 10^3 cells ml^{-1}, the cell concentration in the co-cultured *T. weissflogii* was significantly higher, with 245 ± 43 × 10^3 cells ml^{-1} ($p < 0.05$).

We also compared the final cell concentrations of the outer and inner cultures of *Skeletonema costatum* and *Thalassiosira weissflogii* control cultures. The outer culture and inner culture of *S. costatum* had concentrations of 2150 ± 1930 × 10^3 and 890 ± 780 × 10^3 cells ml^{-1}, respectively. This difference is not significant ($p = 0.18$). Similarly, the outer (152 ± 27 × 10^3 cells ml^{-1}) and inner cultures (152 ± 20 × 10^3 cells ml^{-1}) of *T. weissflogii* are not significantly different ($p = 0.49$).

Profiling of metabolites released by diatom cells

At the end of the experiment (Day 38), we extracted the culturing media for metabolic profiling of the extracellular metabolites based on UPLC/MS. The metabolic profiles were complex, indicating the presence of

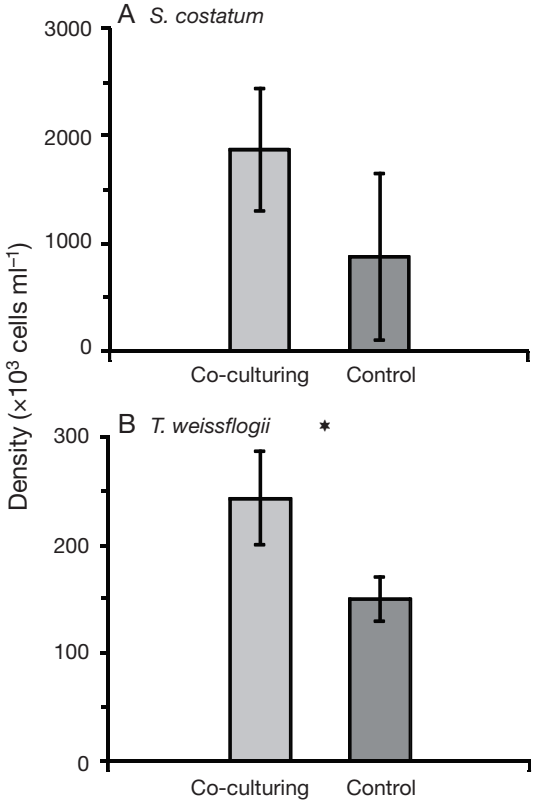

Fig. 2. *Skeletonema costatum* and *Thalassiosira weissflogii*. Cell densities on Day 38 after initiation of the co-culturing experiment. (A) Cell density of *S. costatum* in co-culture with *T. weissflogii* (light grey, n = 2) compared to a control in the presence of *S. costatum* (dark grey, n = 3) and (B) cell density of *T. weissflogii* in co-culture with *S. costatum* (light grey, n = 3) compared to a control in the presence of *T. weissflogii* (dark grey, n = 3). *Significant difference ($p < 0.05$). Values are mean ± SD (n = 3) or ranges (n = 2). The inner cultures of the co-culturing set-up were counted

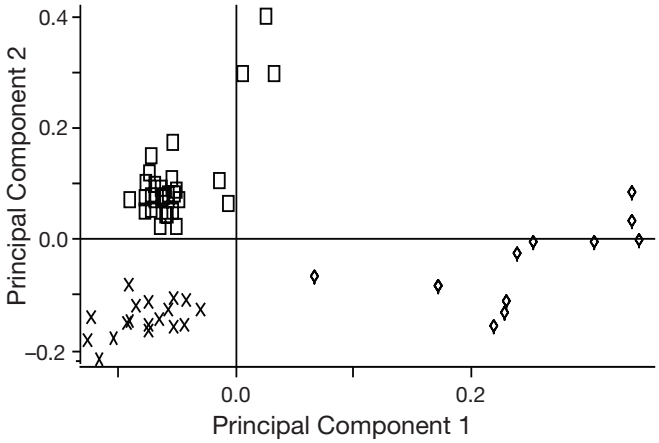

Fig. 3. *Skeletonema costatum* and *Thalassiosira weissflogii*. Resulting scores plot for principal components analysis of liquid chromatography/mass spectometry data of the released metabolites from *S. costatum* (◇), *T. weissflogii* (X) and both species in a co-culturing set-up (□), on Day 38. Distinct clustering was observed among all 3 inoculates, demonstrating differences in the patterns of released metabolites

numerous released metabolites in the medium. We therefore relied on automatic peak processing to form mass-retention time pairs that could be evaluated for differences in intensities of released metabolites. PCA was used to compare the intensity of all these pairs between all chromatograms. The differences between extracellular profiles were visualized using a scores plot of medium extracts. The resulting scores plot shows a separation into 3 groups (Fig. 3). The medium extracts of *Skeletonema costatum* and *Thalassiosira weissflogii* were separated by Principal Component 1, while the medium extracts of the co-cultures were separated from the *S. costatum* and *T. weissflogii* control extracts by Principal Component 2. The *m/z*-retention time pairs responsible for the group separation of the phases were extracted using the corresponding loadings plot, and only those not found in the blank measurements were further considered. Table 1 shows a selection of 8 substances that were exclusively found in the medium extract of *S. costatum*. Similarly, we found 1 substance with a mass-charge ratio of 241 (2 min) that was only detected in the medium extract of *T. weissflogii* control cultures, but not in the medium extract of the *S. costatum* control cultures or in the co-cultures (Table 1).

Table 1. Relative concentrations (given as a function of the intensity of the respective signals in single ion chromatograms normalized to the extracted volume) of 9 selected extracellular substances in the co-culture (n = 5), *Skeletonema costatum* control (n = 2) and *Thalassiosira weissflogii* control (n = 3). Values are mean ± SD (n = 3) or ranges (n = 2). *m/z*: mass to charge ratios of the respective compounds, the time refers to the retention time in the ultra-performance liquid chromatography; ND: not detectable, signal-to-noise ratio (S/N) < 3; traces: S/N < 10; quantified: S/N > 10

Parameter	Co-culture	*S. costatum*	*T. weissflogii*
m/z 121 at 1.45 min	Traces	38.1 ± 2.1	Traces
m/z 239 at 2.86 min	ND	30.2 ± 7.3	ND
m/z 241 at 1.99 min	ND / traces	ND	2.2 ± 0.2
m/z 241 at 4.93 min	ND	9.3 ± 4.0	ND
m/z 269 at 5.73 min	ND	6.6 ± 1.6	ND
m/z 277 at 3.85 min	ND	7.1 ± 4.7	ND
m/z 301 at 2.39 min	ND	11.0 ± 2.2	ND
m/z 309 at 4.49 min	ND	12.8 ± 4.3	ND
m/z 351 at 3.00 min	ND	42.7 ± 13.8	ND

Profiling of cellular metabolites

We used the same methods for the analysis of the metabolic profile of intracellular compounds. Again, the number of detected metabolites led us to use automatic data extraction and PCA to detect the metabolites that are produced in different amounts in cells of co-culturing and control set-ups. The resulting scores plot shows separation between the *Skeletonema costatum* cells in co-cultures and *S. costatum* cells in controls (not shown). This analysis revealed differences between metabolites present in control *S. costatum* cells and co-cultured *S. costatum* cells. Both up- and down-regulation of metabolites in response to co-culturing was observed, and a selection of metabolites is given in Table 2. One example is the significantly higher normalized peak area of a substance with 481 *m/z* at 4.4 min in co-cultured *S. costatum* cells compared to the control cells ($p < 0.001$) (Table 2). In *Thalassiosira weissflogii* cells we were also able to detect significantly different concentrations ($p < 0.05$) of several substances (e.g. *m/z* 527 at 4.2 min) between the cell extracts of the co-cultures and the control cultures (Table 3). Here the concentration of all listed compounds was lower in the co-cultures than in the controls.

Table 2. Selection of 5 intracellular substances that show significant differences between *Skeletonema costatum* in the co-culture (n = 5) and in the control (n = 4, 1 replicate discarded due to contamination). Values are mean ± SD relative concentrations (given as a function of the intensity of the respective signals in single ion chromatograms normalized to the extracted amount of cells). *m/z*: mass to charge ratios of the respective compounds, the time refers to the retention time in the ultra-performance liquid chromatography; **p < 0.01; ***p < 0.001; NS: no significance testable; ND: not detectable, S/N < 3; quantified:S/N > 10

Parameter	Co-culture	Control	Significance
m/z 481 at 4.40 min	1132.0 ± 215.9	353.5 ± 140.2	***
m/z 481 at 4.51 min	261.0 ± 42.4	120.4 ± 71.1	**
m/z 547 at 3.52 min	25.0 ± 4.3	44.4 ± 8.3	**
m/z 654 at 4.83 min	89.5 ± 26.7	24.1 ± 10.4	**
m/z 685 at 6.61 min	95.7 ± 66.4	ND	NS

Table 3. Selection of 5 intracellular substances that show significant differences between *Thalassiosira weissflogii* in the co-culture (n = 5, 1 replicate discarded due to contamination) and in the control (n = 6). Values are mean ± SD relative concentrations (given as a function of the intensity of the respective signals in single ion chromatograms normalized to the extracted amount of cells). *p < 0.05; NS: no significance testable; ND: not detectable, S/N < 3; traces: S/N < 10; quantified: S/N > 10

Parameter	Co-culture	Control	Significance
m/z 509 at 5.18 min	Traces	326.5 ± 225.0	NS
m/z 527 at 4.22 min	176.5 ± 122.2	847.7 ± 378.0[a]	*
m/z 555 at 4.78 min	72.7 ± 41.6	438.4 ± 335.9	*
m/z 581 at 5.00 min	Traces	342.1 ± 240.1	NS
m/z 661 at 6.29 min	171.3 ± 29.3	492.7 ± 229.0	*

[a]One of the 6 replicates was identified as an outlier (Grubbs method) and deleted from the calculations

DISCUSSION

In the present study, we addressed the role of chemical exudates and potential chemical signals on diatom cultures grown under a regime that prevented cell contact but allowed cultures to exchange chemicals. This type of co-culturing experiment is often used in plankton ecology to evaluate the effects of diffusible signals and nutrients (McVeigh & Brown 1954, Jensen et al. 1972, Vardi et al. 2002, Yamasaki et al. 2007). Using this experimental design allowed us to exclude potential effects of cell contact (previously described by Uchida et al. 1995) and to observe only interactions mediated by metabolites and nutrients diffusing through the dialysis membrane. Because the experiment was conducted with larger volumes of culture medium than described by Yamasaki et al. (2007), we were able to sample enough medium to create a comprehensive profile of the metabolites released by diatoms and of the metabolic changes within the cells upon co-culture using LC/MS techniques. In contrast to numerous previous experiments, where culture filtrates from monocultures or partially purified compounds were used to test for allelopathy (e.g. Suikkanen et al. 2005), our method allowed us not only to monitor the action of exuded stable compounds but also to cover unstable compounds. In addition, interactions such as the induced production of allelopathic compounds in the presence of the competitor (e.g. Selander et al. 2006) or indirectly mediated chemical interactions via nutrient effects or secondary interactions involving microorganisms as an additional partner, can be observed. Control experiments, in which the same alga was grown within and outside the dialysis bag, show that there are no significant differences in cell counts between compartments. This suggests that growth conditions are independent of the cul-

ture compartment, and the effects of shading or poor aeration (Pouvreau et al. 2007) that could potentially influence the outcome of the co-culturing can be excluded. Although our culture conditions do not represent the natural planktonic environment due to increased cell counts and artificially enriched nutrient conditions, we can conclude that there is a general possibility of an allelopathic response of diatoms to the presence of co-occurring species (Legrand et al. 2003).

Co-culturing *Skeletonema costatum* and *Thalassiosira weissflogii* had no effect on the growth of *S. costatum*. When cultured in the outer chamber *S. costatum* exhibited similar growth rates independent of the presence of *T. weissflogii* or *S. costatum* in the inner chamber (Fig. 1A). An exponential growth phase, with growth rates comparable to those detected in previous studies using these species (Montagnes & Franklin 2001, Vidoudez & Pohnert 2008), was followed by a short stationary phase and a declining phase in the co-cultures as well as in the control (Fig. 1A). This illustrates that low molecular weight exudates of *T. weissflogii* diffusing through the dialysis membrane do not influence the growth of *S. costatum*. In addition, competition for nutrients, which also diffuse through the membrane, does not influence the performance of *S. costatum*. These results were verified by evaluating cell concentrations in the inner chamber after the end of other co-culturing experiments. No differences in cell concentration of *S. costatum* were observed in controls or in the presence of *T. weissflogii* (Fig. 2A). Such a lack of allelopathic interactions was also found in other co-culturing experiments (e.g. Talling 1957) and under natural conditions, where, e.g. *Heterosigma akashiwo* dynamics during a spring bloom were under nutrient but not allelopathic control (Shikata et al. 2008). In contrast, *T. weissflogii* was affected by compounds exuded from the *S. costatum* culture chamber. Initially, both control cultures and co-cultures of *T. weissflogii* had similar growth rates. However, after a period of stationary growth, the cell density of the co-cultured *T. weissflogii* significantly increased. These cultures eventually reached a cell concentration more than 3 times greater than that in the controls (Fig. 1B). Again, these observations were confirmed because cell concentrations of *T. weissflogii* were significantly higher in the chambers of the co-cultures than in those of the controls (Fig. 2B). Possible explanations for the stimulated growth of *T. weissflogii* include a positive allelopathic effect of one or more compounds that were released by *S. costatum* cultures only during the declining phase. Similar biphasic growth curves can also be observed in cultures of bacteria where switches of cell physiology dependent on quorum sensing might result in increased performance in the medium (van Houdt et al. 2007). That a comparable process depending on signaling in-between individual cells of *T. weissflogii* is responsible for the observed biphasic growth (Fig. 1B) is unlikely, since control experiments with this species alone and previous culturing did not indicate any sign of positive feedback within cultures of this alga. Although care was taken to avoid microbial contamination and cultures were discarded if contaminated, it cannot be fully excluded that other mediating organisms such as an associated bacteria may also be involved in the observed chemical interaction.

Most of the studies concerning allelopathic interactions have focused on negative allelopathic effects (e.g. Gross 2003, Kubanek et al. 2005). Yamasaki et al. (2007) found, for example, that the first species to dominate in a co-culturing experiment with *Skeletonema costatum* and *Heterosigma akashiwo* was generally able to reduce the growth of the other co-cultured species. In contrast, there are only a few examples of positive allelopathic interactions. Recent studies have reported that cyanobacterial filtrates stimulate the growth of a wide range of organisms in a natural phytoplankton community, such as chlorophytes, dinoflagellates and other cyanobacteria (Suikkanen et al. 2005). Bacteria are also known to promote algal growth (Grossart & Simon 2007). Additionally, low concentrations of *Prymnesium parvulum* toxins have a positive effect on the growth rate of other algae (Graneli & Pavia 2006). A stimulatory effect was also observed for *S. costatum* in interactions with the macroalga *Ulva pertusa* (Nan et al. 2004). Nan et al. (2004) observed a growth stimulation of *U. pertusa* when grown in the presence of this diatom. It would be interesting to investigate in future experiments whether *Skeletonema* spp. generally promote the growth of other marine micro- and macroalgae and if this effect is limited to the late growth phases of these algae.

Another possible explanation for the increase of *Thalassiosira weissflogii* cell counts is that *Skeletonema costatum* in the declining phase might liberate nutrients that are exploited for a delayed second increase in cell density (Fig. 1B). Discriminating between allelopathic and nutrient effects is a general problem in allelopathy research (Rengefors & Legrand 2001), and a final answer can only be found through bioassay-guided structure elucidation of the active components. Furthermore, the possibility of the involvement of bacteria that could influence diatom cell counts cannot be fully excluded, even though the utmost care was taken to exclude bacteria and constant monitoring confirmed a low level of contamination in all cultures.

We reasoned that a metabolic profiling approach to monitor the differences in cellular metabolites, as well as those in the medium, might provide insight into the

nature of chemically mediated interactions between diatom species. Metabolic profiling is a well-established method in plant biology for monitoring induced changes in the metabolism of plants due to biotic interactions (von Roepenack-Lahaye et al. 2004). In contrast, only a few attempts have been made to profile the endo- (Allen et al. 2008) or exo-metabolome of diatoms (Barofsky et al. 2009). Direct comparisons of metabolites released by *Skeletonema costatum* and *Thalassiosira weissflogii* using MS techniques and data evaluation based on multivariate statistical analysis support the idea of a species-specific extracellular metabolic profile of both diatoms (Fig. 3, Table 1). The method introduced here allows a rapid separation of metabolites on a reversed-phase column; coupling to a mass spectrometer then gives a profile of numerous eluted metabolites that can be classified based on their retention times and characteristic mass to charge ratios. Using this approach >100 metabolites can be detected at a given time in the culture medium. Thus, multivariate statistical methods had to be used to unravel the quantitative variations of exuded metabolites (Barofsky et al. 2009). We found substances that were exclusively present either in *S. costatum* or in *T. weissflogii* control cultures. Interestingly, none of these species-specific substances were present in the co-culture, indicating an obvious interaction involving released metabolites (Table 1). Inhibition of either biosynthesis or of excretion of these compounds by the competitor might cause the observed change in metabolic profiles. Alternatively, an active uptake or transformation of these particular compounds by the co-cultured diatoms might cause the observed effect. Heterotrophic utilization of organic compounds by diatoms has been discussed as an important survival strategy of diatoms under adverse conditions (Tuchman et al. 2006). Such heterotrophic uptake might indeed be responsible for the promoted *T. weissflogii* growth in our co-culturing experiments. An active uptake of specific phytoplankton-derived metabolites such as dimethylsulfoniopropionate (DMSP) by other phytoplankton species has recently been shown (Vila-Costa et al. 2006). Our methods, based on LC/MS measurements, do not provide any structural information beside the retention times in reversed-phase chromatography and the mass to charge ratios of the $[M-H]^-$ ions. Available databases established for electrospray mass spectrometry metabolomics of higher plants could not be successfully applied to diatom metabolites. Nevertheless, this basic mass spectrometric information allows us to exclude the possibility that the observed metabolites are amino acids or extracellular carbohydrates, which make up the bulk of compounds released from *S. costatum* (Granum et al. 2002). We also found that DMSP or other betains were not among the metabolites that were present in significantly different concentrations between control cultures and co-cultures. We suggest that further structural elucidation of the observed metabolites and direct tests of their biological activity might provide a shortcut for the identification of an active principle when compared to traditional bioassay-guided structure elucidation (Pohnert et al. 2007)

Although cell density of *Skeletonema costatum* was not affected by the co-cultured *Thalassiosira weissflogii*, we still observed a physiological response to the presence of the competitor. This is indicated by a change in the intracellular metabolic profile observed after PCA of data derived from cellular extracts (prominent changes illustrated in Table 2). The change in intracellular metabolic profile indicates that *S. costatum* is capable of perceiving the presence of a competitor by chemical signals that diffuse through the dialysis membrane or of reacting to the altered nutrient availability caused by the co-cultivation. Other compounds, such as those shown in Table 3, from the intracellular extracts of *T. weissflogii* are downregulated in the co-culture. Physiological responses to changing conditions that are reflected in a change of the metabolic profile are well documented in the literature (Ribalet et al. 2007, Van Mooy et al. 2009). The extent to which these changes have a direct impact on ecological interactions is by no means clear, but observation of metabolic fluctuations will enable a targeted screening of biochemical and ecological responses to specific metabolites, as has been successfully demonstrated in the case of dimethyl sulfide and polyunsaturated aldehydes (Pohnert 2005, Steinke et al. 2006).

Despite being conducted in the laboratory under conditions far from those in field situations, our experiments provide evidence that diatoms have the potential to interact with each other via released metabolites. These interactions may result in a complex cross-talk between diatom species that might even be overlaid by contributions from the bacterial community (Grossart & Simon 2007). Our experiments are consistent with other studies showing that exudates of one phytoplankton species can influence the growth of another one (Fistarol et al. 2004, Kubanek et al. 2005, Yamasaki et al. 2007, Wang & Tang 2008). We link altered growth to a modified chemical profile of both the released and intracellular metabolites, thereby showing that diffusible compounds are involved in an interaction not only reflected by changes in cell concentrations but also by changes in the physiology of the cells. This is very much in line with previous observations of induced responses of phytoplankton species to external stimuli (e.g. Selander et al. 2006). Our results show the importance of tests for allelopathy, which allow for direct interactions, as compared with

established approaches, which primarily test the activity of the culture medium of isolated species. It is interesting to note that we observed these types of chemically mediated interactions between diatoms that co-occur in the same habitat.

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4.6 Manuscript F

Daily bursts of biogenic cyanogen bromide (BrCN) control biofilm formation around a marine benthic diatom

Bart Vanelslander, Carsten Paul, Jan Grueneberg, Emily K. Prince, Jeroen Gillard, Koen Sabbe, Georg Pohnert and Wim Vyvermann

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Daily bursts of biogenic cyanogen bromide (BrCN) control biofilm formation around a marine benthic diatom

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The spatial organization of biofilms is strongly regulated by chemical cues released by settling organisms. However, the exact nature of these interactions and the repertoire of chemical cues and signals that micro-organisms produce and exude in response to the presence of competitors remain largely unexplored. Biofilms dominated by microalgae often show remarkable, yet unexplained fine-scale patchy variation in species composition. Because this occurs even in absence of abiotic heterogeneity, antagonistic interactions might play a key role. Here we show that a marine benthic diatom produces chemical cues that cause chloroplast bleaching, a reduced photosynthetic efficiency, growth inhibition and massive cell death in naturally co-occurring competing microalgae. Using headspace solid phase microextraction (HS-SPME)-GC-MS, we demonstrate that this diatom exudes a diverse mixture of volatile iodinated and brominated metabolites including the natural product cyanogen bromide (BrCN), which exhibits pronounced allelopathic activity. Toxin production is light-dependent with a short BrCN burst after sunrise. BrCN acts as a short-term signal, leading to daily "cleaning" events around the algae. We show that allelopathic effects are H_2O_2 dependent and link BrCN production to haloperoxidase activity. This strategy is a highly effective means of biofilm control and may provide an explanation for the poorly understood role of volatile halocarbons from marine algae, which contribute significantly to the atmospheric halocarbon budget.

allelopathy | chemical ecology | marine ecology | signal molecule

Biofilm formation in marine habitats is a rapid and ubiquitous process and most submerged surfaces, natural or man-made, are covered with complex microbial communities. Intense efforts are made to control biofilm formation on industrial surfaces such as ship hulls because this biofouling can result in severe economic loss (1). Among the early settlers, microalgae play a key role in the biofilm development and diatoms, especially, are able to settle on even the most fouling resistant surfaces (2). In this context, it is interesting to observe that certain microalgae can obviously control their microenvironment because the patchy variation in species composition observed around these algae (3–5) cannot be explained by abiotic heterogeneity or bioturbation by grazers (4). This spatial organization of species is characterized by complementary distribution patterns and negative correlation of species densities (4). Allelopathic interactions have been suggested as a possible explanation for such observed patchiness (4). Because biofilms are composed of densely packed cells embedded within a matrix of exuded polymeric compounds, secondary metabolites produced by any cell can efficiently target its neighbors rather than diffusing into the surrounding water column (6).

Studies that focused on interspecific interactions between biofilm-forming diatoms revealed that synergistic (7) and antagonistic (8) interactions are common and can have a strong influence on biofilm performance (7). The underlying chemistry of these interactions is unknown, but several modes of action of allelochemicals on susceptible target cells have been demonstrated, including the inhibition of photosynthesis (9, 10), membrane damage (11), inhibition of enzymes (12), reduced motility (13) and oxidative damage (10, 11, 14). In this study we selected the common biofilm forming diatom *Nitzschia cf pellucida* due to the high allelopathic activity observed in preliminary bioassays. Several *Nitzschia* species are known for their production of volatile halocarbons (15–17) and a first screening revealed that the selected alga is also a rich source of such compounds. The formation of low molecular weight halogenated metabolites is widely distributed in macro- and microalgae which contribute significantly to the atmospheric halocarbon budget (18–20). Local maxima of volatile halogenated metabolites are often observed in coastal regions but the function of these metabolites is poorly understood. Here we directly link the halocarbon chemistry of microalgae to an allelopathic activity by establishing that the natural product cyanogen bromide (BrCN) is highly inhibitory against competitors. This metabolite is released during a short period after the onset of light in quantities sufficient to kill or inhibit the growth of competing microalgae.

Results

Allelopathic Effects of Nitzschia *cf pellucida*. Experiments with cocultures of biofilm forming diatom species revealed that the diatom *Nitzschia cf pellucida* (Fig. 1*A*) exerted strong allelopathic effects. We observed that the naturally co-occurring diatoms *Navicula arenaria* (Fig. 1*D*), *Cylindrotheca closterium* and *Entomoneis paludosa* (Fig. 1*B*) were inhibited and killed after 24-h exposure to relatively low cell densities (7,000–10,000 cells mL^{-1}) of *N. cf pellucida*. Another diatom, *Stauronella* sp. (Fig. 1*C*) was more resistant but was killed within 24 h when exposed to circa 80,000 cells mL^{-1} of *N. cf pellucida*. The mechanism of inhibition appeared to be the same for all species investigated: exposure to *N. cf pellucida* cells resulted in loss of pigmentation, shriveling of chloroplasts and finally cell death (Fig. 1).

Application of nutrient-enriched spent *N. cf pellucida* culture medium on cells of different diatom species induced a collapse of photosynthetic efficiency (Fig. S1). Microscopic investigations showed a massive cell death within 10 h for *E. paludosa* (Fig. 2) and *C. closterium*. The diatom *Stauronella* sp. was again more resistant and was able to maintain its photosynthetic efficiency, but its growth was suppressed for 2 d (measured as the initial

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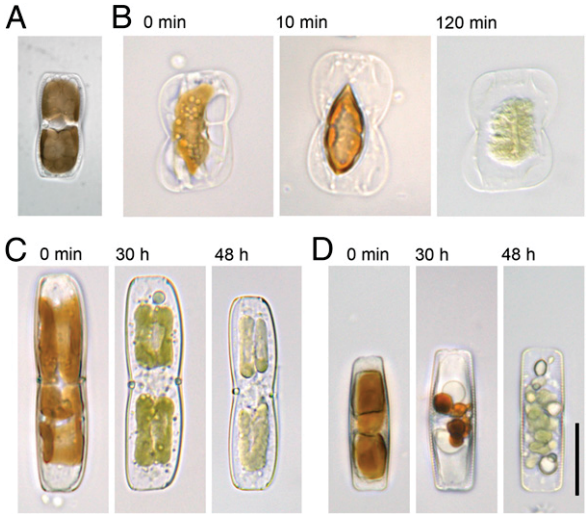

Fig. 1. Antagonistic effects of *N. cf pellucida* on a naturally co-occurring diatom species. (*A*) *N. cf pellucida*. (*B*) *E. paludosa*. Healthy cell (*Left*), cell after 10-min exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and dead cell after 120-min exposure (*Right*) are shown. (*C*) *Stauronella* sp. Healthy cell (*Left*), cell after 30-h exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and cell after 48-h exposure (*Right*) are shown. (*D*) *Navicula arenaria*. Healthy cell (*Left*), cell after 30-h exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and cell after 48-h exposure (*Right*) are shown. (Scale bar: 20 μm.)

fluorescence, F_0, a proxy for algal biomass) after which cell growth at rates similar to the control was restored.

An additional response was detected in *E. paludosa*: *N. cf pellucida*-spent medium induced a loss of motility and cells displayed a strong condensation of the protoplast within 10 min after exposure (Fig. 1*B*). As this species is highly sensitive toward *N. cf pellucida*, we selected it as a model for further bioassay experiments.

The allelochemical potential of *N. cf pellucida* varied dramatically with time of day. Using a bioassay with *E. paludosa* and cell free spent medium from *N. cf pellucida* cultures sampled in time intervals, we showed that allelopathic activity is highest between 2 and 4 h after daybreak when application of spent medium resulted in nearly complete eradication of *E. paludosa* cells (Fig. 2). Six hours after the onset of light, the activity diminished and spent medium had nearly no effect. The proportion of healthy cells increased to nearly 100% toward the end of the night. This striking pattern suggests that labile, reactive or volatile metabolites are responsible for the allelopathic activity. Further studies therefore focused on the characterization of metabolites present 3 h after the onset of light when toxicity was maximal.

Extraction and Structure Elucidation of *N. cf pellucida* Allelochemicals. We performed GC-MS analyses of ethylacetate extracts (EAe) of cell-free spent culture medium and of volatile organic compounds collected by headspace solid phase microextraction (HS-SPME) and revealed the occurrence of 18 different brominated and iodinated volatiles in *N. cf pellucida* cultures. These compounds were a mixture of methylhalogens (CH_3Br, CH_3I), dihalomethanes (CH_2Br_2, CH_2I_2, CH_2ClI*, CH_2BrI*) as well as trihalomethanes ($CHBr_3$, CHI_3, $CHBr_2I$*, $CHBr_2Cl$*, $CHClI_2$*) (EAe and SPME), 1-iodopropane (EAe), di and trihalogenated acetaldehydes (dibromoacetaldehyde*, bromochloroacetaldehyde*, chlorodibromoacetaldehyde*, EAe), and 1,2-dichloroethane (EAe). We identified these compounds based on their retention time and mass spectra using GC-MS and, if not indicated otherwise, compared with commercially available or synthetic (21) standards (compounds marked with an asterisk were identified only by mass spectrometry and retention time). In addition to these metabolites that are known from marine algae (18), we could also detect the volatile and highly toxic cyanogen bromide, BrCN using SPME. A commercially available synthetic standard of this natural product provided material for the confirmation of the structure, quantification and a dose–response assessment in bioassays with *E. paludosa*.

To check if these halocarbons caused the observed allelopathic interactions, we assessed the toxicity of different concentrations of the nine most abundant halogenated compounds and sodium cyanide (NaCN) on the diatom *E. paludosa* (Fig. 3). In these bioassays, cyanogen bromide clearly turned out to be the most toxic halogenated metabolite produced by the algae. The minimal lethal concentration was 2 μM (causing 96% of the *E. paludosa* cells to die within 3 h). BrCN is also by far more potent compared with NaCN, which is active only in concentrations above 40 μM. BrCN concentration in the *Nitzschia* cultures (220,000 cells mL^{-1}) determined by SPME GC-MS with $CDCl_3$ as an internal standard strongly varied over time and was highest 2–4 h after daybreak (up to 7.46 ± 1.77 μM BrCN) (Fig. 2). To ensure that BrCN is also causing the observed allelopathic effects at low *N. cf pellucida* cell densities in the cocultures described above, we measured local concentrations of BrCN in cultures of lower cell densities. Therefore, we sampled small aliquots (2.5 mL) of culture medium just above the diatoms growing at the bottom of the culture vial. Fig. S2 shows that cultures with cells densities even below 20,000 cells mL^{-1} can locally produce cell inhibitory concentrations of BrCN. The concentration of BrCN can thus fully explain the lethal effect of the spent *N. cf pellucida* medium.

No other tested halogenated metabolites were active in the concentration range reached in cultures. Cells of *N. cf pellucida* are more resistant to BrCN and could cope with concentrations

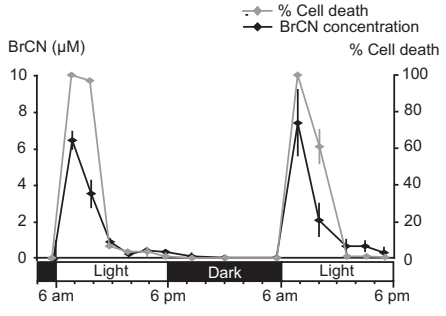

Fig. 2. Time-dependent production of BrCN in *N. cf pellucida* cultures (180,000 cells mL^{-1}) throughout the day. Black line shows BrCN concentrations (μM). Gray line shows the effect of *N. cf pellucida* cell-free spent medium harvested at different time points on *E. paludosa* cells. We measured the effects 10 h after exposure to *N. cf pellucida* filtrate (means ± SD, $n = 3$).

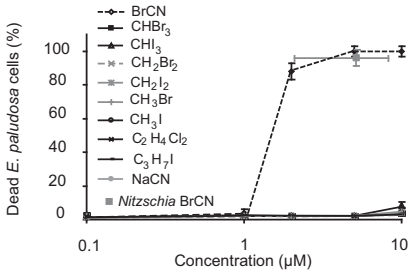

Fig. 3. Dose–response curve for *E. paludosa* for nine halogenated compounds detected in *N. cf pellucida* cultures. The gray square represents the average BrCN concentration in *N. cf pellucida* cultures (3 h after onset of light) and the average response of *E. paludosa* to the *N. cf pellucida* filtrates (means ± SD, $n = 4$).

of up to 16 μM with minor or no growth reduction, and only displayed a reduced growth and photosynthetic efficiency at 32 μM BrCN (Fig. S3).

Light-Dependent BrCN Production and Stability. We show that BrCN production is light dependent: Changing the light regime from a standard 12 h:12 h light/dark rhythm to an extended darkness of 18 h prevented BrCN production (Fig. S4). BrCN production levels could be restored to 76.68 ± 17.38% if cells were exposed to light after 15 h of darkness (instead of 12 h). After a prolonged darkness of 18-h illumination could not trigger BrCN production. Because a pronounced reduction of the BrCN concentration was observed 6 h after the onset of illumination we verified if an abiotic degradation of this metabolite is causing this process. If BrCN was incubated in medium under conditions identical to the culturing we did only observe minor degradation over time. Even after 6 h, more than 80% of the initial BrCN concentration was recovered, excluding abiotic degradation (Fig. S5).

Biotic Interactions and Bacteriacidal Activity. Competing co-occurring species did not affect the BrCN production (Fig. S6). If cells of *N. arenaria* or *E. paludosa* at cell densities of 70,000–80,000 cells mL^{-1} were added to *N. cf pellucida* cultures of 180,000 cells mL^{-1}, no differences in BrCN production were observed [*N. arenaria*: Student's *t* test ($P = 0.9157$), *E. paludosa:* Student's *t* test ($P = 0.4076$)].

In contrast to the pronounced activity against algae, no inhibitory activity is found against bacteria. Agar diffusion assays of up to 64 μM BrCN concentrations and *N. cf pellucida*-spent medium filtrate against six bacterial strains isolated from estuarine intertidal mudflats showed no inhibition zones after up to 8 d of incubation.

BrCN Biosynthesis. To elucidate the source of halogens for the BrCN production we deprived *N. cf pellucida* cultures of bromide and iodide. Omitting these halogens from the culture medium almost completely eliminated the production of BrCN. When we altered the I^-:Br^- ratio in the culture medium from ~1:2,000 in natural seawater to 1:16, production of brominated hydrocarbons ceased and was replaced by the formation of iodinated compounds. Likewise, BrCN production was reduced at the expense of ICN formation, a second natural product. Allelopathic assays revealed that the Br^- and I^- deprived cultures lacked allelochemical activity (Fig. S7). In contrast, spent medium of *N. cf pellucida* grown at high I:Br ratios was even more toxic than spent medium derived from natural seawater and caused massive cell death of *E. paludosa* cells within 45 min after application (Fig. S7).

Because BrCN and cyanogen iodide (ICN) are not known as natural products, we aimed to verify their biogenic origin by addressing their biosynthesis. Therefore, we incubated *N. cf pellucida* cells in culture medium enriched with ^{13}C labeled bicarbonate for 5 d. Analysis of the isotope distribution of the cyanides provided proof for a biosynthetic origin. BrCN and ICN exhibited a ^{12}C: ^{13}C ratio of 1:1.13 and 1:1.18 in BrCN and ICN, respectively, whereas BrCN from cells in natural seawater exhibited the natural ratio of 1: 0.012 (Fig. 4). An axenic *N. cf pellucida* culture enriched with ^{13}C-labeled bicarbonate exhibited a ^{12}C:^{13}C ratio of 1:0.93. GC/MS analysis revealed that isotope enrichment after ^{13}C bicarbonate treatment was also observed for other metabolites.

Biosynthetic considerations of BrCN or ICN suggested the presence of an oxidized halogen species. Known enzymes that could be involved are H_2O_2 consuming haloperoxidases (22). To test whether BrCN production is linked with cellular H_2O_2 production, we assessed the effect of the H_2O_2-decomposing enzyme catalase incubated with *N. cf pellucida* cultures. BrCN production was significantly reduced in the presence of catalase (70.7 ± 13.4% reduction, $P = 0.0002$).

In a second assay for haloperoxidase activity, we added phenol red (phenolsulfonphthalein) at 36 μM to *N. cf pellucida* cultures and spectrophotometrically checked for conversion into brominated phenol blue (3′,3′′,5′,5′′-tetrabromophenolsulfonphthalein). Halogenation of phenol red occurred shortly after daybreak and phenol red concentration decreased to 26.38 ± 2.26 μM (mean ± SD, $n = 5$). Bromophenol blue (8.60 ± 1.86 μM, $n = 5$) was formed within 3 h after daybreak.

Discussion

In this study, we show that the benthic diatom *N. cf pellucida* produces allelochemicals that cause chloroplast bleaching, a reduced photosynthetic efficiency, growth inhibition and massive cell death in naturally co-occurring competing microalgae. The allelopathic compounds are effective even at low cell densities: 8,000 *N. cf pellucida* cells mL^{-1} were sufficient to suppress competitors. Local concentrations of BrCN in dilute cultures with cell counts below 20,000 cells mL^{-1} were sufficient to cause the effects in bioassays. Given the rather slow diffusion processes in water over a biofilm, the local concentrations around *N. cf pellucida* will most likely be even higher. This result demonstrates that the concentration required to trigger activity in bioassays is found in vicinity of the producing cells.

Our work adds to a very limited number of studies demonstrating the molecular basis for chemically mediated interactions between biofilm forming microalgae. Using a combination of chemical analyses and bioassays, we identified the highly reactive metabolite BrCN as the causative agent of the observed activity. This compound has not been previously detected as a natural product. BrCN is highly toxic and has been applied as fumigant and pesticide and was even briefly used as a chemical weapon during World War I (23). BrCN is currently used to fragment proteins by hydrolyzing peptide bonds at the C terminus of methionine residues (24). In addition to this effective metabolite, *N. cf pellucida* also produces a diverse mixture of iodo- and bromocarbons with comparatively lower allelopathic properties. The halomethanes, halogenated acetaldehydes, and iodopropane detected have been reported previously from micro- and macroalgae (22, 25, 26). Cyanogen bromide is hydrolyzed by water to release hydrogen cyanide (HCN), but we can exclude that the allelopathic effects were caused by cyanide alone because hydrolysis is comparably slow and because NaCN caused only minor effects when applied to our bioassay species *E. paludosa*.

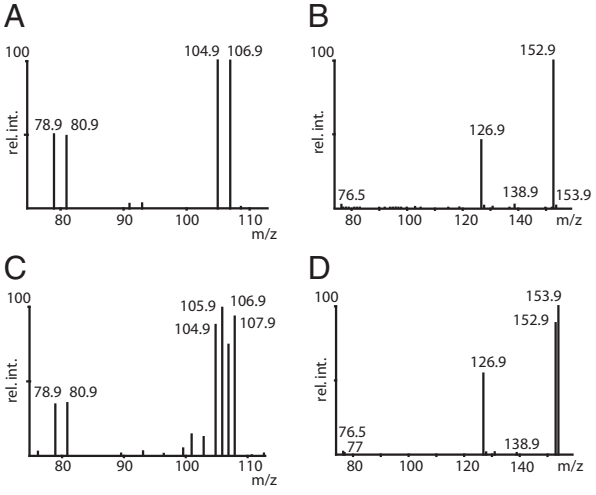

Fig. 4. GC-EI-MS spectra of BrCN (*A* and *C*) and ICN (*B* and *D*). *A* and *B* were harvested from *N. cf pellucida* cultures grown in natural seawater based culture medium. *C* and *D* were from cultures grown in $NaH^{13}CO_3$ enriched medium.

Concentrations of 40 μM NaCN (20 times higher than the active BrCN concentration) caused no short-term (8 h) effect on *E. paludosa* cells.

We observed that BrCN targets competing algae with different efficiency. Whereas *E. paludosa* and *C. closterium* are dramatically affected by this metabolite, leading to reduced photosynthetic activity and growth rate (Fig. S1), *Stauronella* sp. is more resistant. As the producer *N. cf pellucida* itself, this alga shows resistance against the metabolite using hitherto unidentified mechanisms. BrCN production can be seen as a mechanism that is not requiring an inducing partner because its release is independent of the presence of competing diatoms. In contrast to the pronounced effects on certain diatoms, BrCN is virtually inactive against bacteria. Even if incubated over elevated concentrations of 64 μM this metabolite did not affect the growth and morphology of six bacterial strains from intertidal mudflats. BrCN can thus be considered as a metabolite targeted specifically against competing algae.

Because BrCN is not a common natural product, we verified whether it might result from abiotic transformations in the medium or whether it is a true natural product biosynthesized by the alga. ^{13}C-labeled bicarbonate was incorporated into BrCN and ICN in high yields, confirming unambiguously a biogenic origin. Given the degree of labeling of all extracted products after administration of labeled bicarbonate it can be concluded that the CO_2 from bicarbonate is fueling the photosynthesis and the ongoing metabolism leads to a labeling of all metabolites in the algae. The bicarbonate is thus most likely not directly transformed to CN^- but rather via complex metabolic processes. The cyanide source for BrCN is still unclear. Several plants are known to produce cyanides through cyanogenic glucosides (27), but these compounds have never been detected in algae. The green algae *Chlorella* is known to produce cyanides from aromatic amino acids via an amino acid oxidase enzyme system (28), but such mechanism has not been described for diatoms. CN^- production is known from a broad range of organisms, including bacteria, fungi, insects, algae, and plants, as a means to avoid predation (27, 29), but apparently *N. cf pellucida* has found a means to release even more active metabolites by a simple modification.

Application of the H_2O_2-decomposing enzyme catalase significantly reduced the BrCN production in the *N. cf pellucida* cultures, which suggests that haloperoxidase (HPO) activity (22) is involved in the BrCN synthesis. Furthermore, we observed a preference for iodide over bromide incorporation, which corresponds with the halide selectivity for haloperoxidases (30). Also, the absence of ClCN in Br- and I-depleted cultures matches with the halide preference of algal HPO. Lastly, the conversion of phenol red into brominated phenol blue in the *Nitzschia* cultures points to the involvement of haloperoxidase enzymes. These enzymes catalyze the oxidation of halide ions to hypohalous acid by H_2O_2. Hypohalous acid (or a similar oxidized intermediate) can then react with organic substrates that are susceptible to electrophilic halogenation (22). We cannot, however, conclude whether biogenic CN^- (or equivalent) reacts with "Br^+" from the haloperoxidase reaction or if the transformation of halomethanes or other precursors to BrCN is involved in the biosynthetic pathway.

Haloperoxidase enzymes are distributed in marine organisms including Rhodophyta, Phaeophyta, Chlorophyta (18), and Bacillariophyta (16). An important function of HPO is to scavenge harmful H_2O_2 produced during photosynthesis, photorespiration, and other metabolic processes (31). It has also been suggested that HPO of marine organisms are involved in defense mechanisms such as the mediation and prevention of bacterial biofilm formation, but evidence for the involved metabolites was not given till now (32, 33). Here we provide a link between HPO activity and allelopathic potential supporting an ecological role for this enzyme in diatoms.

It is interesting to note that we only detected BrCN during the morning hours in the culture and only if we quickly extracted this reactive metabolite. Given the methodological difficulties in detecting highly reactive metabolites, it would be worth verifying the potential of other microalgae to produce this reactive metabolite, which would most likely not have been picked up in other determinations of halogenated volatiles. The mechanism introduced here could thus have a broader occurrence.

The production of BrCN only within few hours after daybreak coincides with the time span of H_2O_2 production in algal cells due to the Mehler reaction (34). It is clearly light dependent: Prolongation of the dark period also leads to a delayed formation of BrCN. If the dark period is prolonged for 3 h, BrCN production can still be observed. If, however the dark period is prolonged for 6 or more hours, light is not sufficient to elicit BrCN release. Therefore, we conclude that BrCN production is light dependent, but is only possible during a certain timeframe within the day when other physiological prerequirements are in place. Three alternative but not mutually exclusive mechanisms may underlie this phenomenon. First, the observed patterns could be explained by the fact that diatoms exposed to prolonged darkness show an increase in the photoprotective xanthophyll pigment diatoxanthin and higher non-photochemical quenching (NPQ) (35). NPQ dissipates excess absorbed light energy and thereby diminishes the energy arriving at the photosystems and thus less potential for H_2O_2 formation during photosynthesis (34, 36). Second, several algae are known for circadian activities of antioxidative enzymes or low-molecular-weight antioxidants (37–39), often with an increase of antioxidative activities at subjective noon, when oxidative stress is most severe (38). A third potential mechanism is the occurrence of a circadian pattern in the formation of the unknown cyanide source for BrCN production.

A short-term release of a toxic metabolite to suppress growth of competitors might be a highly efficient allelopathic strategy. Like a "molecular toothbrush" BrCN could eliminate the surrounding flora daily after sunrise, leading to increased access to nutrients and light present in the environment and even elevated concentrations resulting from nutrients leaking from killed cells. The clean and nutrient-rich area could then be used by *N. cf pellucida* for effective proliferation in the absence of toxins. This diatom species is more resistant to BrCN compared with its competitors (Fig. S3) but at elevated concentrations it is still sensitive to the toxin. Thus, the short-term toxin burst is an effective means of reducing the risk of autotoxicity and represents a strategy for allelopathic interactions.

In this study, we illustrate that a highly active simple metabolite from diatoms has the potential to promote daily cleaning events around a biofilm-forming diatom. Our results provide a mechanism by which diatoms can generate microscale chemical territoria in which competitors are deterred or killed. Obviously, such strategy contributes to complex microlandscapes maintained by interacting species and may boost the small-scale patchy growth habits of biofilm-forming species. Our results also suggest a potential link between the globally significant emissions of volatile halocarbons released by marine algae and allelopathic activity.

Methods

Bialgal Culture Experiments. The origin of the algal strains and the culture conditions are described in *SI Methods*. Growth interactions between *Nitzschia cf pellucida* and 3 benthic diatom species (*Navicula arenaria Cylindrotheca closterium*, and *Entomoneis paludosa*) were examined by using bialgal cultures as described in *SI Methods*.

Effects of Spent Medium. *N. cf pellucida* spent medium was prepared by filtering exponentially growing cultures (200,000–250,000 cells mL^{-1}) on GF/F filters and subsequently on 0.2 μm membrane filters. This filtered spent medium was enriched with f/2 nutrients and then applied to cells of *C. closterium*, *Stauronella* sp. and *E. paludosa*. In parallel, cells of these species were cultured in f/2 enriched seawater as a control. The effect of *N. cf pellucida* spent medium was monitored by measuring the biomass and

photosynthetic efficiency using Pulse-amplitude-modulated (PAM) fluorescence (see *SI Methods* for details).

Liquid–Liquid Extraction of Allelopathic Compounds and GC-MS Analysis. A total of 150 mL of *N. cf pellucida* spent medium was filtered and extracted three times with 50 mL ethylacetate. The extract was dried with anhydrous sodium sulfate and was concentrated at reduced pressure. GC-EI-MS measurements of the concentrated extracts were performed with a Waters GCT premier (Waters) time of flight mass spectrometer (MS) coupled to an Agilent 6890N gas chromatograph (GC) equipped with a DB-5ms column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness and 10 m Duraguard precolumn, Agilent). The carrier gas was Helium 5.0 with a constant gas flow of 1.0 mL min^{-1}. The source temperature was at 300 °C with an electron energy of 70 eV. The column was held at 40 °C for 2 min, heated up from 40 °C to 150 °C with 5 °C min^{-1}, from 150 °C to 280 °C with a rate of 20 °C min^{-1} and held for 4.5 min. The samples were injected in splitless mode.

Volatile Organic Compounds. Solid phase microextraction (SPME, Carboxen/Polydimethylsiloxane, Supelco) was used to identify the volatile compounds emitted by *N. cf pellucida* cultures. The SPME fiber was exposed for 30 min to the headspace of 82 mL of magnetically stirred filtrate (GF/F filters) of *N. cf pellucida*. For the quantification of BrCN concentrations the SPME fiber was exposed for 5 min to the headspace of 2.5 mL magnetically stirred filtrate (0.2 μm filtered) of *N. cf pellucida*. We used $CDCl_3$ (Eurisotop) (at 0.124 μM) as an internal standard to enable quantification. The extracted compounds were analyzed using a Perkin-Elmer Autosystem XL GC coupled to a Perkin-Elmer TurboMass MS. An Agilent DB-5-MS column was used for separation. The GC was operating isothermally at 70 °C and the MS was recorded in single ion mode. The SPME extraction was calibrated by measuring a dilution series of commercially available BrCN (Sigma Aldrich) with $CDCl_3$ as an internal standard. BrCN concentrations were determined by calculating the GC peak area using standard program peak detection. BrCN peak area was normalized to the $CDCl_3$ peak area and used the calibration curve to calculate the BrCN concentrations in the *N. cf pellucida* cultures.

Bioassays. Bioassays were used to detect the presence of allelochemicals in *N. cf pellucida* spent medium and used *E. paludosa* as the susceptible strain. Cells of exponentially growing *E. paludosa* were inoculated in *N. cf pellucida* spent medium at a final density of 2,000 cells mL^{-1}. After 2 h of exposure to spent medium, we checked for the occurrence of resting cell formation and cell death using an inverted microscope (counting min 300 cells per replicate).

To ensure that our observations of cell death using normal light microscopy is valid, we assessed cell death using the membrane-impermeable DNA-specific stain Sytox (*SI Methods* and Fig. S8).

To check the occurrence of allelochemicals in the ethylacetate extracts, the extracts were concentrated using reduced pressure and finally dried the extract under a nitrogen enriched atmosphere. The residue was dissolved in acetone and added to an *E. paludosa* culture with 2,000 cells mL^{-1} at a final concentration of 1% acetone. This acetone concentration did not affect cell integrity itself within the timeframe of the bioassay.

The toxicity of nine halogenated compounds on the diatom *E. paludosa* was tested at concentrations of 0.1, 1, 2, 5, and 10 μM (Fig. 3). The halogenated compounds were first dissolved in acetone and added to an *E. paludosa* culture with 2.10^3 cells mL^{-1} at a final concentration of 1% acetone. We microscopically checked for dead cells, resting cells, and healthy cells 3 h after application. The same approach was used to check the toxicity of NaCN on *E. paludosa* cells at 2, 10, 20, and 40 μM NaCN.

The toxicity of BrCN on 6 bacterial strains isolated from estuarine intertidal mudflats was tested using agar diffusion assays (ADA, see *SI Methods* for details).

Synthesis of Cyanogen Iodide. ICN synthesis was performed as described (21). 0.25 mmol NaCN (Sigma Aldrich) was dissolved in 0.5 mL of water and cooled it to 0 °C. Iodine (0.25 mmol; Fluka) was gradually added, waiting until the last portion has reacted. The watery solution was extracted three times with diethyl ether, the ether extract was then dried with sodium sulfate, and the ether was removed by a stream of argon. ICN was received as colorless crystals.

Catalase Experiment. The effect of the H_2O_2-decomposing enzyme catalase (600 units bovine liver catalase mL^{-1} dissolved in water, Sigma Aldrich) on the toxicity of *N. cf pellucida* cultures was assessed by adding catalase to the *N. cf pellucida* cultures one hour before the onset of light and the presence of allelochemicals was tested 3 h after the onset of light using the *E. paludosa* bioassay. A control treatment in which we stirred *N. cf pellucida* cultures (analogous to the catalase treatment) one hour before the onset of light was included. Treatments were replicated four times.

Phenol Red Assay. The bromination of phenol red (phenolsulfonphthalein) into brominated phenol blue (3′,3′′,5′,5′′-tetrabromophenolsulfonphthalein) was used as an indicator for haloperoxidase activity (15). Phenol red (30 μM final concentration) was added to *N. cf pellucida* cultures (200,000–250,000 cells mL^{-1}) 3 h after daybreak. Two hours later, phenol red and brominated phenol blue were measured spectrophotometrically at 433 nm and 592 nm, respectively (15). Before the measurements, cells were removed by filtering on a 0.2-μm filter and we adjusted the pH to 6.5 with acetic acid.

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Supporting Information

Vanelslander et al. 10.1073/pnas.1108062109

SI Methods

Algal Strains and Culture Conditions. Diatom strains were isolated from the "Rammekenshoek" intertidal mudflat in the Westerschelde Estuary, The Netherlands (51°26'50" N, 3°38'38" E) on March 3, 2009. Cultures and permanent slides of the cultures used for the experiments are kept in the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium. Clonal cultures were established as described (1). Culture medium was prepared by filtering and autoclaving North Sea seawater enriched with f/2 nutrients (2). Clonal cultures were maintained in a climate room at 19 ± 1 °C and illuminated by cool-white fluorescent lamps at a rate of 50 μmol photons m^{-2} s^{-1} with a light/dark cycle of 12/12 h. Axenic diatoms cultures were obtained by treating an exponentially growing *N. cf pellucida* culture with 400 mg/L penicillin, 100 mg/L streptomycin, 100 mg/L gentamycin and 1 mg/L yeast extract at a salinity of 10 psu during 36 h. Afterward, single cells were isolated by micropipetting and transferred to sterile f/2 culture medium. Iodine enriched culture medium was composed of ESAW artificial seawater (3) supplemented with 45 μM KI. Culture medium enriched with ^{13}C was based on ESAW artificial seawater with 2.07 mM $NaH^{13}CO_3$.

Bialgal Culture Experiments. The growth interactions between *Nitzschia cf pellucida* and 3 benthic diatom species belonging to various genera (*Navicula arenaria*, *Cylindrotheca closterium*, and *Entomoneis paludosa*) were examined using bialgal cultures. Growth experiments were run in polystyrene 24-well cell culture plates containing 2.0 mL f/2 culture medium. Each species was inoculated at a cell density of ∼5,000 cells mL^{-1} resulting in an initial density of ∼50 cells mm^{-2}. Monoculture treatments (inoculated at 10,000 cells mL^{-1}) were included as a control. Cells for inoculations were harvested from monoclonal, exponentially growing cultures. Each treatment was replicated four times. The culture medium was daily renewed (1.0 mL of double strength f/2 medium) to avoid nutrient limitation and hence resource competition for nutrients. Cell densities of each replicate were monitored daily using an inverted microscope (Axiovert 135 Zeiss microscope) by counting a minimum of 300 cells per replicate.

PAM Fluorescence. PAM fluorescence (MAXI Imaging PAM fluorometer) was used to determine the maximum quantum yield of photosystem II (PSII), which is frequently applied as a proxy for photosynthetic efficiency (4). Photosynthetic efficiency was determined as F_v: F_m, where $F_v = F_m - F_0$. F_m is the maximum fluorescence emission level in the dark measured with a saturating pulse of light (emission peak at 450 nm, 2,700 μmol photons $m^{-2}s^{-1}$, 800 ms). The initial fluorescence F_0 was used as a proxy for biomass (5).

Cell Death Assay. To ensure that our observations of cell death using normal light microscopy are valid, we assessed cell death using the membrane-impermeable DNA-specific stain Sytox (5 mM stock in DMSO, Molecular Probes, Invitrogen). Sytox is commonly used as an indicator for dying or dead cells (6). Fluorescence microscopy was performed using a 495-nm excitation filter(Leica DMIL LED Fluo). Eight samples with a varying number of living and dead cells of *E. paludosa* were selected for both light microscopy countings of living and dead and for the Sytox green fluorescence assay. There was a strong relation between the live: dead ratio of both countings (Fig. S8).

Agar Diffusion Assays. The toxicity of BrCN on six bacterial strains isolated from estuarine intertidal mudflats was tested using agar diffusion assays (ADA). *Rhodovulum sulfidophilum* (LMG 5201), *Phaeobacter inhibens* (LMG 22475), *Marinobacter flavimaris* (LMG 23834), *Bacillus marisflavi* (LMG 23072), *Alcaligenes aquatilis* (LMG 22996), *Flexibacter tractuosus* (LMG 13172) were tested against 2, 4, 8, 16, 32 and 64 μM BrCN solutions and *N. cf pellucida* filtrate sampled 3 h after daybreak. Inhibition zones were monitored after 3–8 d at 25 °C. All assays were performed in triplicate and were compared with solvent controls.

1. Chepurnov VA, Mann DG, Vyverman W, Sabbe K, Danielidis DB (2002) Sexual reproduction, mating system, and protoplast dynamics of *Seminavis* (Bacillariophyceae). *J Phycol* 38:1004–1019.
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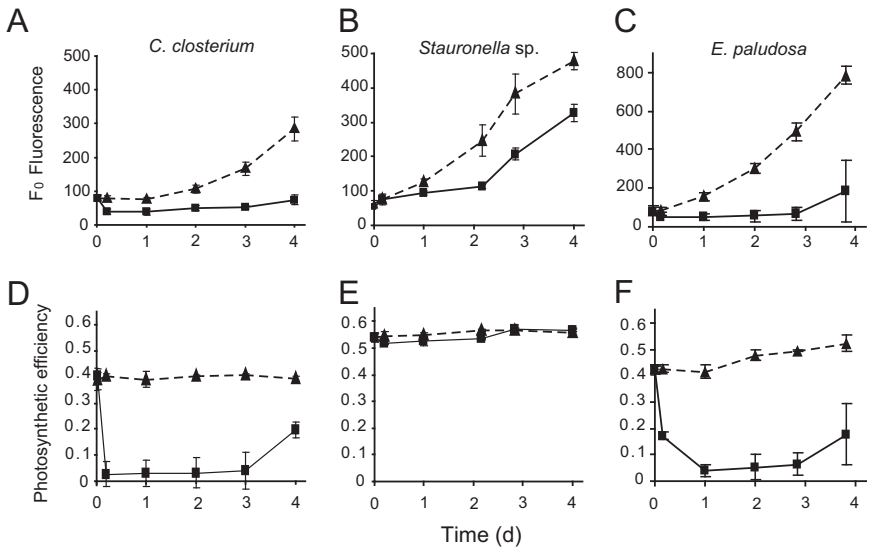

Fig. S1. Effects of nutrient enriched spent cell-free medium of *Nitzschia cf pellucida* on the biomass (*A*–*C*; measured as initial fluorescence F_0) and photosynthetic efficiency (*D*–*F*) (F_v:F_m using PAM fluorescence) of three naturally co-occurring diatom species. Species were cultured with f/2 nutrient-enriched seawater (black triangles with dashed lines) and with f/2-enriched spent medium of *N. cf pellucida* (filled squares and solid lines). (*A* and *D*) *Cylindrotheca closterium*. (*B* and *E*) *Stauronella* sp. (*C* and *F*) *Entomoneis paludosa*. (Means ± SD, n = 4).

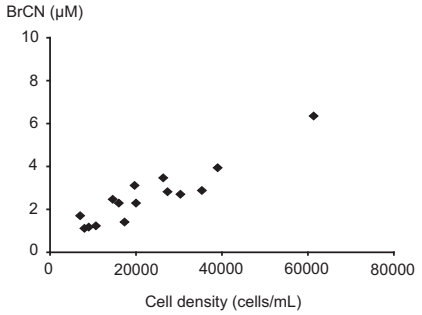

Fig. S2. Local BrCN concentrations in *N. cf pellucida* cultures with low cell densities.

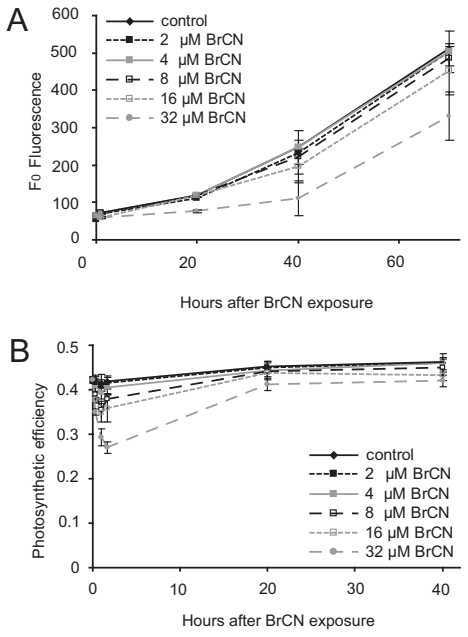

Fig. S3. Effect of BrCN on growth (*A*, measured as F_0) and the photosynthetic efficiency (*B*) of *N. cf pellucida*. (Means ± SD, n = 4).

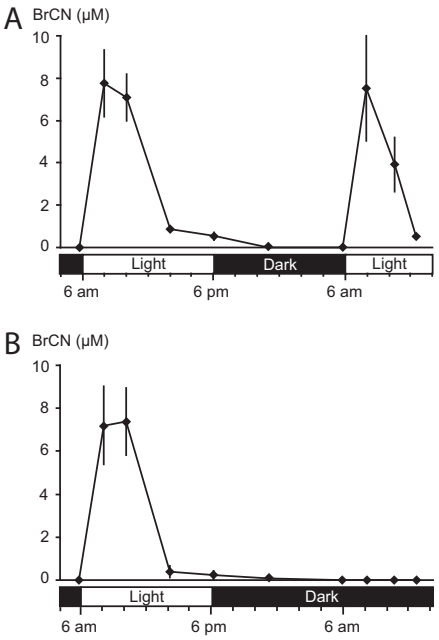

Fig. S4. Light-dependent production of BrCN in *N. cf pellucida* cultures (180,000 cells mL^{-1}). (*A*) BrCN production during a 12 h:12 h light:dark cycle. (*B*) BrCN production in *N. cf pellucida* cultures with a 12 h:12 h light/dark cycle followed by a prolonged darkness of 18 h.

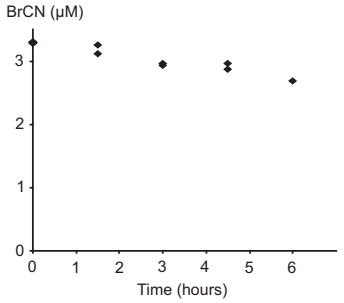

Fig. S5. Degradation of BrCN in illuminated seawater over time.

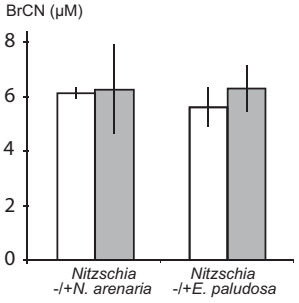

Fig. S6. Effect of competing co-occurring species on BrCN production. White columns represent BrCN concentrations in *N. cf pellucida* monocultures (180,000 cells mL^{-1}) and gray columns depict BrCN concentrations in cocultures of *N. cf pellucida* monocultures (180,000 cells mL^{-1}) and *N. arenaria* (70,000–80,000 cells mL^{-1}) or *E. paludosa* (70,000–80,000 cells mL^{-1}).

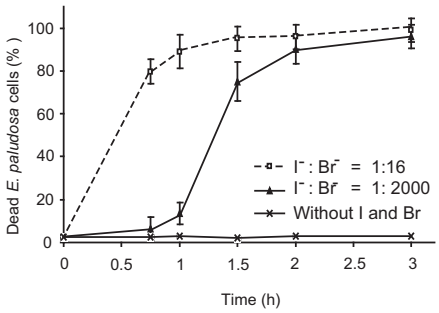

Fig. S7. Cell death in *E. paludosa* following exposure to filtrate of *N. cf pellucida* grown in media with different I^-: Br^- ratios (1:16 and 1:2,000) and in absence of I^- and Br^-. (Means ± SD, n = 4).

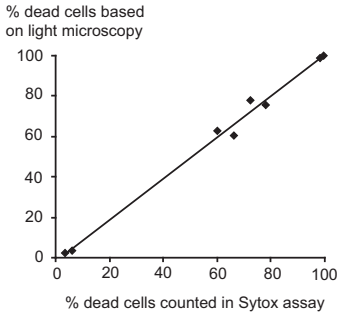

Fig. S8. Cell death assessment using the Sytox green fluorescence assay vs. cell death observed by light microscopy. Significant linear regression is plotted (P = 0.0000002).

4.7 Manuscript G

Production and role of volatile halogenated compounds from marine algae

Carsten Paul and Georg Pohnert

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Production and role of volatile halogenated compounds from marine algae†

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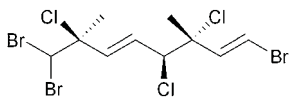
Marine organisms are an important source of volatile halogenated natural products. Marine algae, in particular, contribute significantly to the global budget of halogenated hydrocarbons that play an important role in climate functioning. However, despite the large amounts of halogenated metabolites from algae, we know relatively little about their function in the producing organism. In this *Highlight*, we discuss the current knowledge of volatile halogenated compounds from algae, with a focus on biosynthesis, algal physiology and chemical ecology. We also briefly discuss geochemical aspects arising from the release of halogenated natural products from micro- and macroalgae.

1 Introduction

Ever since the pioneering work by R. E. Moore, volatile halogenated products from marine algae have attracted the interest of marine researchers from multiple disciplines.[1] It was recognized early on that a single algal species could be responsible for a highly diverse assortment of volatile halogenated metabolites. For example, the edible odoriferous red seaweed *Asparagopsis taxiformis* releases a complex mixture of more than 120 halogenated metabolites containing less than five carbons in the longest chain.[2–4] Although relatively simple volatile terpenes have been recognized as halogenated algal natural products, halogenated monoterpenes such as the polyhalogenated **1** were first discovered in the digestive gland of the sea hare *Aplysia californica*.[5] Shortly afterwards, it was identified that the red alga *Plocamium pacificum* was the true source of **1** and other related polyhalogenated metabolites, thereby suggesting that the sea hare sequesters these halogenated metabolites from the algae for its own chemical defense.[6]



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The aim of this *Highlight* is to discuss aspects of biosynthesis, algal physiology, and chemical ecology of algal metabolites together with geochemical aspects. We have excluded cyanobacteria-derived metabolites, which are often of high molecular weight, but instead refer the reader to several comprehensive reviews.[7–10] We aim to provide a picture of the function of volatile halogenated metabolites for the producing alga and their role as bulk metabolites in the oceans. For further reading on specific topics we can recommend overviews on the biosynthesis of halogenated natural products,[11–13] on mechanistic aspects of halogenating enzymes[14] and on general aspects of marine natural products of environmental relevance,[15] as well as reviews on halogenated metabolites from brown algae[16] and red algae.[2]

2 Occurrence and biosynthesis of volatile halogenated metabolites from algae

The production of halogenated metabolites is a common feature of marine micro- and macroalgae. Brown, green, and red seaweeds produce a variety of halogenated metabolites from different pathways including halomethanes, short-chain hydrocarbons, terpenes, and phenols.[17] Out of all the marine seaweeds, red algae (Rhodophyceae) possess the highest abundance of unique biosynthetic pathways for organohalogen production.[2] In contrast, microalgae are predominant producers of halomethanes, and only few structurally more complex halogenated metabolites have been reported from phytoplankton. In this section, we give an overview of the most important classes of volatile halogenated metabolites from algae, while more comprehensive compilations of halogenated algal metabolites can be found in dedicated reviews.[2,16,18]

2.1 Halomethanes

Halomethane production is ubiquitously observed in marine algae. Chlorinated, brominated and iodinated halomethanes, as well as mixed structures, have been reported in screenings. In addition, the degree of halogenation is varied, with many reported examples of methyl halides, dihalomethanes, haloforms and carbon tetrahalides.[1,17–20] Besides the more complex halogenated metabolites, single species of red algae often produce highly diverse mixtures of volatile halomethanes that include $CHBr_3$, $CHBr_2I$, $CHBrI_2$, CHI_3, $CHBr_2Cl$, $CHClBrI$, CH_2Br_2, CH_2BrI, CH_2I_2, and CBr_4.[1,2] In a survey of Arctic macroalgae, it was observed that brown and green algae generally exhibited a higher release of these organohalogens compared to red alge.[21] Bromoform was the predominant metabolite isolated from brown and green algae, reaching production rates of up to four μg per g of algal biomass per day.[20] Microalgae are also effective producers of this metabolite, and the subsurface maximum of bromoform observed in the tropical eastern Atlantic Ocean can

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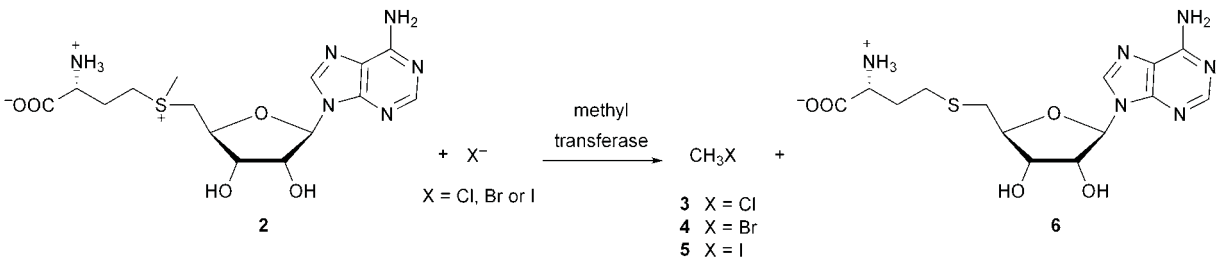
be attributed to a phytoplanktonic source.[22,23] In general, the ability to produce halocarbons is widespread in unicellular algae. A recent survey of phytoplankton cultures consisting of three different classes revealed that diatoms, coccolithophorids and a chlorophyte all produce chloromethane, bromomethane and bromoform in differing quantities.[24] Marine plankton belonging to the Cryptophyceae, Dinophyceae, Prasinophyceae and Prymnesiophyceae, as well as cyanobacteria, have also been reported as halomethane producers.[25–27]

The biosynthesis of methyl chloride, methyl bromide and methyl iodide **3–5** is generally carried out with methyl transferases. These enzymes methylate nucleophilic halide anions by employing *S*-adenosyl-L-methionine **2** (SAM) as the methyl donor (Scheme 1).[28–30] The crystal structure of a plant SAM-dependent halide methyltransferase has given further insight into the halogenation mechanism in *Arabidopsis thaliana*.[31] A model for substrate/nucleophile binding and reaction at the active site has revealed that the reactive sulfonium methyl group orients into a large cavity. This seems to be the reason for the observed promiscuous nature with respect to a variety of nucleophiles. The enzyme promotes reaction most efficiently with thiocyanate, compared to the halides.[31] Experiments with a partially purified methyl transferase from the microalga *Pavlova pinguis* revealed that a single enzyme can transfer chloride, bromide and iodide to SAM.[32] Methyl halide transferases have a surprisingly low affinity towards halides, which is reflected by their high Michaelis–Menten constants.[30,33] However, this might be compensated by relatively high halide concentrations in algal tissue compared to low concentrations of SAM. It also has to be noted that relative amounts of the respective halomethanes detected in seawater and in the atmosphere do not necessarily correlate with the total number of biosynthesized molecules, because abiotic transformations such as nucleophilic substitution reactions can readily transform methyl halides.[34]

The biosynthesis of polyhalomethanes such as $CHCl_3$ or CH_2Br_2 is catalysed by haloperoxidases (HPOs).[35] These enzymes can contain iron or vanadate as co-factors, and are categorized on the basis of the most electronegative halide that can be oxidized. Thus, chloroperoxidases (ClPO) oxidize chloride, bromide and iodide, while iodoperoxidases (IPO) only oxidize iodide. Only the central aspects of haloperoxidase chemistry are mentioned herein, but there are several reviews dealing in detail with enzymatic properties, reaction mechanisms and distribution of haloperoxidases.[13,14,35,36] The most dominant class of haloperoxidases among marine algae are bromoperoxidases (BrPO), which are mostly vanadium-dependent, and have been found in all types of algae including Chlorophyta,[37,38] Rhodophyta,[39,40] Phaeophyta,[41–43] and Bacillariophyta.[44]



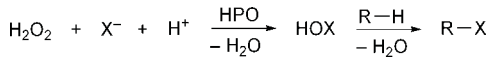
Scheme 1 Biosynthesis of methyl halides employing a methyl halide transferase.

Carsten Paul

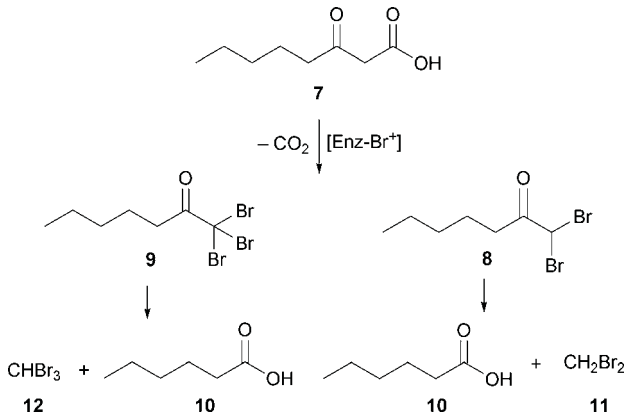
Carsten Paul (born 1983) graduated in 2008 from the Friedrich Schiller University in Jena with a diploma in chemistry (environmental chemistry). After a short-term internship with Dr. Paul Jensen at Scripps Institution of Oceanography in La Jolla, USA, he returned to Jena where he was awarded a graduate fellowship by the Jena School for Microbial Communication (JSMC). He is currently completing his Ph.D. studies under the guidance of Professor Pohnert. His research interests are chemically mediated interactions of microalgae with bacteria and other microalgae.

Georg Pohnert

Georg Pohnert obtained his Ph.D. at the University of Bonn on the pheromone chemistry of brown algae. In 1997 he started as a postdoc at Cornell University, where he studied the E. coli *phenylalanine receptor site. He then moved to the Max-Planck-Institute for Chemical Ecology, where he started his independent research on algal defense reactions. In 2005, he was appointed as professor at the Ecole Polytechnique Fédérale de Lausanne. In 2007 he accepted a Lichtenberg professorship and chair of bioorganic analytics at the Friedrich Schiller University in Jena. Currently his research interests focus on the chemical ecology of micro- and macroalgal chemical communication and the oxylipin chemistry of defense reactions.*



Scheme 2 Simplified haloperoxidase reaction with organic substrates.



Scheme 3 Biosynthesis of polyhalomethanes.

Vanadium- and iron-dependent ClPO, on the other hand, are most commonly found in fungi,[45] while no vanadium-dependent ClPO has yet been characterized from algae. Recently it was shown that marine prokaryotes also have the genetic potential for vanadium-dependent ClPO production.[46] During the haloperoxidase reaction, H_2O_2 is used to oxidize halide anions by a two-electron oxidation yielding the corresponding hypohalous acid. This reactive X^+ intermediate can then be used to halogenate electron-rich organic substrates (Scheme 2). In algae, the formation of dibromomethane **11** and bromoform **12** proceed through the precursor 3-ketooctanoic acid **7** (Scheme 3). Following its decarboxylation, bromination yields the intermediates 1,1-di- **8** and 1,1,1-tribromo-2-heptanone **9**. These are then subjected to non-enzymatic hydrolysis, yielding the respective halomethanes and hexanoic acid **10**.[40,47] The release of oxidized halogen species by extracellular BrPO, and subsequent reaction with dissolved organic matter in seawater, may represent an alternative mechanism for the formation of polyhalomethanes.[33]

2.2 C_2–C_9-halogenated hydrocarbons

In macroalgae, the production of longer chain-length halogenated hydrocarbons is typically observed. Halogenated alcohols, acetaldehydes and acetones as well as mono- and polyhalogenated ethanes, propanes, butanes and pentanes have been reported from red and brown algae.[48–50] The red alga *Asparagopsis taxiformis* is a prolific resource for these metabolites as well as for the production of unsaturated and otherwise functionalized hydrocarbons.[2] A selection illustrating the high structural variability is given in Fig. 1.[1–4] However, to date, no experiments on the biosynthesis of these metabolites have been reported, but it can be rationalized that rather unspecific HPOs are involved in key steps leading to this high structural diversity.

Besides these small molecules, functionalized hydrocarbons with longer chain length are also produced by macroalgae. A well-studied example are the red algae of the genus *Bonnemaisonia*, which produce polyhalogenated heptan-2-ones **27–31**,

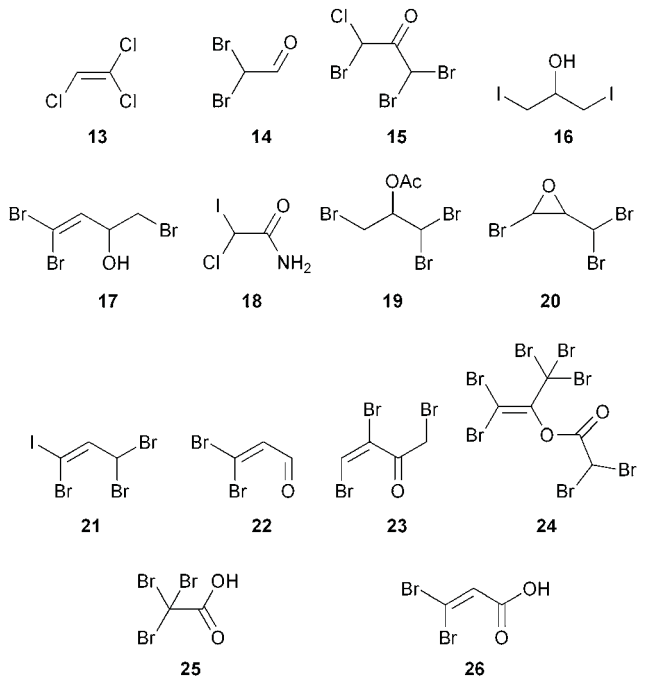
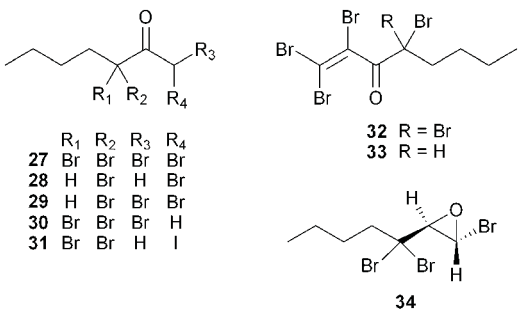


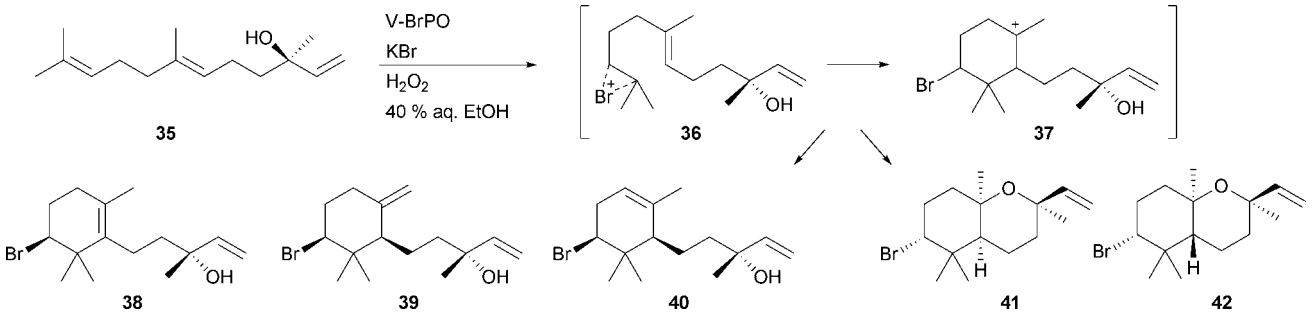
Fig. 1 Short-chained algal halocarbons representing a high variability of chemical functionalities.

1-octen-3-ones **32** and **33**, and the halogenated epoxide **34**.[51] Labeling experiments showed that these metabolites are derived from acetate and that labeled palmitate is also incorporated with a high percentage rate.[52] Due to the ambiguous results, however, only a tentative connection to fatty-acid biochemistry can be drawn, because it cannot be clarified whether anabolic or catabolic processes are involved in the formation of heptanone and heptenone derivatives. Polyhalogenated octenones have been detected from another Bonnemaisoniaceae species, *Delisea fimbriata*, which is also a source of halogenated lactones (see Section 4.2).[53]



2.3 Halogenated terpenes

Many red algae are producers of halogenated terpenes, and only a few of these metabolites have been reported from other groups of algae. The structures of most algae-derived halogenated terpenes can be rationalized on the basis of well-known principles of terpene biosynthesis with additional steps involving halogen peroxidases as formal cation donors.[35,54] Acyclic

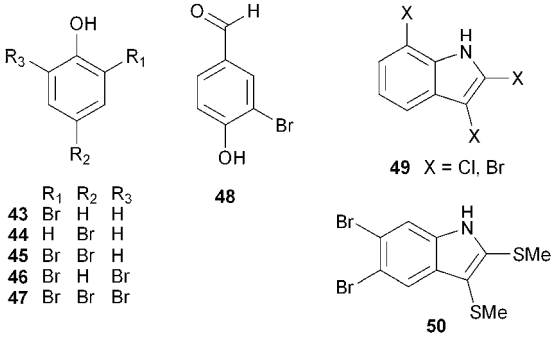


Scheme 4 Proposed bromonium-initiated cyclization in terpene biosynthesis.[55,56]

terpenes can be formed by electrophilic reaction of X^+ with electron-rich carbon centers of the isoprene subunits (Scheme 4). The resulting cations can then react with additional halogen anions to give multiply-halogenated products. X^+ ions can also initiate the biosynthesis of cyclic terpenes, which involves additional enzymatic or non-enzymatic internal cyclization reactions of cationic intermediates (Scheme 4).[55,56] This principle has been demonstrated using purified BrPOs from the red algae *Corallina officinalis*, *Laurencia pacifica*, and *Plocamium cartilagineum*.[56] When (*E*)-(+)-nerolidol **35** reacts with BrPO in the presence of bromide and hydrogen peroxide, a mixture of bromoether, bromoalcohol, bromohydrin, and epoxide species is produced. Among them are the natural snyderols **38–40** and 3β-bromo-8-*epi*-caparrapi oxides **41** and **42** (Scheme 4). The application of these simple biosynthetic principles allows the formal construction of hundreds of cyclic and noncyclic chlorinated and brominated terpenes found in the red algae.

2.4 Halogenated aromatic metabolites

Macroalgae are also known for their production of halogenated aromatic metabolites. Due to the electrophilic character of the chemical equivalent of X^+, which is generated by XPO, these enzymes play the central role in the bromination of aromatic metabolites. Simple halogenated aromatic metabolites are very common, which was illustrated by a screening of 49 species of marine red, green and brown macroalgae for the key seafood flavor components 2- and 4-bromophenol **43** and **44**, 2,4- and 2,6-dibromophenol **45** and **46**, and 2,4,6-tribromophenol **47**. All five bromophenols were found in 62% of samples, four in 32% of the samples, and three in the remaining 6% of samples. In most cases, **47** was found as the dominant metabolite.[57] The biosynthesis of these bromophenols was addressed using the green alga



	R_1	R_2	R_3
43	Br	H	H
44	H	Br	H
45	Br	Br	H
46	Br	H	Br
47	Br	Br	Br

48

49 X = Cl, Br

50

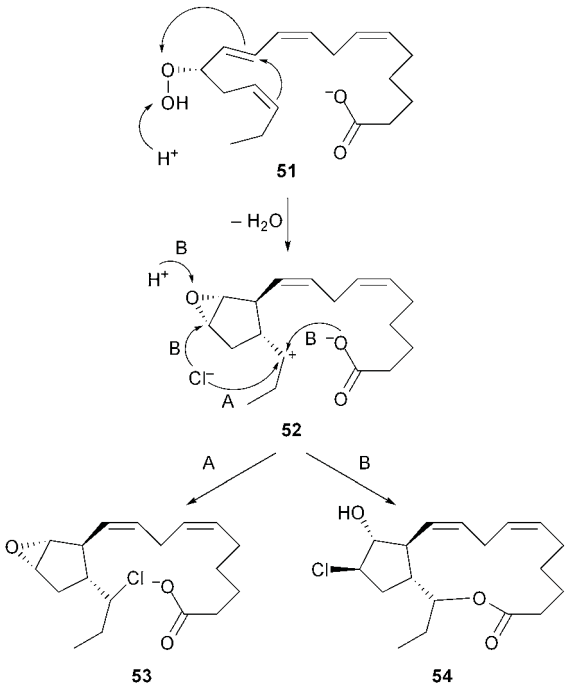
Ulva lactuca. Crude cell extract containing BrPO transformed 4-hydroxybenzoic acid to **47**.[58] 3-Bromo-4-hydroxybenzaldehyde **48** is produced from tyrosine in a chloroplast-enriched fraction of the red alga *Odonthalia floccosa*.[59] Halogenated indoles **49** and **50** with strong antifungal activity have been detected in several red algae as well.[60,61] Most other halogenated aromatic metabolites from algae, like diiodotyrosine or halogenated phloroglucinols and phenols, are of higher molecular weight and are not within the scope of this review.[16]

2.5 Oxylipins

Halogenated oxylipins are rarely found in marine algae. These metabolites arise from the initial oxidation of unsaturated fatty-acids by lipoxygenases. The resulting intermediate fatty-acid hydroperoxides may be transformed in reactions involving a nucleophilic attack of a halide. Chlorinated fatty-acid-derived metabolites named egregiachlorids A–C (*e.g.*, egregiachlorid A **53**) were isolated from the brown alga *Egregia menziesii*.[62] Even though the biosynthesis was not explored in detail, a pathway was hypothesized that involves the initial oxidation of stearidonic acid to a hydroperoxy fatty-acid **51** by a 13-lipoxygenase, cyclization to the cyclopentyl cation **52** and subsequent nucleophilic attack by a chloride anion yielding the chlorinated oxylipins **53** and **54** (Scheme 5). A related pathway was later suggested for several other C_{18} oxylipins from the brown alga *Eisenia bicyclis*.[63] Recently, a new enzymatic halogenation mechanism *via* a hydroperoxide halolyase was established in the marine diatom *Stephanopyxis turris* (Scheme 6).[64] This diatom can transform C_{20} fatty-acids such as eicosapentaenoic acid **55** with a lipoxygenase to form hydroperoxide intermediates such as **56**. Cleavage of the intermediate is presumably assisted by a nucleophilic attack of a chloride anion, yielding chlorinated octadienes **57** and **58** and (5*Z*,8*Z*,10*E*)-12-oxo-5,8,10-dodecatrienoic acid **59**. Evidence for enzyme participation is given by the high enantiomeric excess of the optically active chlorinated product **57**.[64]

3 Geochemical impact of halogenated metabolites from algae

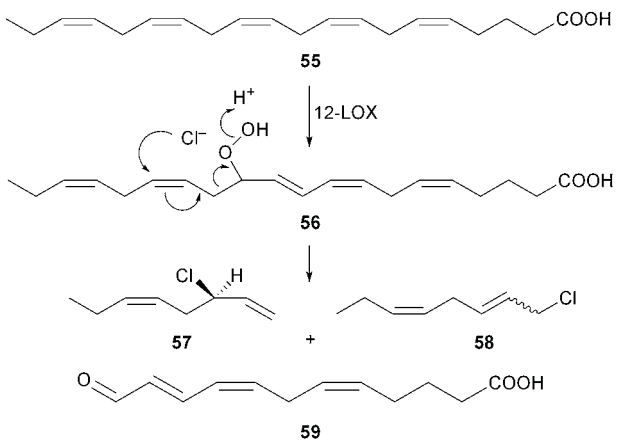
Natural and anthropogenic emissions of halogenated compounds have attracted much attention due to their role in atmospheric chemistry. Halocarbons or inorganic halogens such as iodine influence the radiation budget of the earth and the oxidation power of the atmosphere. The major impact of these



Scheme 5 Postulated pathway for fatty-acid-derived halogenated oxylipins.[63]

compounds on atmospheric chemistry can be seen in their contribution to tropospheric and stratospheric ozone depletion.[65] In the polar regions, the impact of natural halogenated volatiles may be more significant than in other regions of the world due to their close proximity to the major zone of ozone destruction. In consequence a lot of research activity focused on investigation of algal halocarbon emissions from Arctic and Antarctic regions.[66,67]

Brown algae belonging to the order Laminariales are strong accumulators of inorganic iodide. These algae represent a major pump in the global biogeochemical cycle of iodine, and are among the major source of iodocarbons in the coastal atmosphere.[68]



Scheme 6 Halolyase-mediated transformation of eicosapentanoic acid to chlorinated octadienes.[64]

Certain volatile halocarbons can be clearly attributed to natural production while others are exclusively produced from anthropogenic activity, but several compounds are produced by both biogenic and anthropogenic sources. It is proposed that the most dominant natural halogenated metabolite is methyl chloride, which is released by marine algae, as well as some higher plants and fungi (Table 1).[69] As far as the ocean flux of halocarbons to the atmosphere is concerned, then estimated values vary greatly – the source strength for methyl chloride of the open oceans is estimated between 380 and 650 kilotonnes per year.[70,71] However, from the viewpoint of stratospheric ozone depletion, methyl bromide, with its high ozone chemistry potential, is the more detrimental metabolite. This compound is not only released from natural sources but is also produced and widely used as an industrial fumigant. The estimation of the natural production and release rates of this marine halomethane is also speculative (Table 1). It has been suggested that the combined emissions from micro- and macroalgae contribute substantially to the amounts of bromine in the global cycle; perhaps in the same order of magnitude as anthropogenic sources.[72] Methyl bromide emission of the oceans have been estimated around 35 kilotonnes per year by Khalli *et al.*,[73] whereas Lobert *et al.* claim that there is no general supersaturation of methyl bromide in seawater and that the oceans may thus be a net sink (rather than a source) of methyl bromide.[74] Bromide emissions from the oceans are only indirectly related to algal productivity because other sources and sinks for this metabolite have to be considered such as methyl bromide release by sediments as well as bacterial or abiotic degradation.[75] Other shorter-lived halocarbons from algae are significant in tropospheric and potentially stratospheric chemistry as well. For example, $CHBr_3$, CH_2Br_2, and CH_3I are highly supersaturated in the ocean. It can therefore be speculated that because of their massive contribution to halocarbons in ocean waters, algae are also significant contributors to halometabolites found in the atmosphere.[76]

Both micro- and macroalgae are considered as relevant producers of halogenated methanes and structurally related climate relevant metabolites. Since macroalgae are restricted to the seashore, their production influences the concentration of halocarbons in seawater mainly close to the coast. Their halogenated metabolites may have a substantial impact locally, but on the global scale, only a minor contribution to the overall monohalomethanes is estimated from kelp and other macroalgae.[77] However, the production of polyhalogenated bromomethanes, which are predominantly produced by macroalgae, contribute significantly to the global budget.[78] In contrast, microalgae belonging to the phytoplankton are not restricted to

Table 1 Estimated release rates of CH_3Cl and CH_3Br from biotic and anthropogenic sources (calculated after ref. 69)

	Estimate (kilotonnes/year)	
Source	CH_3Cl	CH_3Br
Oceanic	650[70]	35[73]
Macroalgae	0.14	0.06
Total biotic	3350	83
Anthropogenic	386	48

the coastal zone and are responsible for a significantly higher share of global primary production compared to macroalgae. This means that phytoplankton have greater importance for the global fluxes of halocarbons, even though their net production rates may be considerably lower compared to macroalgae.[25]

Correlation of halocarbon concentration patterns in air and seawater with other parameters can be used to estimate the impact of algae on halocarbon production.[79] An exemplary study that correlated the halogenated compounds iodoethane, 1-iodobutane, 1-chlorobutane, 2-chlorobutane, dichloromethane, chloroform, and tetrachloroethene with pigments, which are indicative of phytoplankton blooms, was conducted in Menai Strait, UK.[80] Partial least-squares modeling of the complex data set highlighted the importance of microalgae on the signature of halogenated metabolites during their spring blooms, whereas macroalgae and sediments dominated as sources for these volatile compounds during non-bloom conditions.[80] Ship-borne measurements during a crossing of the Southern Indian Ocean was also used to correlate organohalogen production to chlorophyll concentrations, thereby supporting the significance of phytoplankton from the open ocean as a halomethane source.[81] Such observations match well with results from numerous field and laboratory studies on single macroalgal species or microalgal cultures.

4 Function of halogenated metabolites from algae

4.1 Algal physiology

Despite the massive production rates, the physiological and ecological roles of methyl halides are largely unknown. Based on observations of high methyl halide production rates of the salt marsh plant *Batis maritima*, it was proposed that methyl halide release can be a means to regulate the halide concentration of the plant tissue.[28] However, estimations of methyl halide production in the kelp *Macrocystis pyrifera* suggest that only a small proportion can be excreted *via* this pathway, and this would most likely not be sufficient for the regulation of intra-algal chloride concentrations.[77] The idea of a "metabolic accident" in which ubiquitous free halide ions are non-specifically methylated by methyl transferases that fulfill other metabolic purposes has also been brought forward.[33] A distinct metabolic function of CH_3Cl could be shown in fungi. There, the biosynthesis of methyl esters is linked to CH_3Cl as the methyl donor. These findings are supported by the fact that the formation of both esters and CH_3Cl were inhibited by SCN^-.[82,83] However, no similar mechanism is reported for algae. This brief overview shows that much additional work is needed to elucidate the role of methyl halides. Perhaps in the near future, the emerging tool of gene deletion experiments in combination with metabolomic techniques and bioassays could provide the first answers regarding the specificity and role of methyl transferases in algae.

In contrast to the nucleophilic substitution reactions involving free halide anions, the haloperoxidase reaction consumes H_2O_2 (Scheme 2). This can be shown by the external addition of H_2O_2 that leads to an increased production of polyhalogenated methanes in macroalgae.[84,85] The halocarbon metabolism is thus closely coupled to oxidative processes, with several physiological consequences for the producer. Pedersen *et al.* suggested that halocarbons are side products in the breakdown of surplus hydrogen peroxide in algal cells under oxidative stress.[85] Several observations support this hypothesis. Release of $CHBr_3$ and CH_2Br_2 by the kelp *Macrocystis pyrifera* was reduced in the darkness. H_2O_2 is produced by photoautotrophs during the Mehler reaction in the chloroplasts and BrPO, consequently, might serve as a way to rid the cells of this harmful product. Since H_2O_2 additions partially restored the ability to produce these metabolites during darkness, this halocarbon production is apparently limited by oxidant supply.[72] Furthermore, the addition of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which causes a reduced electron flux and consequently less H_2O_2, also diminishes the release of $CHBr_3$.[86]

Haloperoxidases, in particular IPO, also play an essential role in the iodine metabolism of *Laminaria digitata*. This kelp is an effective iodide accumulator and was harvested for the retrieval of this element.[87] IPO is known to play a crucial role in the uptake of iodine by oxidizing iodide from the seawater to HOI with the aid of H_2O_2.[87] In the cells, HOI is reduced to iodide, where it presumably serves the organism as an inorganic antioxidant.[68] In accordance with this hypothesis, gene expression analysis revealed the induction of two IPO coding genes after induction of *L. digitata* by oligoguluronates that are known to trigger signaling cascades involving reactive oxygen species (ROS).[88] The brown alga *Ectocarpus siliculosus* (for which the genome was recently sequenced) also accumulates halides, although to a significantly lower level.[89] This difference was reflected in the genome where only one bromoperoxidase was detected. In contrast, large families of haloperoxidases are found in kelps. Interestingly, the *Ectocarpus* genome encodes 21 putative dehalogenases, which may serve to protect the alga against halogenated compounds produced by co-occuring kelps.[89]

In contrast to these bulk chemicals released by algae, the physiological reasons for the production of other halogenated metabolites are often less clear. It was suggested that BrPO-mediated oxidation of phenolic polymers in the brown alga *Fucus serratus* is involved in adhesive formation and cell-wall strengthening.[90] If model oxidation reactions involving BrPO, polyphenols and H_2O_2 were carried out in the presence of bromide or iodide, differing polymer properties resulted.[91] It is, however, not clear how the halogen influences polymer formation. Two mechanisms could be envisaged involving either the bromination of phenols followed by their oxidation and polymerization, or the enzymatic generation of oxidized bromine species, which can further react with the nucleophilic phloroglucinol repeating unit.[90] A majority of the other halogenated secondary metabolites observed in macroalgae may perhaps be involved in the interaction of the alga with its environment, as summarized in the next section.

4.2 Chemical ecology

Halogenated metabolites attracted the early interest of natural product chemists from the perspective of pharmacological significance. This was motivated by the observation that halide substitutions of natural products often result in an alteration of their pharmacological properties.[13,92,93] This modulation of activities may also play a significant role in the function of halogenated metabolites in an ecological context.

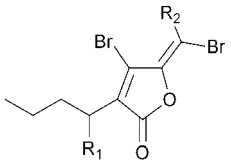
Besides its function in the regulation of reactive oxygen species in intact algae, bromoform might also contribute towards the alga's chemical defense. The observation that the surface of dead cells of the red alga *Corallina pilulifera* were covered with diatoms, while bromoform-producing cells were not, motivated further studies in this direction.[94] Indeed, bromoform inhibits diatom growth at concentrations somewhat higher than the bromoform emissions by the algae.[94] However, *C. pilulifera* produces additional halogenated methanes such as CH_2Br_2 and $CHBr_2Cl$ that might have additional effects and could explain the experimental outcome. This observation is of great relevance for general antifouling mechanisms of algae, since production of the halomethanes is widespread among micro- and macroalgae.

It has also been observed that other fouling organisms are affected by volatile halogenated metabolites. Paul *et al.* measured the antimicrobial activity of bromoform and dibromoacetic acid from the red algae *Asparagopsis armata*.[95] These metabolites were active against six bacterial strains, including two marine *Vibrio* species. Further evidence for the role of brominated metabolites as antifouling agents was achieved by omitting bromide from the culture medium, which suppressed the metabolic capacity to produce brominated compounds. Algae that lacked brominated secondary metabolites exhibited increased bacterial colonization compared to algae cultured in a bromide-containing medium.[95] Microscopic investigation of *A. armata* revealed that halogenated metabolites are stored in specialized gland cells that maintain a physical connection with the outer cell wall. These structures could be responsible for the observed release of bromoform and dibromoacetic acid into the surrounding environment.[95] Dibromomethane, which is for example produced by the green alga *Ulvella lens* and by the red alga *Lithophyllum* sp., induces larval metamorphosis in the sea urchin *Strongylocentrotus nudus*.[96,97] The toxicity of bromoform on marine mussles, shrimp and fish was tested by Gibson *et al.*, who found LC_{50} values of 7–50 ppm in short-term toxicity assays, and also observed altered behavior of shrimp and fish upon exposure to bromoform.[98]

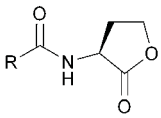
Compared to the extensive literature on antifouling activities of halomethanes from macroalgae, little is known about activities in plankton interactions. However, because phytoplankton cells are also challenged by bacteria and other microorganisms, as well as by competing phytoplankton, similar processes might be expected.

Antibacterial activity has additionally been observed from extracts of *Bonnemaisonia hamifera*. In the field, this filamentous red alga exhibits lower epibacterial abundance than other co-existing algal species. The surface extract, which is obtained by briefly dipping the alga in hexane, inhibits bacterial growth of several marine bacteria.[99] Additional bioassay-guided fractionation revealed 1,1,3,3-tetrabromo-2-heptanone **27** as the active compound that inhibits the growth of ecologically relevant fouling bacteria isolated from algal surfaces at natural concentrations. Furthermore, the biofouling on artificial surfaces that were treated with **27** was significantly reduced.[100]

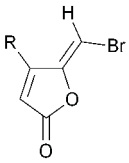
A completely different strategy that does not rely on antibiotic activity of natural products for the defense against bacteria was established with the red alga *Delisea pulchra*. This alga is capable of interfering with bacterial communication using brominated furanones **60–63**, which suppress bacterial development on its surface. Initial observations indicated that variations in furanone concentration are inversely correlated with bacterial abundance of the alga. Nevertheless, ecologically relevant concentrations of the metabolites did not have antibiotic activity. However, these metabolites affected mobility and attachment of bacteria, which pointed towards the disruption of bacterial communication.[101]



60 R_1 = H; R_2 = Br
61 R_1 = H; R_2 = H
62 R_1 = OAc; R_2 = H
63 R_1 =OH; R_2 = H



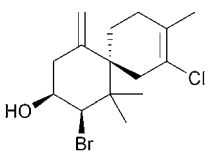
R = alkyl, 2-oxoalkyl...
64



65 R = H
66 R = Br

Gram-negative bacteria use excreted acylated homoserine lactones (AHLs) **64** for decision-making in a process termed quorum sensing.[102] The concentration of AHL in the environment of bacterial cells regulates their metabolic activity and behavior and thereby influences the settlement success of the fouling organisms. An essential protein in this communication mechanism is LuxR. When AHL reaches a threshold concentration, it becomes bound to LuxR, and this complex activates transcription of operons encoding relevant enzymes for metabolic reactions.[102] The red alga *D. pulchra* produces **60–63** that are structurally related to the quorum-sensing regulator AHL. These furanones inhibit bacterial colonisation by preventing binding of AHL to LuxR or by displacing bound AHL from the LuxR complex.[103] This leads to an accelerated degradation of the LuxR-like protein and a disruption in quorum-sensing mechanisms.[104,105] Since AHL-based quorum sensing is a widely distributed mechanism not only limited to marine fouling bacteria, these metabolites also have the potential for the treatment of infectious diseases.[106] Meanwhile, synthetic derivatives **65** and **66** of the *Delisea* furanones with enhanced quorum-sensing inhibitory properties are available and in use for medicinal purposes.[107] Further investigations of this well-studied model alga revealed multiple ecological functions for the polybrominated furanones. These metabolites also inhibit the settlement of four epiphytic algae that are representative of the fouling community in the environment of *D. pulchra*.[108] Further studies gave evidence that these furanones also deter feeding of local herbivores,[109] completing the broad spectrum of their biological activities.

Besides bacteria and epiphytes, algal surfaces are also challenged by settlement of larvae, and halogenated secondary metabolites are also active against these organisms. Barnacle larval settlement was inhibited by polyhalogenated monoterpenes isolated from the red alga *Plocamium costatum*,[110] and the brominated and chlorinated sesquiterpene elatol **67** from *Laurencia rigida* is active against other invertebrate larvae.[111]



67

The localization and release mechanisms of active compounds that comprise an ecological function in surface interactions of algae with epibionts are crucial. Early studies were conducted on the brominated sesquiterpene β-snyderol, the major terpene from the red alga *Laurencia snyderae*, which binds 77% of the organic bromine of the alga. Dispersive X-ray fluorescence spectroscopy was used for the selective detection of Br-containing species, which can be localized using this technique. It has been observed that intracellular vesicles called *corps en cerise* contain elevated amounts of this metabolite.[112] A recent study demonstrated that these halogenated terpenoid-containing vesicles can travel to the cell-wall region of the alga.[113] Interestingly, this process, which delivers the terpenoids to the algal surface, can be induced by the presence of fouling bacteria, which is the first report of an induced exocytosis of secondary metabolites from algae.[113] Such induced mechanisms might help to reduce the costs affiliated with secondary metabolite production, thereby increasing the performance of the algae.[114] Related storage systems are also reported for other halogenated metabolites, such as brominated furanones **60–63** from *D. pulchra* or the halogenated heptan-2-ones **27–31** from *B. hamifera*.[53,115] These algae localize the brominated metabolites in specialized gland cells that mediate the release of bioactive molecules onto the surface of the alga (Fig. 2).[116] Such gland cells have also been identified in the red algae *Asparagopsis* (Falkenbergia stage). Light-microscopy techniques revealed that a threshold bromide concentration in the medium is necessary to form and maintain these vesicle cells.[117] Further studies established stalk-like structures connecting gland cells with the outer wall of the pericentral cells, and might provide a mechanism for the transfer of metabolites to the algal surface.[118] Such storage-and-release systems have so far only been reported from red algae.

A critical point in chemical ecology is the determination of the ecologically relevant concentration that is encountered by organisms co-occurring with the producer. In antifouling assays, the surface concentration is considered to be the relevant factor. Establishing this concentration is not trivial, and offers challenges for analytical chemists. Some methods are based on the extraction with an organic solvent such as hexane,[119] but these methods are limited to low-polarity solvents, since the algal cells should not be destroyed by the solvent treatment. Recent studies employed desorption electrospray ionization mass spectrometry imaging (DESI-MS) techniques to demonstrate a surface-associated chemical defense of the red algae *Callophycus serratus* against the fungal pathogen *Linda thalassiae*.[120] On the surface of this alga, a patchy distribution of bromophycolides was observed with concentrations that were sufficient to maintain a chemical defense.[120] The ability of DESI-MS to reveal spatial distribution patterns of bioactive molecules at micrometer resolution has a definite potential to improve our understanding of chemical interactions on algal surfaces.

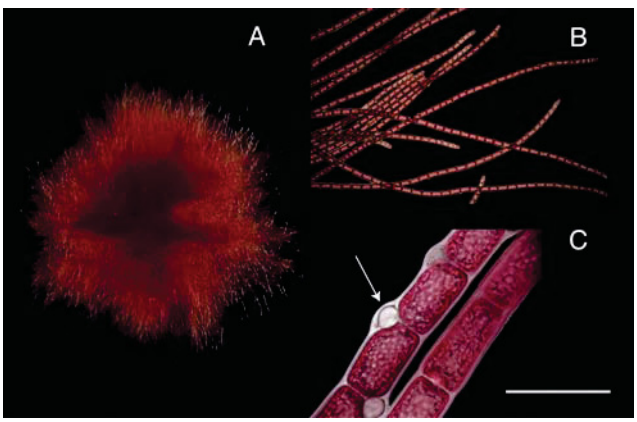


Fig. 2 *Bonnemaisonia hamifera*: (A) in the tetrasporophytic phase; (B) filaments; and (C) a one-layer-thick filament. The arrow in panel C points to a gland cell that stores bioactive halogenated compounds. Scale bars: A = 1 cm, B = 700 μm, C = 60 μm. Re-printed with permission from *Marine Ecology Progress Series*.[116]

The activity of halogenated metabolites from algae is, however, not limited to fouling and defense against pathogens. Herbivory, which can have a major impact on algal performance, is also influenced by this compound class.[121] The involvement of halogenated metabolites, in particular brominated secondary metabolites, in chemical defense of red algae have also been brought forward by Paul *et al.*[122] The authors tested the consumption rate of different algae using the generalist *Hyale nigra*. They compared the consumption rate of filamentous algae with cellular inclusions containing halogenated metabolites to algae without gland cells, and found a higher potential of chemical defense in algae with inclusions. The removal of bromide from the media of *A. armata*, an alga with cellular inclusions, resulted in a clearly reduced chemical defense activity against the herbivore *H. nigra*, thereby suggesting the involvement of brominated compounds in the defense of this species. The specific activity of secondary metabolites is demonstrated by numerous bioassays using volatile halogenated compounds and a broad spectrum of marine herbivores. Macroalgae are capable of deterring herbivore feeding with halogenated terpenoids including monoterpenes,[123–125] sesquiterpenes[121,126,127] and brominated hydroquinones.[121]

5 Conclusion

Marine algae are a rich source of volatile halogenated metabolites, in particular, halomethanes which make algae a substantial contributor to the global budget of these molecules. Nevertheless, little is known about their physiological function and their roles in ecological interactions. Unfortunately, it is very difficult to address such aspects for metabolites that are ubiquitous in organisms and their environment. This is especially true for phytoplankton where their overall concentrations in the water column might be relevant, as well as locally elevated amounts of the metabolites in the immediate vicinity of the producing cells. In some cases, results will be difficult to rationalise by the accepted models of plankton ecology. New concepts involving both physiological consequences as well as considerations of chemical ecology will be required to produce new explanations for the potential costs and functions for the producer. In the near future, the availability of whole-genome sequences for several micro- and macroalgae, coupled with emerging transformation and silencing techniques, will open new routes to an in-depth exploration. In strong contrast to simple methyl halides from micro- and macroalgae, considerably more is known about the elaborate halogenated natural products found in many

macroalgae for which functions in chemical ecology can often be clearly defined. Due to the presence of halogen atoms, these metabolites often have exceptionally high biological activities and can aid in chemical defense or act as antifouling agents for the producing organism. Often these biologically active metabolites are produced in high quantities. Especially in dense kelp forests or in dense red algal populations, these metabolites can be very abundant, and might not only play a role for the producer itself but also cause cascading effects. Such complex interactions influencing whole ecosystems still await exploration.

6 Acknowledgements

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5 Discussion

A paper published in the early 1960's first introduced the "paradox of the plankton" and raised the question "how it is possible for a number of species to coexist in a relatively isotropic or unstructured environment all competing for the same sort of materials" (Hutchinson 1961). Even though this question cannot be definitively answered it spurred a number of research fields. Several authors have suggested allelopathy as an essential driving force to sustain this biodiversity (Gross 2003; Legrand *et al.* 2003). The interactions between different diatom species have aroused certain interest with the observation that biologically active compounds are exuded by diatoms. However, neither a description of the effect of these exudates on the metabolism of diatoms nor a detailed chemical characterization of the active compounds has yet been performed.

Bacteria, due to their high abundance and metabolic potential, are also likely to interact with diatoms but only few active bacterial compounds affecting diatom growth are described. The effects of diatom compounds on bacterial communities are similarly poorly understood. PUA, a suite of diatom derived compounds, have been shown to affect bacterial growth and metabolic activity in laboratory experiments but so far these results were not verified in field experiments or situations which closely mimic the natural environment.

Traditionally, bioassay guided fractionation, after the development of elaborate bioassays, has been used to tackle the identity of the bioactive compounds. Even though this procedure is still a valid method, the application of metabolomics in marine chemical ecology has been suggested as an alternative approach (Goulitquer *et al.* 2012). Previously to this work, UPLC-MS and GC-MS based metabolomics protocols were developed and thus can be applied to questions concerning allelopathy of diatoms.

Co-culturing techniques to investigate interactions in non-contact situations

It is possible that interactions among diatoms and between diatoms and bacteria are detectable on different levels. For example, if researchers only look for an altered diatom growth they probably miss more subtle interactions that occur on the level of

changes in the metabolism in one or both interaction partners. The scope of studies can be broadened by the additional use of a more sensitive method such as mass spectrometry based metabolomics. This method can thus detect interactions that might not be observable by monitoring simply the algal growth. The influence of grazers on the production of specific metabolites (Selander *et al.* 2006) and algal morphology (Bergkvist *et al.* 2012; Long *et al.* 2007; Selander *et al.* 2011) is well documented while the influence of microorganisms, including other diatoms and bacteria, on algae is far less well understood. This might at least partly be due to methodological limitations. For example, an investigation of the influence of bacteria on the metabolism of diatoms requires that both partners can exchange molecules that might be functional in that interaction. In parallel it has to be guaranteed that both organisms can be investigated separately from each other in order to be able to determine the metabolism of one a single cell type. This can be achieved with a setup consisting of two culture chambers which are separated by a membrane.

In order to perform such co-culturing experiments, I first used dialysis tubes with a molecular weight cut off (MWCO) of 12 kDa. After filling the tubes with cultures of the diatom *T. weissflogii* and closing them, these dialysis tubes were placed in glass flasks which were filled with cultures of *S. costatum* (**Manuscript E**). To prevent differences that might occur between cultures that were inside or outside the dialysis bag I additionally inoculated the dialysis bags with *S. costatum* and placed them in glass flasks filled with *T. weissflogii*. The metabolic profiles of these co-cultures were then compared to the corresponding mono-cultures consisting of the same species inside and outside of the dialysis tube. A similar non-contact co-culturing approach was previously applied in plankton ecology to investigate the effect of diffusible compounds on different phytoplankton species (Jensen *et al.* 1972; McVeigh and Brown 1954; Yamasaki *et al.* 2007) because it has several advantages in comparison to conventional methods such as the addition of cell free filtrate or one pot mixed cultures. First, non-contact co-culturing can discriminate between effects caused by diffusible substances and effects caused by direct cell contact. Indeed, the alga *Gyrodinium instriatum* cultured in direct contact with the dinoflagellate *Heteracapsa* sp. experienced reduced growth accompanied by the loss of flagella and girdles which was not observed after the application of *Heteracapsa* sp. cell free filtrates (Uchida *et al.* 1995). Additionally,

experiments using cell free filtrates as done by several investigators e.g. (Fistarol *et al.* 2005; Suikkanen *et al.* 2005) have trouble detecting effects which are caused by unstable substances. The non-contact co-culturing technique described in **Manuscript D** is, however, capable of monitoring the effect of unstable substances. Finally, the production of certain metabolites might be induced by diffusible, potentially unstable, substances from co-occurring organisms as shown for the paralytic shellfish poison production in *Alexandrium minutum* (Selander *et al.* 2006).

Nevertheless, even though it offers several appealing advantages, the use of dialysis bags (**Manuscript E**) has some drawbacks. Until now, there has been no quantitative data available to describe the diffusion efficiency of metabolites relevant to plankton. Thus, to some extent it remains uncertain if the setup used is entirely suitable for allowing chemical signals to pass between cultures of diatoms. Further, the use of dialysis tubes might result in altered growth conditions between cultures inside and outside the tubes. In addition, the dialysis tubes cannot be easily opened or sampled which increases the need for suitable control cultures and complicates the interpretation of the obtained results.

Hence, there is a demand to establish an easy operable co-culturing device which can overcome the drawbacks presented above. **Manuscript D** presents the development of a co-culture device which eliminates these problems. This setup consists of two culturing chambers, separated by a 0.22 µm membrane, that provide identical growth conditions. Both chambers contain an additional opening which can be used for inoculation and sampling. In contrast to commercially available devices which harbor only limited volumes of approximately 5 mL (Yamasaki *et al.* 2007), the developed setup can be used with a maximum of 500 mL per culturing chamber offering enough sample volume for comprehensive metabolic surveys.

A prerequisite for the monitoring of chemically mediated interactions is that metabolites involved in interactions diffuse between culture chambers at a sufficient rate. I tested the diffusion of several relevant metabolites. When nitrate, an essential macronutrient for phytoplankton growth, was added to only one culturing chamber, tests 24 h later showed equal concentrations in both culture chambers. Similar results were obtained for DMSP, a metabolite with multiple functions in the plankton (Pohnert *et al.* 2007; Seymour *et al.* 2010; Steinke *et al.* 2002). Further, the more nonpolar heptadienal

reached also equal concentrations in both chambers after 24 h. However, the equilibrium was at roughly 30% of the inoculated concentration suggesting a loss of heptadienal e.g. due to its volatility or by adsorption to the glass or to the membrane. Thus, the setup facilitates the diffusion of metabolites with a broad range in polarity which suggests that the diffusion is sufficient for most compounds even though in this investigation only a limited number of substances were tested. Further, the co-culture device offers similar growth conditions between different setups which ensure a high reproducible culture growth. In summary, this setup offers great advantages in comparison to previous handmade devices and can be applied to a variety of different types of experiments such as metabolomics based investigations.

Impact of co-existing organisms on diatom growth and metabolism

The co-culture approach combined with MS based metabolomics was performed for both diatom-diatom and diatom-bacteria interactions. For the investigation of diatom-diatom interactions I used *S. costatum* and *T. weissflogii*, two algae commonly used in plankton research e.g. (Casotti *et al.* 2005; Sarno *et al.* 2005). During the experiment, the cell abundance of *S. costatum* in mono- and co-cultures did not differ at any time point, suggesting that *T. weissflogii* exudates do not influence *S. costatum* growth (**Manuscript E**). In contrast, *S. costatum* had a growth dependent effect on the growth of *T. weissflogii*. In the early stages of the experiment including exponential and beginning of stationary growth of both algae, *S. costatum* had no detectable effect on *T. weissflogii* growth. However, during the late stationary growth the cell concentration of *T. weissflogii* in co-cultures increased significantly in comparison to *T. weissflogii* in control cultures. The cultures in the dialysis bags could be sampled only at the end of the experiment and revealed an enhanced cell growth of *T. weissflogii* in co-cultures verifying the stimulating effect of *S. costatum* exudates on *T. weissflogii* growth. Often studies dealing with allelopathic interactions consider only negative interactions as allelopathic. However, the outcome of such interactions varies depending on multiple factors and can be very complex. For example, in the interaction between *S. costatum* and *Heterosigma akashiwo* the alga first dominating in a co-culturing experiment can inhibit the growth of the other (Yamasaki *et al.* 2007). Further, the allelopathic effect of *S. costatum* strongly depends on the growth phase of *S. costatum* with late stationary

and declining phase cultures being the most potent concerning the inhibiting capability against *H. akashiwo* which is in accordance to the growth stage dependent effect oberserved in **Manuscript E**. Noteworthy, in another investigation filtrates inhibiting the growth of *H. akashiwo* had a growth promoting effect on *Thalassiosira* sp. (Yamasaki *et al.* 2011) which indicates a species specific effect and is again in accordance with the results obtained here. A growth promoting effect of *S. costatum* was also observed on the macroalga *Ulva pertusa* (Nan *et al.* 2004) which suggests that the growth stimulation of *S. costatum* is more prominent than previously thought.

Besides the effect on growth I looked for changes of the metabolic profile of both intra- and extracellular compounds using UPLC-MS. By investigating the extracellular compounds surrounding diatoms, it became obvious that both diatom species are surrounded by a unique chemical sphere, which is in accordance with previous results (Barofsky *et al.* 2009). Here, the pattern of metabolites released did not only differ between different diatoms but also changed in response to the co-cultured interaction partner. For instance, I found several substances that were only present in the culture medium of *S. costatum* grown alone, but not in the medium of the co-culture. Similarly, one substance present in the medium of *T. weissflogii* was also not present in the co-cultures indicating an interaction between species which affects the metabolism of the algae (**Manuscript E**). The reason for that observation can be diverse. For example, an inhibition of the biosynthesis or of excretion of specific compounds could cause the changes observed. Additionally, uptake of the released substances by the co-cultured diatom would change the metabolic profile and potentially indicate a heterotrophic or mixotrophic life style. A heterotrophic life strategy involving the utilization of a wide range of organic substrates including carbohydrates and amino acids has been shown for freshwater benthic diatoms under reduced light (Tuchman *et al.* 2006). Unfortunately the chemical identities of these relevant compounds in **Manuscript E** remain unknown. I aimed to deduce structural elements by performing MS/MS experiments as well as accurate mass measurements. Nevertheless, the amount of substance was not sufficient to obtain reliable data. I further tried to perform large scale culturing of *S. costatum* to obtain a sufficient amount of biomass. Here, I could not recover the mass-retention time pairs I previously discovered to be of significance (**Manuscript E**; Paul, unpublished data). This might have multiple reasons. For example the culturing was performed in a

different device and extracted at a single time point. The metabolites produced by diatoms intra- and extracellularly depend strongly on the age of a culture (Barofsky *et al.* 2009; Myklestad *et al.* 1989; Vidoudez and Pohnert 2011) which makes the recovery of the previously detected metabolites highly challenging. However, it seems unlikely that the relevant compounds were amino acids or carbohydrates, substances that make up a significant part of released metabolites in diatoms (Granum *et al.* 2002), because these classes of compounds should have not be retained on the solid phase extraction cartridges and should show a different behavior during the chromatographic separation. Additionally, zwitterionic substances such as DMSP which *T. weissflogii* can take up highly efficiently (Spielmeyer *et al.* 2011) can be excluded due to the same reason.

In summary, even though no effect on *S. costatum* growth could be observed the alga responded to the co-culturing as indicated by a change of the intracellular metabolic profile (**Manuscript E**). Several substances from *S. costatum* cells were found in higher concentrations in the co-cultures than in the controls which might indicate a general stimulation of the algal metabolism. In contrast, all metabolites differentially regulated in *T. weissflogii* are down regulated in co-cultures in comparison to controls. Metabolic responses to abiotic environmental factors have recently been described. For example, diatoms can alter their membrane lipid composition during phosphorous limitations by exchanging phosphorous containing lipids with non-phosphorous containing lipids (Van Mooy *et al.* 2009). Further, a whole cell response including processes such as mitochondrial electron transport and nitrate assimilation was also shown for iron limitation in *P. tricornutum* (Allen *et al.* 2008) as another example of an altered diatom metabolism upon abiotic stress.

In order to identify relevant compounds, I performed a GC-MS based metabolomics approach instead of UPLC-MS. GC-MS is usually executed with electron impact ionization with 70 eV which enables the characterization of substances based on the masses of the fragments obtained. Further, extensive databases are available from plant metabolomics experiments (Wagner *et al.* 2003) which should enable the identification of many metabolites, especially from algal primary metabolism. Thus, this approach is suitable to investigate the impact of bacteria on the cellular primary metabolism of diatoms.

In this investigation I co-cultured the diatom *T. pseudonana* with the bacterium *Dinoroseobacter shibae* (**Manuscript D**). *T. pseudonana* was the first diatom with a completely sequenced genome (Armbrust *et al.* 2004) and *D. shibae* is a ubiquitous algal symbiont which also has a fully sequenced genome (Wagner-Döbler *et al.* 2010). I aimed to evaluate the effect *D. shibae* has on *T. pseudonana* growth as well as to monitor the metabolic response of a diatom cell to co-occurring bacteria.

During the first days of the experiment (day 0 to 3) the growth of *T. pseudonana* in co- and monocultures did not differ from each other. However, during the last two days of the experiment (day 4 and 5) the cell abundance of *T. pseudonana* cultured with the bacterium was significantly higher than in mono-culture controls. There are many potential explanations for that enhanced diatom growth. Bacteria are known to produce growth promoting factors (Cole 1982; Croft *et al.* 2005; Seyedsayamdost *et al.* 2011b) and *D. shibae* specifically was shown to enhance algal growth by the production of Vitamin B1 and B12 (Wagner-Döbler *et al.* 2010). Further, one might expect different nutrient concentrations at some point between controls and co-cultures because both culturing chambers in controls were inoculated with *T. pseudonana* but only one in co-cultures. Still, a nutrient limitation in *T. pseudonana* controls is unlikely to be an explanation for the enhanced growth in co-cultures. I monitored photosystem II (PSII) efficiency in both treatments. PSII efficiency is used as diagnostic tool to detect nitrogen, phosphorous and silicate limited microalgae (Kolber *et al.* 1988; Lippemeier *et al.* 1999; Liu *et al.* 2011). I could not detect any differences in the PSII efficiencies between diatoms in controls and co-cultures, suggesting that no nutrient limitation occurred. Further, a test of nitrate concentrations in the media revealed no difference between both treatments at more than 300 µM which should not be limiting (Spielmeyer and Pohnert 2012). Thus, the cells in both treatments had a similar physiological status which is a requirement for a sensible comparison of the metabolic profiles. However, the algal abundances were different for both treatments and needed normalization before a comparison of the metabolic profiles could be conducted. I used the same amount of extracted cells in all samples in order to achieve a normalization based on the cell abundance.

The comparison of the metabolic profiles revealed that no substances in mono-cultures were up-regulated in comparison to co-cultures. These results suggest that *D.*

shibae does not inhibit the biosynthesis of any intracellular substance. In contrast, several substances were found in higher concentration in diatom cells grown in the presence of *D. shibae* than in *T. pseudonana* cells grown without *D. shibae*. **Manuscript D** gives a list of these tentatively identified substances. These compounds include some short length organic acids, sugars, and especially amino acids. However, from this experiment it cannot be concluded if this increased cellular concentration of amino acids is due to increased production resulting from a chemical bacterial signal or due to an uptake of amino acids released by bacteria from the culture medium. In general, it is known that diatoms such as *Thalassiosira* sp. and *Phaedactylum* sp. can take up amino acids from the surrounding medium (Admiraal *et al.* 1984; Admiraal *et al.* 1986; Flynn and Wright 1986). Further, the importance of amino acids in diatom-bacteria interactions was recently stressed for the benthic diatom *Fragilaria pinnata*. Here, the pool of dissolved free amino acids was significantly altered in the presence of bacteria. For example, the histidine concentration was higher in axenic cultures than in diatom-bacteria co-cultures, while the concentration of isoleucine was much higher in co-cultures than in axenic controls (Bruckner *et al.* 2011). Thus, it seems possible that the enhanced intracellular amino acid concentration detected in my experiment is a consequence of an altered pool of dissolved free amino acids which can be taken up. Regarding the uptake mechanism, an active assimilation seems more likely for the uptake because the concentrations in the surrounding medium are presumably too low to facilitate a passive diffusion (Hellebust and Lewin 1977).

Interestingly, the metabolite picolinic acid, a tryptophan catabolite, was also found in higher concentration in co-cultures. Although it's function is unknown, previous studies have suggested that related metabolites play a role in bacterial-algal interactions. A picolinic acid derivative isolated from marine bacteria called thallusin was necessary for the cell differentiation in the macroalga *Monostroma oxyspermum* (Matsuo *et al.* 2005). Further, picolinic acid can also act as a siderophore by chelating iron and thus might potentially be involved in a symbiotic interaction.

Taken together, these results suggest that complex fluxes of metabolites exist among diatoms species as well as between diatoms and bacteria. The heterotrophic utilization of organic substrates is a well known concept in plankton ecology (Hellebust and Guillard 1967). The uptake of organic compounds might, however, not be limited to

metabolites as sugars (Neilson and Lewin 1974) and amino acids (Rivkin and Putt 1987) but rather a variety of excreted substances could be utilized by diatoms. The uptake is dependent on irradiance, with a higher uptake rate during the dark (Hellebust 1971). Assuming one cell produces an excess of photosynthetic products during day light periods these can be taken up again during dark periods. Thus, the excretion of organic substances might not only serve as signals in chemical interactions but also as an extracellular storage place of organic substrates. In case competing species are also present there might be not only a competition for nutrients such as phosphate but also for previously released metabolites. In an environment with such an enhanced competitive character the use of inhibiting allelopathic substances would most likely be even more favorable.

Interactions of diatoms with the model bacterium *K. algicida*

The metabolic profiling approach has allowed for the detection of subtle interactions based on extracellular and intracellular metabolic responses to biotic stimuli. In addition, a bioassay guided fractionation approach was used in order to determine what compounds elicit a growth or metabolic response from diatoms.

K. algicida, a marine bacterium originally isolated from a *S. costatum* bloom, inhibited the growth of *S. costatum* (Sohn *et al.* 2004) but the inhibitory substance and regulation mechanism for the production of this active substance had not been previously described. Recently, the genome of *K. algicida* was sequenced and it has been suggested that polyketide synthases are involved in the algicidal activity of *K. algicida* (Lee *et al.* 2011). To follow this hypothesis, I performed a preliminary investigation on the activity of the polyketide gene cluster of a *K. algicida* culture that inhibited the growth of *S. costatum* using real time polymerase chain reaction (RT-PCR) (Paul and Behnken, unpublished results). These results revealed that the polyketide gene cluster was not active and thus polyketides are unlikely to contribute to the observed algicidal effect.

Subsequently, I used a bioassay guided fractionation approach in order to characterize the growth inhibiting substance(s) produced by *K. algicida* (**Manuscript A**). I determined that the active substances had a molecular weight of more than 30 kDa pointing to an involvement of proteins in the interaction between *S. costatum* and *K.*

algicida. I hypothesized that such proteins might well be proteases because these enzymes are known to mediate interactions between bacteria and phytoplankton. For instance, the action of bacterial proteases to inhibit the growth of *S. costatum* was shown for the γ-proteobacteria *Pseudoalteromonas* sp. (Lee *et al.* 2000). Further, proteases were also responsible for the motility reduction of the dinoflagellate *Lingulodinium polyedrum* (Mayali *et al*. 2008). Applying protease inhibitors and using commercially available protease demonstrated clearly that proteases, released by *K. algicida,* are at least partially responsible for the bacteria's ability to suppress diatom growth. The use of such diffusible substances for algicidal activity is a common phenomenon for γ-proteobacteria e.g. (Imai *et al.* 1995; Mayali and Azam 2004). For bacteria of the *Cytophaga-Flavobacterium-Bacteroides* phylum, such as *K. algicida,* this mechanism is far less common although a few exceptions exist (Doucette *et al.* 1999; Skerratt *et al.* 2002). However, releasing diffusible substances to inhibit algal growth might be not energetically efficient unless a regulation mechanism governs the timing of the release. For example, releasing the inhibitory proteases only in the presence of the target algae would be an efficient way to kill it. However, *K. algicida* releases the protease irrespective of the presence of *S. costatum* as target algae (**Manuscript A**). Another possible mechanism to decrease the biosynthetic costs of the algicide production is a metabolic switch dependent on bacterial density as known from quorum sensing (QS) (Fuqua *et al.* 1994). Here, after reaching a threshold autoinducer concentration a genetic cascade starts which results in the production of compounds which benefit the bacterial populaiton such as extracellular enzymes. Using *K. algicida* conditioned cell free filtrates, fresh bacterial cultures initiated protease release significantly earlier than in untreated control cultures (**Manuscript A**). These results indicate that *K. algicida* releases an autoinducer during growth and thus the protease release is likely governed by QS.

In gram negative bacteria such as *K. algicida* these autoinducers are often N-acyl homoserine lactones (AHL) (Waters and Bassler 2005) which regulate the pathogenicity in bacteria such as *Pseudomonas aeruginosa* (Jones *et al.* 1993; Valade *et al.* 2004). Indeed, the importance of AHL in the production of an algicidal pigment called PG-L-1 (Nakashima *et al.* 2006a) by a marine bacteria named MS-02-063 has lately been demonstrated. Here, the pigment production decreased after the application of the AHL

inhibitor β-cyclodextrin suggesting the involvement of AHL in the PG-L-1 production (Nakashima *et al.* 2006b). However, in a standard methylene chloride extraction (Morin *et al.* 2003) in combination with GC-MS measurements which typically result in AHL specific fragments (Cataldi *et al.* 2004) there was no evident found for the presence of AHL in *K. algicida* filtrates.

In general, gram positive and negative bacteria are known to use also other types of autoinducing substances such as oligopeptides, γ-butyrolactones, quinolones or borate derivatives (Waters and Bassler 2005; Williams *et al.* 2007). Further bioassay guided fractionation of the *K. algicida* conditioned medium should reveal the substance *K. algicida* exploits for the QS regulating system. Indeed, *K. algicida* had only a 93 % 16S rRNA sequence similarity with the most related bacterial species when published (Sohn *et al.* 2004) and thus one might speculate that this genus might use a class of compounds for a QS regulation system which has not yet been associated with such a function.

From an ecological perspective it is important to assess the specificity of the active compounds. In case of *K. algicida* the proteases did not only inhibit the growth of *S. costatum* but also a variety of diatoms including *T. weissflogii* and *P. tricornutum*. Interestingly, the diatom *C. didymus* was not affected by the released proteases (**Manuscript A** and **Manuscript B**). Such algicidal specificity may have a dramatic impact on the relative abundance of algal species within the plankton community, but so far the reasons for resistances as observed for *C. didymus* have remained obscure.

In a first attempt to elucidate the reason for that resistance I looked for a detoxification of the bacterial protease by *C. didymus* (**Manuscript A**). Exposure of the bacterial filtrate to *C. didymus* did not change the effect of the filtrate on *S. costatum* suggesting that no active detoxification exists. In another approach I aimed to investigate the protease activity of *K. algicida* filtrates after applying them to *C. didymus* and *S. costatum* cultures. Here, I used a fluorescent based method for the quantification of protease activity (Jones *et al.* 1997) (**Manuscript B**). The results showed that the protease activity remained constant for *S. costatum* cultures while the protease activity in *C. didymus* cultures increased significantly, indicating that there is an induced protease release by the resistant diatom *C. didymus* due to the presence of bacterial substances. To verify this hypothesis I aimed to profile the protease released

by *C. didymus* in response to bacterial exudates. Therefore the algal proteinaceous exudates were concentrated by a factor of approximately 100, applied to a gel electrophoresis and specifically proteases were detected by zymography. This technique facilitates lower detection limits for proteases than standard coomassie staining (Paul, unpublished data) and is thus suitable for the profiling of released proteases. This approach revealed that *K. algicida* exudates induce a massive release of algal protease by *C. didymus*. Further fractionation of the bacterial filtrate revealed that bacterial proteins including proteases trigger that response in *C. didymus*. Overall, results suggest that resistant diatom *C. didymus* releases its own protease in response to proteins from *K. algicida* while the susceptible diatom *S. costatum* does not show such induced physiological response. Such a response might well be involved in the resistance mechanism of *C. didymus* giving it an ecological advantage with respect to susceptible species.

It is tempting to assume that the algal protease release is involved in the resistance mechanism by the degradation of bacterial protease, but final experimental evidence is lacking, primarily due to methodological limitations. Final evidence would be an active detoxification by *C. didymus* which can be proven by the application of *C. didymus* conditioned medium to *S. costatum* as performed in **Manuscript A**. Unfortunately, if the proteases produced by *C. didymus* also inhibit the growth of *S. costatum* as seems likely, that approach is unsuitable for this purpose but emphasizes the potential impact of such induction on the plankton community. From an ecological point of view such strategy seems plausible. If *C. didymus* degrades bacterial proteins it needs to avoid that other algae such as *S. costatum* benefit from that degradation. That can be easily prevented if the competing species are killed at the same moment. Another approach to prove the function of induced proteins would involve the detection of bacterial protease with and without the addition of algal protease to determine whether bacterial proteins are degraded by algal proteases. Preliminary results of such approach did not give any evidence for such a mechanism. However, those experiments only focused on proteases but other enzymes might be also involved in the deleterious effect of *K. algicida* (**Manuscript A**). Thus, it would be certainly better to cover the effect on the whole range of proteins released by *K. algicida*. Furthermore, such approach also entails several difficulties. For example, the incubation of bacterial filtrates with *C. didymus*

only occurred for some hours, and was not in the range of culturing times (several days). Further, an experiment that involves the simple mixing of two protease solutions would exclude the impact of living diatoms cells. Thus, while future experiments should try to finally link the protease induction with the resistance of *C. didymus* against *K. algicida*, it might be necessary to develop more methods or to concentrate bigger volumes in order to obtain a higher sensitivity.

The scientific community has gained a greater appreciation for the general influence of bacteria on the performance of diatoms in recent years. For example, production of transparent exoploymer particles (TEP) and coomassie blue stainable particles (CSP) by *Thalassiosira rotula* was strongly depending on the presence of bacteria (Grossart and Simon 2007). For *T. weissflogii* this TEP production, which is again dependent on the presence of co-existing bacteria, was further linked to aggregate sinking velocity (Gaerdes *et al.* 2011) highlighting the significance of diatom-bacteria interactions for element cycling processes. Lately, it was also demonstrated that the bacterium *Escherichia coli,* commonly used as a models species, induced protease production in *P. tricornutum* (Bruckner *et al.* 2011) although the ecological relevance of this interaction is unclear. The results presented in **Manuscript B** extend the previous knowledge of diatom-bacteria interactions because none of these studies had characterized the substances eliciting a response in the alga.

While in the example of *K. algicida* the function of the induced protease release remains to some extent speculative, the induction of intracellular proteases in diatoms is better documented. For instance, a heat shock of 30°C induces intracellular proteases in *Chaetoceros compressum* (Kinoshita *et al.* 2001). Similarly, the diatom *T. weissflogii* induces a protease production upon light and nitrogen stress which was suggested to be involved in an autocatalyzed cell death (Berges and Falkowski 1998). In accordance, intracellular cysteinyl aspartate-specific caspase orthologues called metacaspases were suggested to trigger programmed cell death in marine phytoplankton which has been suggested to guarantee the survival of algal cells (Bidle and Bender 2008; Bidle and Falkowski 2004). Similarly the induced protease release in response to biotic interactions might be advantageous for *C. didymus* if bacterial proteins are degraded or competing diatom species are inhibited in growth.

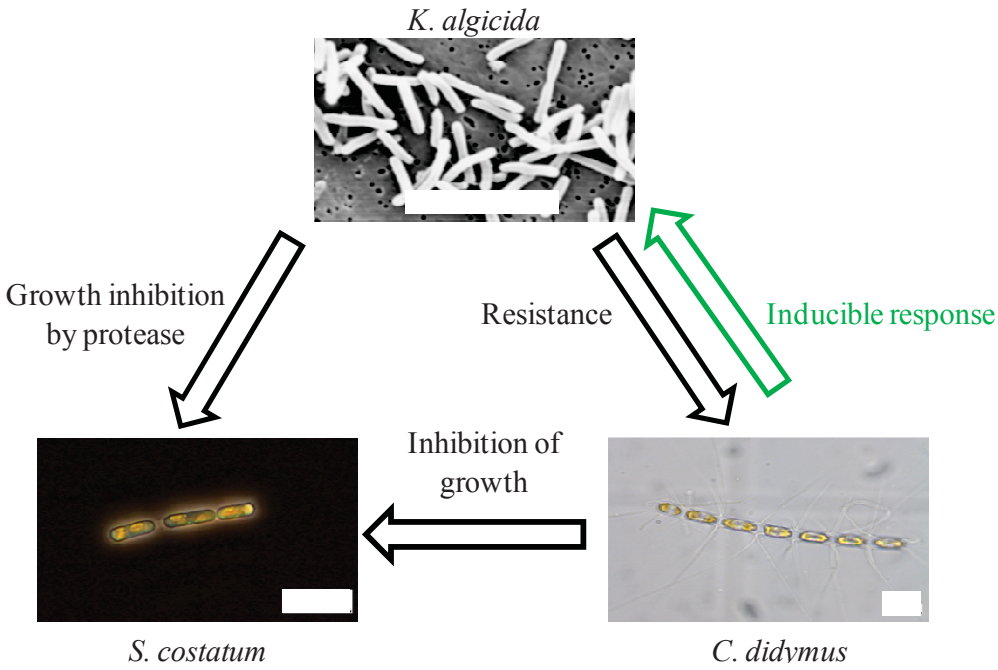

Figure 5: Summary of the different interactions of K. algicida *with the diatoms* S. costatum *and* C. didymus*. Scale bars: 5 µm for* K. algicida *(Sohn* et al. *2004) and 20 µm for both diatom species.*

In summary, *K. algicida* interacts with different diatoms in various ways (**Figure 5**). Susceptible diatoms such as *S. costatum* encounter growth suppression by bacterial proteases with a release governed by a QS regulation mechanism. In contrast, *C. didymus* is not affected and releases its own protease in response to bacterial enzymes. This induction has an influence on *S. costatum* growth and might serve as a bacterial protease degradation mechanism to enable *C. didymus* to survive the stress encountered by bacterial proteases in diatom-bacteria interactions.

Influence of diatom derived PUA on bacterial community structure

Diatoms might not only influence the bacterial community by the release of high molecular weight enzymes but also by low molecular weight secondary metabolites. PUA, produced by diatoms after wounding (Pohnert 2000) and released during late stationary growth (Vidoudez *et al.* 2011a; Vidoudez *et al.* 2011b; Vidoudez and Pohnert 2008), are acknowledged to have an effect on the bacterial community structure in laboratory experiments using often elevated concentrations (Adolph *et al.* 2004; Balestra *et al.* 2011; Ribalet *et al.* 2008). However, a verification of that effect had not

previously been performed under conditions similar to those in the natural environment. To achieve these conditions, mesocosm experiments are especially well suited because they are more representative of the natural environment than fully controlled laboratory experiments and better manageable than non-controlled field experiments (Escaravage *et al.* 1996; Grice and Reeve 1982).

To elucidate the effect of PUA on the microbial community, including bacteria and virus abundance at natural and artificially high PUA concentrations, we used indoor mesocosms with a fully controlled light regime and temperature conditions. Each tank was filled with approximately 2000 L of natural seawater which contained the natural microbial community of that specific side of the Bothnian Sea. The experiment consisted in total of 4 treatments: two different *S. marinoi* inoculations (SKE1 and SKE2), one seawater control and one seawater treatment which was supplemented with PUA at natural and above natural concentrations during different time periods. After the addition of nutrients and inoculation of the tanks with two different *S. marinoi* strains a bloom of this species developed which species composition was, beside *Skeletonema* sp., similar to what is naturally found in the Bothnian Sea (Ask *et al.* 2006). Also the intensity of the diatom bloom, measured as chlorophyll a concentration (**Manuscript C**), is comparable to previous diatom dominated mesocosm experiments (Jonasdottir *et al.* 2011; Riemann *et al.* 2000) illustrating that the experiment represented ecologically relevant conditions well.

The first PUA addition was intended to reach a final concentration of 1 nM of heptadienal and octadienal. At the first sampling after the PUA addition only a fraction of the added PUA was found. That is not surprising because PUA have a high volatility, might be degraded by abiotic or biotic factors or might be adsorbed by the mesocosm container. However, after repeated addition concentrations close to 1 nM were detected and were similar to concentrations found in nature (Vidoudez *et al.* 2011a; Vidoudez *et al.* 2011b). Later on, during another PUA addition period, higher additions yielded detectable concentration of maximal 600 nM and 200 nM for heptadienal and octadienal respectively. This was designed to simulate above natural concentrations as used in most laboratory experiments (Adolph *et al.* 2004; Ribalet *et al.* 2008). The controls had negligible PUA concentrations in the beginning of the experiment and at no point were PUA concentrations greater than 1 nM detected being in the same order of magnitude

than the low PUA treatment. The *S. marinoi* treatments yielded, as intended, different PUA release profiles with SKE2 releasing roughly twice as much PUA as SKE1 while nearly no PUA was found in control mesocosms. Thus, the different treatments had different concentrations of PUA which facilitate the elucidation of the effect of PUA on the microbial community (**Manuscript C**).

In order to describe the effect of PUA on the microbial community an obvious parameter to evaluate is the abundance of bacteria and viruses. The data clearly demonstrate that PUA have no effect on the growth of viruses and bacteria. For example, the virus abundance in control and PUA treatment did not differ during the whole experiment demonstrating that PUA do not influence virus abundance. For bacteria, the abundance in the PUA and the control treatment did also not differ at any time between the treatments, even at 600 times the natural concentration, clearly pointing to at most a very minor effect of PUA (**Manuscript C**). Interestingly, in both *S. marinoi* treatments later on during the growth a significant lower bacterial abundance than in the control and PUA treatment was measurable. This might indicate that *S. marinoi* interacts with bacteria by the production of antimicrobial substances, a phenomena that was described previously for *S. costatum* without knowing the active chemical substance (Terekhova *et al.* 2009).

However, the effect of PUA can be overlooked if only the total bacterial abundance is concerned. A deeper understanding can be achieved if the relative changess of specific bacterial strains are also considered. A technique which can estimate the effect on natural bacterial assemblages is terminal-restriction fragment length polymorphism (T-RFLP) (Marsh 1999; Osborn *et al.* 2000). After multivariate data evaluation, e.g. with cluster analysis, the similarity between different samples can be estimated. In our mesocosm experiment the effect of PUA on the bacterial community was easily evaluated by comparing the T-RFLP profiles of control with PUA samples. These samples had, however, a high similarity even at high PUA concentrations strongly suggesting that PUA do not influence the bacterial community composition. Interestingly, at one sampling point one *S. marinoi* treatment had a unique bacterial community proposing that diatoms indeed shape actively its surrounding bacterial community but not by employing PUA (**Manuscript C**). Such temporal changes in bacterial community compositions during diatom blooms have been previously

described. For example, in a *Thalassiosira* sp. dominated mesocosm and in field experiments the composition of the bacterial community changed over time (Riemann and Middelboe 2002; Riemann *et al.* 2000). That bacterial communities in diatom cultures dynamically change was also shown by inoculation of a natural bacterial assemblage with two different axenic diatom species. Here, after a certain time both diatom species harbored different bacterial communities which were different from the starting assembly for both species (Grossart *et al.* 2005). While this example illustrates the effect of different diatom species, our results indicate that the bacterial diversity is even altered on a strain level. Nevertheless, that effect was only a short-term phenomenon (**Manuscript C**). In contrast to these results, researchers found also stable bacterial-algal associations (Schäfer *et al.* 2002). Such stable bacterial compositions might be observable if the temporal resolution of the measurements is not high enough and the short term fluctuations are missed.

The way that diatoms alter the bacterial community is still not completely understood but certainly dissolved organic matter plays a major role. The composition of the organic substances released is often a characteristic signature for a certain diatom species (Myklestad 1974) which also varies depending on the growth stage of the algae (Myklestad 1995). Further, the pool of organic substances which can be utilized by heterotrophic bacteria depends on the presence of other bacteria and on the available nutrient concentration for the algae (Grossart and Simon 2007). Further, in a metabolomics approach algal exudates varied significantly during culture growth. Even when the identities of these compounds were not elucidated the authors suggested that the detected substances are previously unknown metabolic excretions because standard amino acids and saccharides were excluded (Barofsky *et al.* 2009). Thus, the chemical sphere around an algal cell might be highly variable offering different ecological niches for bacteria. Assuming that bacteria can utilize this carbon pool in different ways (Tada *et al.* 2011), the area surrounding diatoms would be characterized by complex and highly variable bacterial communities. In accordance, it was recently shown that the availability of algal derived substrates determines the bloom of different bacterial populations by providing a series of ecological niches (Teeling *et al.* 2012).

Halogenated substances of diatoms in an chemical ecology context

Besides PUA, which are debated to have a signaling function in multiple interactions (see above and **Introduction**), diatoms are also capable of releasing halogenated compounds which can be encountered by co-existing organisms and are thus of interest in the context of chemical ecology. These substances include C1 substances with a varying degree of halogenation such as methylhalides and polyhalomethanes (Colomb *et al.* 2008; Gribble 1992; Hense and Quack 2009). Although they are structural closely related, the biosynthesis of methylhalides and polyhalomethanes differs. While the first class of compounds is synthesized via a methyl transfer using *S*-adenosyl-*L*-methionine (Wuosmaa and Hager 1990) the later is catalyzed by haloperoxidases (Beissner *et al.* 1981; Butler and Carter-Franklin 2004). The knowledge of the occurrence, relevance for algal physiology and ecology, as well as the geochemical impact of algal halogenated compounds from micro- and macroalgae is synthesized in **Manuscript G**. Interestingly, in contrast to macroalgae where several halogenated metabolites are known to play a central role in the algal ecology, there is only a limited knowledge about comparable functions in microalgae.

In **Manuscript F** we showed that the benthic diatom *Nitzschia* cf. *pellucida*, belonging to a genus known to produce a series of halogenated substances (Moore *et al.* 1996), can inhibit the growth of competing organisms by the release of active substances into the surrounding medium. A survey of the metabolites released revealed the presence of a series of halogenated substances such as bromoform and iodoform which did not inhibit the growth of competing algae. A further GC-MS screen unveiled BrCN to be released by *N.* cf. *pellucida* which biotic origin was proven by C^{13} labeling experiments. Using *Entomoneis paludosa* as target algae in bioassays the minimum lethal concentration was estimated to be 2 µM which is lower than the BrCN concentration found in cell free filtrates. BrCN can consequently explain the full activity of cell free *N.* cf. *pellucida* filtrates which is the first time that halogenated microalgal compounds have been shown to be active against competing species. BrCN was previously unknown as a natural product and its biosynthesis had not been addressed before this study. However, the origin of the bromine is most likely an oxidation of bromide with the aid of haloperoxidases. Because haloperoxidases are H_2O_2 dependent, after the application of the H_2O_2 decomposing enzyme catalase the

BrCN concentration was reduced. Further, brominated and iodinated substances were more abundant than chlorinated substances by far (only one substance found) in *N.* cf. *pellucida* cultures. These results are consistent with the fact that bromoperoxidases, capable of oxidizing bromide and less electronegative halogens, are known from algae (de Boer *et al.* 1986; Moore *et al.* 1996; Theiler *et al.* 1978) but no chloroperoxidases, known from fungi and potentially from marine prokaryotes (Morris and Hager 1966; Winter *et al.* 2007), have been identified. Thus, the chlorinated substance $C_2H_2Cl_2$ might originate from abiotic conversions, for example by nucleophilic substitution.

The source of the cyanide is less clear. Cyanogenic glycosides are known from plants (Vetter 2000). These glucosides are synthesized starting from amino acids and release HCN upon the activity of a β-glucosidase and α-hydroxynitrilases (Moller 2010). The green algae *Chlorella* was also shown to release HCN with the aid of an amino acid oxidase system using aromatic amino acids as substrates (Pistorius *et al.* 1977). No similar system has been described so far from diatoms. Further, the active substance is BrCN rather than the hydrolysis product HCN, since NaCN showed only minor effects in the bioassay suggesting a new biosynthetic pathway. The mode of action of BrCN cannot be explained with our experiments. Current protein sequencing approaches use BrCN to hydrolyze proteins at the C-terminus (Mortvedt *et al.* 1991). Thus, BrCN might be involved in protein degradation processes within the target algae.

Interestingly, chlorinated oxylipins originating from a hydroperoxide halolyase-mediated fatty acid transformation from the diatom *Stephanopyxis turris* were recently identified (Wichard and Pohnert 2006). Preliminary bioassays to test the activity of these substances, however, did not show any activity against competing diatoms (Fiedler, unpublished data). Still, further research should be performed to track down the physiological and ecological relevance of these chlorinated compounds.

Constraints of allelopathic interactions – physical and evolutionary aspects

Even though this thesis can, in conjunction with other recent studies, help to better understand allelopathic interactions, the relevance and ecological advantage of chemically mediated interactions in the plankton has often been questioned. Two major points of those critics are first the physical limitations involved in the transport of compounds from the emitting to the receiving cell and second how mechanisms such as

allelopathy might have evolved. In that last part of that thesis I would like to evaluate these constraints with regards to the results presented and to general considerations.

A basic requisite for allelopathic interactions is the transport of any active substance from the emitting to the receiving organism. Measurements that try to measure the distance how far active metabolites travel away from the emitting cell are so far missing due to a lack of suitable analytical methods. Further, in contrast to terrestrial chemical ecology, marine chemical ecology delivers the identity of active allelopathic substances far less often (Zimmer and Zimmer 2008) which is an additional challenge for the prediction or characterization of the chemical space around an algal cell. Still theoretical modeling studies have been performed to estimate the significance of allelopathic interactions. For instance, the transport of compounds away from macroscopic organisms is characterized by a relatively rapid advection processes and high to medium Reynolds number (Wolfe 2000). In contrast, at small scales which apply for photosynthetic protists, the environment is characterized by low Reynolds numbers (laminar flow) and molecules are transmitted between cells by the slow process of diffusion. That raises the question of how likely is it that substances excreted by one cell in nature are encountered by target cells. As soon one substance is exuded it diffuses slowly away from the cell building a concentration gradient and creating a chemical envelope around the cell. Clearly, experiments performed in this thesis do not reproduce such natural conditions because cell free filtrates test only the bulk concentration and co-culture investigations were shaken during the experiment creating turbulence in the media. Further, PUA additions in mesocosms created average PUA concentrations in the range of ecological relevant concentrations but natural existing gradients around an emitting cell could not be simulated with such approach.

Currently, no experimental data exist which can describe such chemical envelop on a spatial scale. Recently however, such gradient of allelopathic substances was modeled in dependence of the cell density (Jonsson *et al.* 2009). Here it was demonstrated that at a cell density of 1 cell mL^{-1} (pre-bloom conditions) the local concentration 3 radii from an allelopathic cell is around 100 times higher than the bulk concentration. However, because the encounter rate of producing and receiving cell were estimated to be too low, the authors concluded that allelopathy cannot be helpful for microalgal cells to initiate a bloom. Nevertheless, at high cell densities allelopathy could well be of significance, for

example by prolonging an algal bloom. In such a bloom scenario the abundance of co-existing bacteria such as *K. algicida* might also be highest. If the active substances, such as proteases in that case, are only excreted once a certain cell density is reached (**Manuscript A**) the encounter rate of proteases and co-occurring algal cells is most likely high enough to be of significance. These algae cells are subsequently lysed which leads to the liberation of nutrients which would be advantageous for the bacteria.

These estimations are only applicable in pelagic environments were the active substance can be diluted into a homogeneous environment. In benthic environments the conditions are probably more favorable for chemically mediated interactions and competition for space might be more important. Here diatoms, for example *N.* cf. *pellucida* (**Manuscript F**), live in a milieu which is rich in organic matter such as extracellular polysaccharides. As active substances are excreted they may remain in the organic mucus which decreases the diffusion away from the cells. In such way benthic cells can inhibit the growth of competitors and defend their microhabitat.

Without doubt more investigations, experimental and theoretical, are needed to characterize the relevance of allelopathy in bloom and pre-bloom situations. These studies should specifically try to estimate the concentration gradient of known allelochemicals (e.g. BrCN). In combination with dose-response curves obtained in bioassays it should be possible to estimate the biologically active chemical space around a producing cell. Further, modeling studies can subsequently calculate the likelihood of susceptible cells encountering allelopathic compounds, and how long they are likely to be exposed.

Although researchers have been considering the question of to what extent allelopathy is involved in processes that shape the plankton community, a more fundamental question is how allelopathic interactions evolved and can persist in pelagic environments. Lewis wondered how such interactions can be stable if the benefit from the production is shared with non-producers (cheaters) (Lewis 1986). In response, it has been suggested that cells which contain the same genetic material, as is the case for asexually dividing diatoms, can be treated as a multicellular organism (Flynn and Irigoien 2009) which would change the ecological interpretation. Recently, however it was modeled that the time mother and daughter cells need to separate in a turbulent flow is less than 1 minute and thus it is unlikely that cells of the same clone are

neighboring cells for a long time constraining a clone selection in evolutionary processes (Jonsson *et al.* 2009). However, the calculation is based on a turbulent flow which is not necessarily the case in plankton ecology. Thus, similar studies based on a laminar flow would be of a high scientific value.

In general, the production of any substance may make sense if the benefit outweighs the production costs. The benefit for producing cells might be obvious if competing cells are killed or inhibited in growth. However, costs-benefit balances as performed in terrestrial plants (Baldwin 1998) are yet not completed for allelopathic diatoms. Thus, the estimation of the production costs is pure speculation. Even though it is widely accepted that the production of infochemicals is costly due to biosynthetic machinery necessary to produce allelopathic compounds, one should consider the production costs if the applied enzymes have multiple functions. For instance, bromoperoxidases involved in BrCN production (**Manuscript F**) consume H_2O_2 which is harmful for an algal cell. Thus, it has been suggested that one function of haloperoxidases is to scavenge H_2O_2 (Manley 2002). Interestingly, BrCN production can only be observed during the morning hours, the time when H_2O_2 productions during the Mehler reaction was shown to be highest in the marcoalgae *Ulva rigida* (Collén *et al.* 1995). This suggests that such enzymes have multiple functions and the costs are thus may be not as high as expected. The production of allelochemicals might have been rather evolved as a sustainable use of resources that are available within an algal cell anyway.

6 Conclusion and outlook

The aim of this thesis was to investigate chemically mediated interactions of diatoms with bacteria or other diatoms. For diatom-bacteria interactions there is only a very limited knowledge of which compounds, produced by either diatoms or bacteria, are involved in these interactions. Furthermore the specificity and regulation mechanism of these interactions was not satisfactorily addressed.

To tackle these issues I characterized bacterial proteins that inhibit the growth of several non-toxic diatom species. I showed for the first time that the release of those proteins is regulated by the bacterial cell density in a process as known from quorum sensing. Interestingly, the diatom *C. didymus*, not inhibited by the bacteria, showed a physiological response to the bacterial proteins and released its own proteases. Even though it is tempting to assume that this response is involved in resistance mechanism this hypothesis cannot be confirmed by experimental evidence. Thus future research should try to further evaluate the significance of that induced response. All these examples result from investigation of pair-wise interactions. It would also be of immense interest to investigate how such response alters the complete phytoplankton community and may increase diatom diversity. Thus future studies may also look at how other diatoms and bacteria react to *C. didymus* protease induction.

Additionally, we tested the effect of diatom derived PUA on the bacterial community. We now can negate the possibility that PUA, as postulated previously, shape the bacterial community. Instead, other yet unidentified factors, potentially other temporarily variable organic exudates, seem to have a greater influence. Future research should evaluate the underlying mechanisms by which the diatoms change bacterial community composition in more detail. Are, for example, multiple substrates available that can be utilized by different bacteria with different efficiency or are broad or narrow-spectrum antibiotics exuded? If the growth of certain bacteria is especially promoted it would be interesting to evaluate whether these same bacteria also support diatom growth in order to form mutualistic relationships.

Diatoms may not only receive signals from bacteria but also from other diatom species that produce allelopathic compounds. BrCN is one such compound and presents the first example of halogenated allelopathic compounds produced by diatoms. Surely, there are several remaining open research questions on that topic. For example, how is the biosynthesis of cyanide accomplished, what is the target of BrCN, and how is auto-toxicity avoided? Thus, this research opens up several exiting research projects which need to be addressed. For example, the target of BrCN can be discovered using metabolomics approaches. Therefore, one might add BrCN in lethal and non-lethal concentrations to the target algae and investigate the metabolic response with high temporal resolution.

Metabolomics has also been suggested to be helpful for a better understanding of several aspects in chemical ecology. In this thesis I wanted to use this technique to explore the effect of bacteria and diatoms on the physiology of diatoms. Therefore, I successfully developed a cultivation device which enables the co-cultivation of planktonic species without direct cell contact. Such device had not previously been established and can now serve for a variety of experiments in aquatic chemical ecology.

I subsequently used co-culturing approaches to investigate diatom-diatom and diatom-bacteria interactions. The results suggest that the metabolism of a diatom cell depends on the presence of co-existing organisms. Thus, there might be a complex exchange of metabolites between members of the planktonic community, many of which may be used as a carbon source. Future research should try to elucidate the chemical structure of yet unidentified metabolites and verify how these substances affect the interacting organism. Once the structures of bioactive compounds are elucidated, aspects of these compounds' biosynthesis, fate, transport and use by other cells can be addressed.

In summary, the research presented adds valuable data for an enhanced understanding of chemically mediated interactions of diatoms by identifying signal molecules from bacteria and diatoms. Further, for the first time metabolomics approaches were used to investigate the effect of biotic stimuli on diatoms. The results on allelopathy regulation, specificity and intra- and extracellular metabolite variability may help to better explain the "paradox of the plankton".

7 References

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8 Curriculum Vitae

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SCIENTIFIC WORK:

9/2008-10/2008	Internship at Scripps Institution of Oceanography (Paul Jensen), San Diego, USA □ Isolation and cultivation of marine bacteria □ Molecular techniques (PCR, T-RFLP)
9/2006-12/2006 and 5/2007-7/2007	Lab Assistant, "Leibniz Institute for Natural Product Research and Infection Biology", Department of Biomolecular Chemistry, Jena, Germany

- Prep HPLC for natural product isolation

3/2007-4/2007	Internship, "Thuringian State Institute of Environment and Geology", Jena, Germany - Sample preparation for platin-group elemental analytics

FOREIGN LANGUAGE COMPETENCE:

English	Fluent, speaking and writing
French	Basic skills

EXPERIENCE:

Student Seminars	Structure elucidation with IR spectroscopy Chemical Ecology Structure elucidation using MS, NMR and IR HPLC courses
Field experience	Mesocosm experiment, Umea, Sweden 2011
Conferences	Participation at several international conferences, (ASLO 2009, Nice, France; ASLO 2011, San Juan, Puerto Rico, FEMS 2011 as invited lecture, Geneva, Switzerland)
Teaching skills	Certificate for adult education "LehreLernen"
Additional education	Introduction for working in a GxP environment

9 Selbstständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbstständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, 21. November 2012

Carsten Paul