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Chemical signaling in diatom-parasite interactions

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List of Abbreviations

ANOVA	Analysis of Variance
CG	*Coscinodiscus granii*
CW	*Coscinodiscus wailesii*
EPS	Extracellular Polymeric Substances
ESI	Electrospray Ionization
GC-MS	Gas Chromatography Mass Spectrometry
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography Coupled with Tandem Mass Spectrometry
MeOH	Methanol
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PRM	Parallel Reaction Monitoring
QC	Quality Control (pooled sample)
Rt	Retention Time
SEM	Scanning Electronic Microscopy
spp.	species pluralis
TIC	Total Ion Current
TEP	Transparent Exopolymer Particles
UHPLC-HR-MSMS	Ultra High Performance Liquid Chromatography High Resolution Mass Spectrometry

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1. Introduction

Phytoplankton constitute the basis of the marine food web and are surrounded by a broad spectrum of microorganisms, forming an intricate network where individuals compete for essential resources like nutrients, light and territories.[1] The importance of marine microbes in ecosystem pathways such as photosynthesis and degradation of organic matter was first emphasized in 1974 by Pomeroy.[2] Microorganisms are abundant throughout the world's oceans and are largely involved in CO_2 respiration by decomposing phytoplankton and releasing CO_2 into its surroundings.[3] The interactive community structures of phytoplankton and microorganisms are assumed to strongly influence metabolic fluxes in marine environments and to regulate biogeochemical cycles.[4,5]

Photosynthetic, unicellular algae can establish mutualistic interactions where both host and symbiont benefit from each other (**Figure 1**). In contrast, opportunistic microorganisms such as bacteria, viruses or eukaryotes can infect and kill algal cells, limiting the growth and performance of algal populations.[6] Microalgae can produce allelopathic compounds for mediating such complex interactions.[7] Both predatory attacks (e.g. grazers) and pathogenic infection are a major threat to photosynthetic organisms in aquatic and terrestrial habitats. However, the mechanism of algal defense has received little attention in contrast to terrestrial plants.

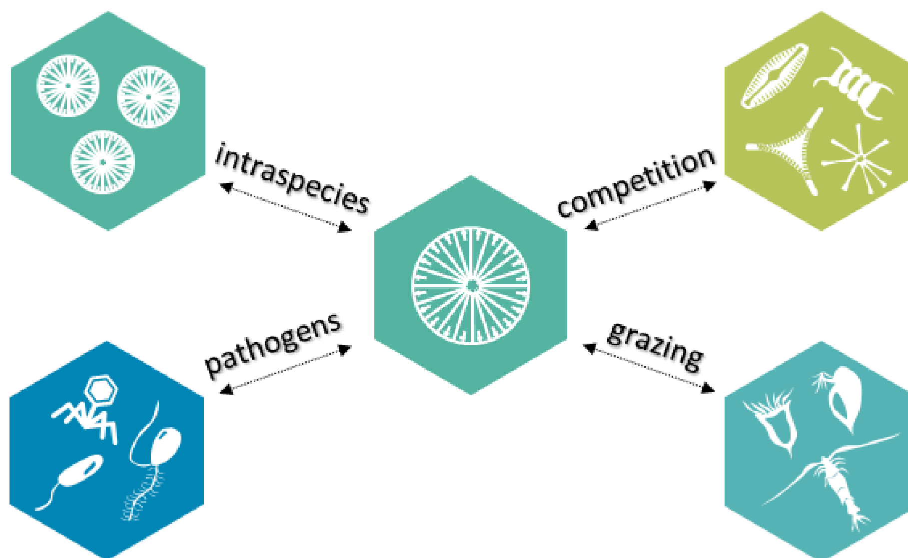

Figure 1 Depiction of the diverse interactions that diatoms form with other microorganisms. Adapted from Ianora *et al.* (2011)[1]

1. Introduction

Pathogens of algae can severely alter population dynamics and indirectly influence processes in the aquatic ecosystem, modeling biogeochemical cycles and atmospheric chemistry.[8] The research gaps of algae-pathogens research are broad in comparison with the information available on plant-pathogens interactions. Interestingly, the same taxonomic groups of pathogens can be found in both the marine and terrestrial ecosystems, including bacteria, fungi, oomycetes, viruses. In plants, the synthesis of small metabolites like the hormones salicylate and jasmonate plays a key role in the defense against pathogenic attacks.[9] Following the initial response, plants can utilize primary metabolites like carbohydrates, organic acids, amines/amino acids, and lipids. Secondary metabolites such as phenols, terpenoids, indoles, alkaloids, and glucosinolates can be induced and serve as signals against pathogens and for maintaining the plant immunity.[9] Secondary compounds which possess antimicrobial activity are categorized as phytoalexins and can be induced upon wounding or pathogenic attack. Pathogen Associated Molecular Patterns (PAMPs) are pathogen elicitors targeting host extracellular recognition sites and triggering the biosynthesis of antimicrobial metabolites at the site of infection.[9] For instance, in the plant model organism *Arabidopsis*, the production of the phytoalexin camalexin is induced by various pathogens including the oomycete *Phytopthora*.[10] Common examples of oomycete patterns that elicit immune responses in plants are β-glucans (carbohydrates), elicitins (proteins) and eicosapolyenic acids (fatty acids).[11]

One of the main tools to identify defense-related compounds is metabolomics, which aims to systematically study the unique chemical fingerprint that cellular processes leave behind. The metabolome is defined as all the metabolites produced in a biological cell, tissue, organ or organism, which are the end products of cellular processes.[12] In recent years, metabolomics approaches gathered a striking interest to analyze plant pathogenesis and discover new strategies in controlling plant diseases with the advantage of a high throughput analysis.[13] The application of metabolomics strategies enabled the elucidation of the plant immune responses of *Nicotiana tabacum* attacked by fungi and oomycetes.[9] Furthermore, the metabolomics tools also contributed to the field of algal chemical ecology to identify chemicals signals such as mating pheromones and defensive oxylipins.[14, 15] The research gaps of parasites in marine chemical ecology are partly due to the chemically complex matrix surrounding aquatic organisms and the resulting low concentrations of bioactive metabolites, making it particularly difficult to detect such signals.[1]

1. Introduction

In their natural environment algae growth is limited by its close association with surrounding bacteria, viruses, fungi and protists.[6] The structurally diverse group of protists is comprised of eukaryotic microbes with no relation to animals, plants, and fungi but are able to infect a broad range of hosts.[16,17] Among the pathogenic protists are the oomycetes, which are filamentous eukaryotic microorganisms and are ubiquitous throughout the terrestrial and marine ecosystems.[18] They belong to the kingdom of Stramenopiles and are genetically related to diatoms and brown macroalgae (**Figure 2**). During the process of sporogenesis, oomycetes produce biflagellated, free-swimming zoospores which distinguish them from true fungi.[18] These infectious zoospores can be attracted by susceptible hosts and bound to the host cell surface. After development in the tissue and conversion into a branched sporangium, the production of new zoospores by sporogenesis can occur.[19] The ability of short adaptation to their host-defense mechanisms makes oomycetes very hard to control, driving the search for novel fungicides and disease control. Indeed, some oomycetes species such as *Phytophthora* sp. require sterols from their hosts that they cannot synthesize themselves to build on their reproductive form, and most antifungal substances do not target this compound class.[20, 21] Oomycetes belonging to the group of *Phytiaceae*, the causal agents of downy mildew, are one of the most widespread pathogens parasitizing plants.[22, 23] The model species from the genus *Phytophthora* is thoroughly investigated to unravel plant responses to parasite infections.[24, 25] Research in the field of crop pathogens shows that these groups of oomycetes are able to modulate the immune reaction of their hosts by secreting effector proteins granting a successful invasion by the parasite.[26, 25] However, the elucidation of chemical signalling in oomycetes is still poorly understood for other species.[27] Studies have shown, that the concentration gradients of the isoflavones daidzein and genistein found in soybean root exudates can elicit a chemotactic response of *Phytophthora sojae* zoospores to direct them towards the optimal infection sites.[28, 29]

Other prominent examples of oomycete infection are observed in the marine ecosystem. The oomycete from the lineage of *Saprolegnia* is a widespread pathogen of fish and the causal agent of Saprolegniosis, known as water mold disease, which can decimate many aquaculture fields.[30]

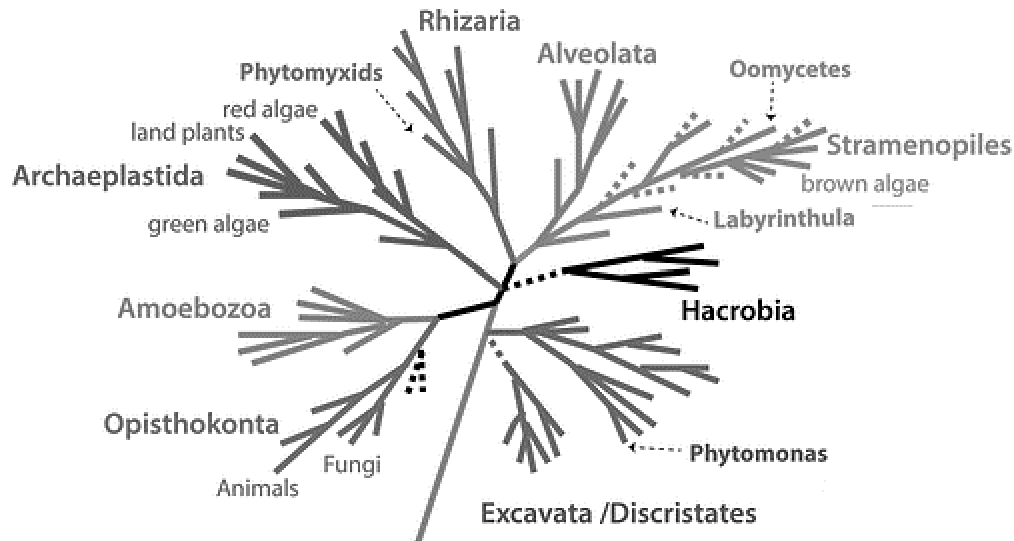

Figure 2 A schematic eukaryotic tree of life depicting the phylogenetic relation of oomycetes. Adapted from Schwelm *et al.* (2017)[17]

The most devastating pathogen of seaweed aquaculture is the cosmopolitan oomycete *Eurychasma dicksonii*, which can infect more than 45 brown algae species under laboratory and natural conditions.[31] These infections can cause crop losses of up to 30 % generating a growing interest in studying these organisms which have major impacts on the aquaculture economy.[32, 33] Due to its worldwide distribution, the intracellular parasite *Eurychasma* has been successfully established as a model organism for studying the pathogenesis in brown macroalgae under laboratory conditions. The infection mechanism of *Eurychasma dicksonii* is analogue to those of *Phytophthora*, with the attachment of a zoospore and invasion of tissue *via* the production of a haustorium.[33] Zoospores of *Eurychasma* can infect a single cell, as shown for the filamentous seaweed *Ectocarpus siliculosus*.[34] However, whether *Eurychasma* zoospores are chemotactic has not yet been determined. The algae react to the infection by thickening its cell wall through enhanced production of the polysaccharide β-1,3-glucan.[33] Furthermore, microbial infection of the brown algae *Laminaria digitata* induces the biosynthesis of reactive oxygen species (ROS) and halogenated compounds such as bromoform ($CHBr_3$).[34, 35]

The phytoplankton can also be infected by many parasitic organisms. With few exceptions, marine oomycetes appear host-specific to one microalgae genus.[36] The most accurately described parasites in terms of structural properties and life-cycle are the oomycetes *Lagenisma coscinodisci* and *Ectrogella perforans* that can infect diatoms.[37]

1. Introduction

A number of studies address the question of whether parasites are essential drivers in marine population dynamics.[38] The presence of parasitism is believed to pressure phytoplankton into developing genetically diverse communities[39, 40] and therefore parasites may be the driving force behind the exceptional species richness in phytoplankton communities.[41] Moreover, parasites were found of great importance in structuring marine ecosystems and could sustain grazing of an inedible algal bloom.[42, 43] Kagamie *et al.* presented, that zoospores produced by infection of the large, inedible diatom *Asterionella formosa* provided an attractive food source for zooplanktonic grazers.[39] The effect of parasitism on higher trophic levels was also evaluated with bioinformatic simulations.[44] It was suggested that parasites are recognized as a linkage, connecting trophic levels by turning inedible diatom lipids into edible food sources for grazers.[45] Parasites can also terminate algal bloom, as demonstrated for the oomycete *Ectrogella perforans* in marine diatoms *Licmophora* sp. or with protists *Parvilucifera* spp. infecting toxic dinoflagellates *Alexandrium minutum*.[46, 47] Parasitism can also cause epidemics in aquaculture of microalgae, as revealed for the freshwater astaxanthin-producer *Haematococcus pluvialis* infected with a parasitic chytrid.[48]

Diatoms are the most abundant phytoplankton group. Their photosynthetic activity accounts for up to 20 % of the global oxygen production. Diatoms contribute largely to carbon fixation and are a highly diverse species in microplankton communities that can form blooms.[49, 50] In addition, diatoms are the oceans' major contributors of biogenic silica thanks to their siliceous cell wall, the frustule.[51]

The diatom frustule possesses regular perforations throughout the shell, responsible for the diatoms remarkable optical properties and uniqueness in the planktonic community.[52] Furthermore, diatom-derived natural products have gained increasing attention for therapeutical usage[53] as well as application in the field of nanotechnology.[54] The organic matrix surrounding these shells shows a high abundance of sugars and proteins, which protects the frustule from disintegrating in the surrounding seawater.[55,56] It was suggested that this mechanical barrier may serve as a constitutive defense mechanism of diatoms.[57] In contrast to these findings, the pores which differ in size and space between them, are assumed to play a role in the infection process by providing an entrance to parasites, but this has never been proven experimentally.[52]

1. Introduction

Diatoms divide vegetatively by successive mitosis, a process that decreases their cell size over time until a critical size threshold is reached.[55, 58] To avoid death, diatoms must perform sex and meiosis which occurs in the spermatogonangium and which restores the original cell size. This process enables diatoms to generate genetic diversity in terms of modulating phenotypes, overexpression of certain genes and biosynthesis of novel chemical compounds. The Red Queen Hypothesis states that biological diversity is driven by a constant evolutionary arms race through the co-evolution of closely associated hosts and parasites, favouring the maintenance of sexual reproduction within populations.[59]

Diatoms are unable to actively escape unfavorable conditions and developed different strategies to cope with biotic stressors. Defense mechanisms can be physiological (production of secondary metabolites), morphological (shell thickness, pores) or behavioural (sinking, accumulation, resting stage formation).[60-62] Chemical defense strategies were long suggested to be only constitutive for phytoplankton, meaning that active metabolites are constantly produced and not triggered by wounding or pathogenic attacks.[57] This strategy harvests many disadvantages for the organism, as the permanent production of these metabolites can affect growth, reproduction and can be costly in energy. Furthermore, the dilution effects of the aqueous surrounding stand in contrast to a high metabolic cost of the permanent production of bioactive compounds. Whereas dynamic strategies like the inducible defense, e.g. only activated when needed and not consuming energy for sustainable storage, can minimize the energy consumption.[63]

The first report on the activation of chemicals through wounding in microalgae was discovered in the diatom *Thalassiosira rotula.* Upon wounding, unsaturated aldehydes are synthesized and might ward off these attacks.[64] These defense compounds are produced by enzymatic cleavage of fatty acids, leading to the production of α, β, γ, δ – unsaturated aldehydes (PUAs).[65] Further defense-related production of oxylipins was reported in the diatom species *Cerataulina* upon grazing.[61] Laboratory studies monitoring the growth of marine bacteria after exposure to three PUAs revealed that bacteria strains of the *Roseobacter* species elicited increased sensitivity towards diatom-produced PUAs, whereas other species showed no inhibition of growth.[66]

Moreover, enhanced production of PUAs in diatom cultures was reported during cell lysis or under exposure to external stressors[1, 67], raising further questions on the overall role of chemical mediators in microalgae communities.

Model organisms for diatom-parasite studies are still insufficient. This is mainly due to limitations in sampling techniques, distinguishing parasitic species and difficulties in cultivating these in the laboratory. Furthermore, they require specialist observations for microscopic detection.[68] Little is known about the impact of host-parasite interactions on marine diatoms and the classes of molecules involved in defense.[6] At this stage, information about such mechanisms is very poor and researchers are currently in the process of discovering new oomycete species which infect diatoms such as *Pseudo-nitzschia* sp.[37] Ongoing work on these pathosystems under laboratory conditions is crucial for understanding phytoplankton responses to an ever-changing environment. As well as gaining insights into the evolutionary role of parasitism and their potential role as top-down control in diatom populations and their link to trophic pathways at a global scale (**Figure 3**).

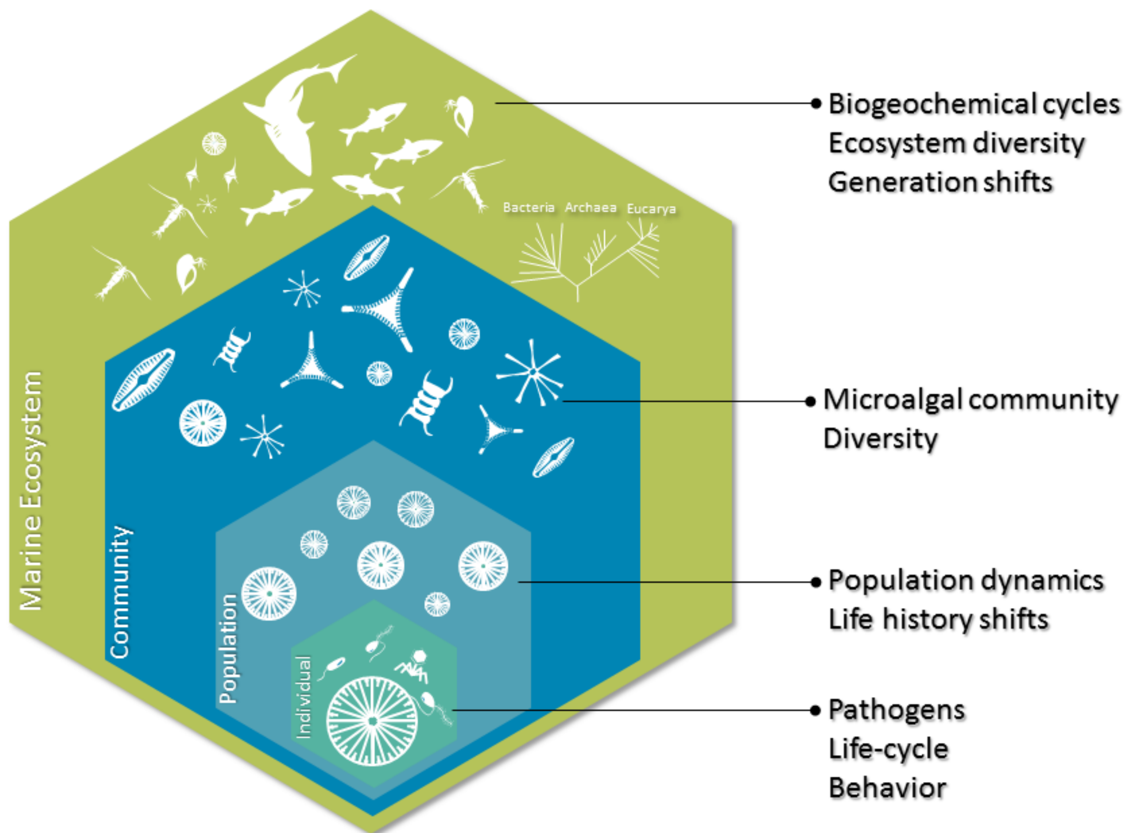

Figure 3 Trophic levels in the marine community and the place of phytoplanktonic microalgae. Adapted from Fernández (2017)[69]

The presented limitations stress the central importance of developing methods for investigating such interactions to detect chemical compounds linked to host-parasite interactions.

One of the well-described oomycete models of marine diatoms is *Lagenisma coscinodisci*, with the infection process described in 1978.[19, 70] This parasite was reported at multiple locations worldwide, parasitizing the widely distributed diatom genus *Coscinodiscus*.[71] The prevalence of infestation by this oomycete can be particularly high, reaching numbers of up to 41.9 %.[72, 73] The long-term maintenance of a host-oomycete dual-culture was successfully established recently (**Figure 4**).[74] The infection was investigated with comparative metabolomics to highlight the up-regulation of algal metabolites that serve the parasite in spreading its zoospores in the species *Coscinodiscus granii*.[75] To date it remains unsolved, whether the zoospores of *Lagenisma* choose their hosts depending on cell size or growth stage, whether infection happens upon random encounters or if the potential hosts emit a chemical gradient attracting the parasite. Even the possibility of adelphotactic behavior, e.g. cell-attached zoospores recruit others by emitting attractants, has to be considered. In particular, the molecular effectors causing some diatom to be susceptible and others to be resistant against zoosporic parasites are not yet known.

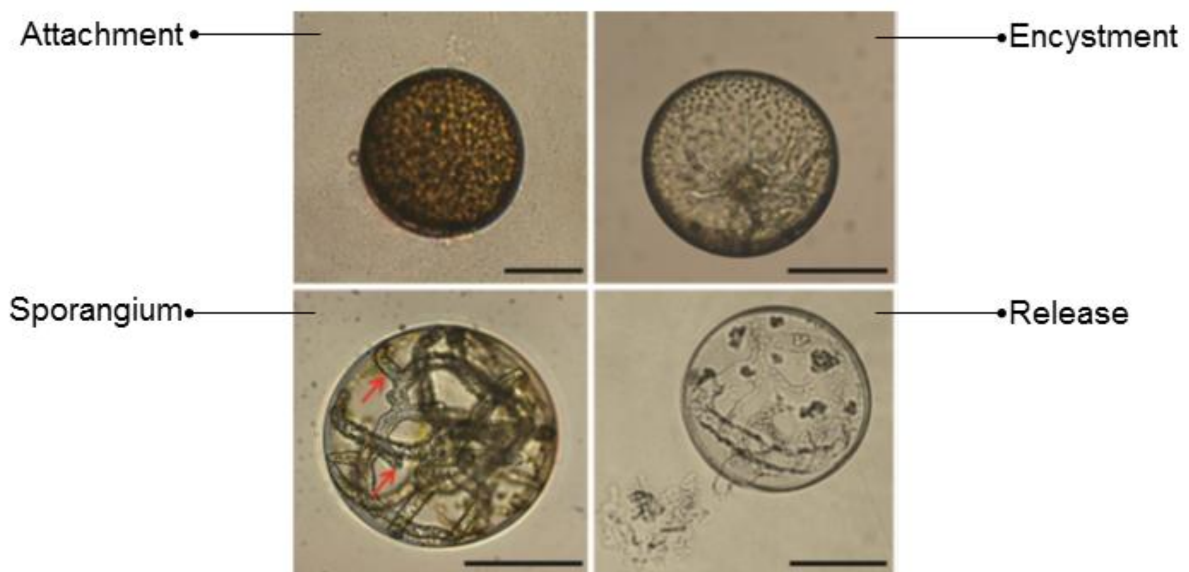

Figure 4 Depiction of the oomycete *Lagenisma* in its algal host. Scale bars equal 50 µm. Reproduced from Vallet *et al.* (2019)[75]

The giant diatom *Coscinodiscus wailesii* is an invasive species introduced to the north sea initially reported in 1977 (Edwards *et al.*)[76] in the English Channel. Interestingly, *C. wailesii* became resistant to *L. coscinodisci* after sexual reproduction[74], raising questions about how the resistance was acquired and whether low-molecular metabolites are involved in the defense. Here, I want to apply current approaches in the field of marine chemical ecology to elucidate the mechanisms of resistance in *Coscinodiscus wailesii* to the oomycete *Lagenisma coscinodisci*.

Two major experimental approaches for studying interactions of different species within a community are available. Incubating cells in the filtrates of another species can provide information on the presence of infochemicals exuded into the medium and help to determine whether these chemicals are mediating such interactions. Within this thesis, filtrate experiments were performed according to Bigalke and Pohnert[77]. They investigated whether the resistance of the diatom *Chaetoceros didymus* against the algicidal bacterium *Kordia algicida* is occurring during the incubation with filtrates of the susceptible diatom *Skeletonema costatum*. It has to be taken into account, however, that these approaches do not represent natural conditions. To further assess the induction of small molecules between species, non-contact dual-cultivation are commonly used.[8, 77, 78] The experimental setup deployed in this thesis was reported in several studies related to species-species interactions, mimicking more natural conditions. For instance, the non-contact inoculation of two microalgae strains (*S. costatum* and *Heterosigma akashiwo*) led to the observation of growth inhibition.[79] Additionally, Scholz *et al.* assessed the induction of host-susceptibility by monitoring the behavior of susceptible and resistant diatom host species *Navicula, Nitzschia* and *Rhizosolenia* to chytrid parasites.[65]

The above-mentioned techniques can deliver information on the presence of diffusible chemicals in the surroundings of the diatom, representing the diatoms exometabolome. These methods, however, lack information about processes happening inside cell (the endometabolome) upon infection. Therefore, to effectively characterize intra- and extracellular metabolite profiles, the application of metabolomics tools is needed. Paul *et al.* efficiently coupled dual-culture experiments to metabolic profiling for monitoring the involvement of chemical mediators in phytoplankton species interactions.[80] In every metabolomics approach, the experimental setup is a key factor in receiving conclusive data. Re-occurring limitations in biological sample analysis are the volume of extracts as well as the concentration and stability of the metabolites. Data acquisition can be performed by using gas- or liquid chromatography coupled with mass spectrometry (GC-MS or LC-MS).

1. Introduction

Coupling chromatographic methods to mass spectrometry enable the qualitative analysis of specific compounds isolated from sample extracts and deliver information about polarity (reflected by the retention time) or volatile properties.

LC-MS is used for determination of non-volatile compounds, macromolecular compounds (lipids) and polar compounds. The application of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) enables the structure identification based on characteristic spectral information. Successive fragmentation delivers high-resolution mass spectra which enables compound identification based on the specific metabolomic fingerprint. Multivariate statistical methods serve to identify similarities or differences of metabolites between treatments. A current problem which metabolomics strategies are facing is the identification of metabolites, as the databases of metabolites are still growing and are considered insufficient for valid compound identification. For further structural elucidation and validation of novel chemical structures, several follow up methods like nuclear magnetic resonance (NMR) and infrared spectroscopy (IR) are required, most often after purification of the pure product from large-scale cultures. Although compounds with specific mass to charge ratio can be assigned to formula, final validation of compounds can only be reached by measuring standards and comparison of retention times.[81]

2. Objectives of the Thesis

Algal communities have been shaped throughout evolution by an always present competition for resources and being the target of pathogenic attacks. To cope with a constantly changing environment and parasites pressure, microalgae possess diverse defense strategies including molecular processes, which are understudied. In this thesis, we applied different experimental approaches to investigate the mechanisms of resistance and susceptibility in the pathosystem model of diatom-oomycete *Coscinodiscus-Lagenisma*.

After the establishment of a host-parasite model under laboratory conditions, the giant diatom *Coscinodiscus wailesii* became resistant after sexual reproduction whereas *Coscinodiscus granii* is still very susceptible to the parasite infection since 2017. This thesis aims to unravel if the diatom resistance is mediated through diffusible substances, what is the metabolic response of *C. wailesii* in response to *Lagenisma*'s exposure and if parasitic zoospores are chemotactic. Considering that defense mechanisms in microalgae may be of morphological, behavioral or physiological nature, we carried out a series of experiences with different experimental settings to answers our questions.

Chemotaxis setups were developed and assessed for behavioural assays with *Lagenisma* zoospores. Non-contact co-cultivations were performed to assess if some substances were released in the surrounding medium from the resistant algae that can impact the infection performance. The infection rate and growth performance of susceptible diatom were recorded when treated with exudates of the resistant algae. The endo- and exo-metabolome of the resistant diatom challenged by the oomycete was investigated by solid-phase and cell extractions. Metabolic profiles were analyzed with ultra-high-pressure liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HR-MS). The structure identification was carried out with tandem mass spectrometry for the most significant metabolites that distinguished challenged cells from unchallenged.

3. Material and Methods

3.1 Materials

All solvents used were UHPLC-grade purity and were purchased from SIGMA-ALDRICH Chemie GmbH (Munich, Germany).

3.2 Microbial strains and growth conditions

Two different host strains from the species of the marine diatom *Coscinodiscus* spp. were used for further experiments. The strains *Coscinodiscus granii* and *Coscinodiscus wailesii* were both isolated in Helgoland during the summer blooms of 2017 and 2016, respectively, (**Figure 5**) and their identity was determined by microscopic observations and DNA sequencing.

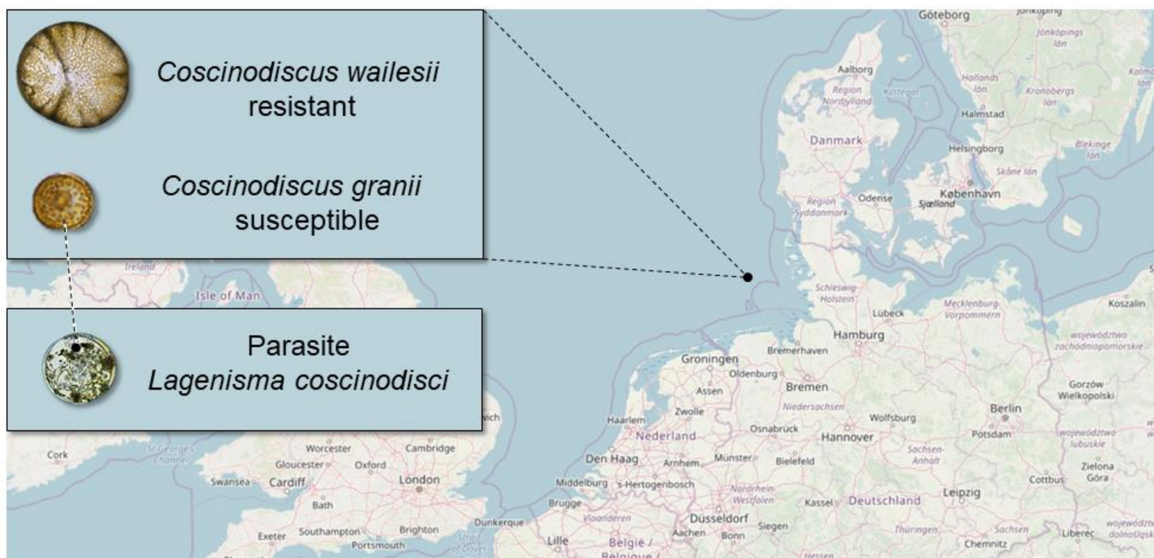

Figure 5 Depiction of the sampling location of the diatom *Coscinodiscus* spp. and the oomycete *L. coscinodisci*. All strains isolated in Helgoland during the summer bloom. The parasitic strains are maintained in the most susceptible host *C. granii*. [www.openstreetmap.org/copyright, © OpenStreetMap-Contributors]

All the strains of *Lagenisma* available were isolated and maintained from the hosts *C. granii,* excluding strain LagC2 isolated from *C. radiatus.* The algal cultures used in the experiments were maintained in tissue-flasks in silica-enriched Guillard's (f/2) Marine Water Enrichment Solution (SIGMA-ALDRICH Chemie GmbH, Munich, Germany) medium.

The thermoregulation and light/dark cycle was 16° C for 14 hours daylight and 12° C for 10 hours in darkness. All the following experiments were carried out in biological triplicates, when not mentioned otherwise. Cultures were observed with a stereomicroscope (binocular visiScope®, VWR International GmbH, Pennsylvania, US). The cell density and number of sporangia were determined using a Sedgewick Rafter chamber (Pyser-SGI, Kent, UK). The growth rate was calculated using the following formula:

$$growth\,rate = \frac{\ln(\frac{Nt}{N0})}{\Delta t} \qquad (1)$$

Nt and N_0 represent the cell densities at time t and time 0, and Δt describes the time difference between the measurements in days.[82]

3.3 Microbiology experiments

3.3.1 Assessment of the *Lagenisma* virulence

To determine the most virulent strain of the oomycete *Lagenisma coscinodisci*, an infection experiment was performed on the susceptible diatom *C. granii*. A culture of susceptible *C. granii* grown for 12 days to reach the end of the exponential phase was infected with *L. coscinodisci* strains (LagC7A, LagC7B, LagCGMix, LagCG, LagC2). To reach an initial cell-density of 100 cells ml^{-1}, aliquots of *C. granii* culture were pipetted in 6-well plates (Sarstedt, Nümbrecht, Germany) and filled up to 5 ml with f/2 medium. 30 µl of each *Lagenisma* strain was added in each well and the infection rate was determined after three days, according to the following formula:

$$infection\,rate = \frac{number\ of\ sporangia}{total\ number\ of\ cells} x\ 100 \qquad (2)$$

To assess the susceptibility of *C. wailesii* against different strains of *Lagenisma coscinodisci*, 1 ml of diatom cells grown for five days in f/2 were pipetted in 40 µm nylon mesh strainer (Corning®, Durham, USA) in 3 cm diameter petri dish containing 11 ml of fresh medium. The initial concentration was 19 cells ml^{-1}. 1 ml of *C. granii* cells five days-infected with either *L. coscinodisci* strain LagCG, LagC2, LagC7A, LagCGmix, LagC7B were added to the outer part of the strainer, reaching a final concentration of 32 cells ml^{-1}. The cells were observed for three weeks and microscopy pictures were taken after ten days with a digital microscope Keyence VHX 5000.

3.3.2 Non-contact dual cultivation and exudate treatment

Non-contact dual cultivation of *C. granii* and *C. wailesii* in 5 cm diameter Petri dishes. 40 µm Corning® nylon mesh cell strainer were used to physically separate both species from another but allowing the diffusion of water-soluble chemicals and the *Lagenisma* zoospores. All the cells from *C. granii* culture cultivated in 300 ml f/2 for 15 days were collected in a cell strainer and transferred into new f/2 medium. The cells were counted and 5 ml were transferred to Petri dishes at a final cell concentration of 200 cells ml^{-1}. 5 ml at 200 cells ml^{-1} of either *C. granii* or *C. wailesii* were added into strainers. 20 µl of *C. granii* infected with *L. coscinodisci* strain Lag C7B were added to cells, while 20 µl of f/2 medium was added to the control cells. Incubation was conducted for five days using a shaker set at 50 rpm. Cells were counted after homogenization.

To investigate allelopathic effect of diatom *C. wailesii* and *Lagenisma* infection performance in *C. granii* treated with filtrates, susceptible cells were co-incubated in the exudate of the resistant ones. After six days of cultivation in monoculture, *C. wailesii* cells (23 cells ml^{-1}) were harvested with 40 µm nylon mesh. The filtrates without diatom cells were further filtrated onto a 1 µm mesh pluriStrainer (Pluriselect Life Science, Leipzig, Germany) to remove cell debris, but not the bacteria. *C. granii* cells were added to 30 ml of *C. wailesii* filtrates at a final concentration of 100 cells ml^{-1}. Five biological replicates were performed and the control consisted of treatment with cultivation in SW with or without parasite. After six days, the total amount of cells was counted after homogenization and the infected cells (sporangia, early- and late-stage) were determined.

To assess if a substance present in *C. wailesii* exudates can inhibit *Lagenisma* infection, *C. granii* cells treated by axenic or non-axenic filtrates were infected with 1 ml of three days infected cells. Seven days old cell *C. wailesii* culture grown in 400 ml of f/2 (9 cells ml^{-1}) and 30 ml were filtrated on 40 µm nylon mesh to obtain the xenic exudates. 30 ml were sterile-filtrated with a 0.22 µm filter to yield the axenic exudates. 250 ml of *C. granii* cultures grown for four days in f/2 were filtrated on 40 µm nylon mesh and all the cells were pipetted in 45 ml of fresh medium.

The determined cell density was 272 cells ml^{-1}. 0.5 ml of this stock were added to 4.5 ml of seawater medium (no nutrients supplementation), of the axenic and xenic *C. wailesii* exudates. The initial cell count at the start of the experiment was 37 *C. granii* cells ml^{-1}. The

cells were counted after seven days incubation and pictures were taken with a digital microscope Keyence VHX 5000.

3.3.3 Isolation and identification of the non-obligate bacteria associated with diatoms *Coscinodiscus* spp.

The non-obligate bacteria associated with *Coscinodiscus* spp. strains were isolated by striking 1 ml of culture filtrate and cells on f/2 agar and marine broth agar plates, and whenever need purity was achieved by striking single colonies onto new plates. Bacterial materials were sampled in RNAlater and DNA extractions were performed with a kit following the manufacturer's instructions. The Reverse and Forward primers were **1492r** and **27f**, respectively, targeting the 16S rDNA gene. The 16S rDNA sequencing was done by Eurofins and the sequences were aligned with the software DNASTAR Lasergene. Sequences similarity matching was achieved with the public database using BLASTn tool and high similarity (Megablast) option, excluding uncultured/environmental sample sequences. The taxonomic identity was assigned whenever over 99% similarity was found with database sequence with a published reference.

3.3.4 Investigation of the exo- and endo-metabolome of *C. wailesii* with comparative metabolomics

A 14-day-old culture of *C. wailesii* grown in silica enriched f/2 medium was filtrated to recover the exudates and the cells for endo- and exo-metabolome investigations.

Cells were transferred into a culture flask with 160 ml f/2 with a final cell concentration of 40 cells ml^{-1}. Five replicates for treated and untreated cultures were performed. For the infection of cells, 4 ml of the parasite strain LagC7B were added and 4 ml f/2 medium for control samples. Cells were monitored with microscopy to check the presence of infected cells until day 11, the day of extraction. To confirm the origin of the metabolites identified, *C. wailesii* cells were collected, added to 100 ml of ^{13}C labelled $NaH^{13}CO_3$ medium and incubated for ten days. The process was repeated twice on the same cells to ensure correct labelling.

3.3.5 Chemotaxis assessment

An 11-day old culture of *C. granii* (8 000 cells ml^{-1}) was used to inoculate 30 µl of the most virulent parasite strain LagC7B. After nine days, the infected cells were filtrated and excluded to recover the actively swimming zoospores. These were fixated with lugol 0.1 % and counted in a Neubauer counting chamber (depth 0.100 mm, 0.0025 mm^2).

The total zoospore density (zoospore ml^{-1}) was estimated with the following formula:

$$Zoospore\ Concentration = \frac{Total\ number\ of\ zoospores}{Number\ of\ squares} x\ 10000 \quad (3)$$

The extracts of *C. granii* cells and exudates were prepared by adding to 500 µl of DMSO (0.01 %) to each aliquoted extract to reach an initial concentration of 0.1 mg ml^{-1}. 50 µl of each dissolved extract was transferred in 0.2 ml PCR-tubes (Eppendorf, Hamburg, Germany). A solution of 2 % agarose gel (dissolved in distilled H_2O) was prepared by heating for 20 seconds in the microwave and cooled down to 40 °C. 50 µl of the soluble agarose gel was quickly transferred into the PCR tubes and thoroughly mixed. Glass capillaries (50 µl) were immediately loaded with the compound mixture by capillarity to prevent agarose from solidifying.

The capillary end was closed with food-safe silicone grease and then stored horizontally on dry tissues. The chemotaxis chambers were assembled according to the description (**Figure 6**).

The loaded capillary was inserted into the chemotaxis device and the chamber was filled with 300 µl of zoospore suspension. Microscopic observations with an inverted microscope (Axiovert 200, Zeiss, Germany) were performed directly after loading the chamber and pictures were taken at the start and after 10 min without changing microscopic parameters.

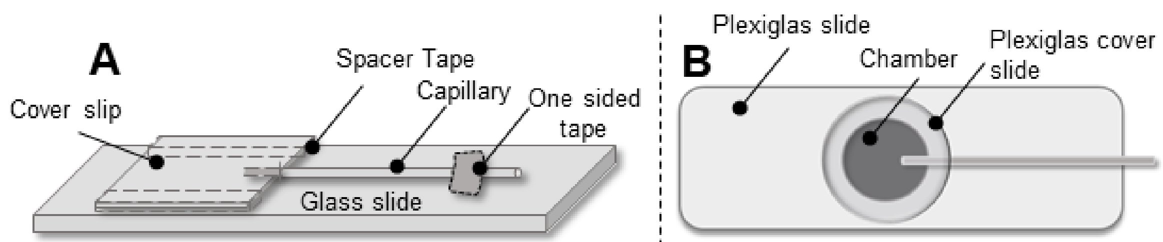

Figure 6 Setup of capillary assay chambers used for chemotaxis assessment. Chamber was generated by applying spacer tapes with the same height as capillary. **A** Glass-chamber builds with coverslip and spacer tape. **B** Two-component Plexiglas-chamber with embedded circular chamber holding zoospore suspension. The chamber was assembled by sticking together both components with silicone grease

3.4 Metabolic profiling

3.4.1 Extraction methods and sample preparation

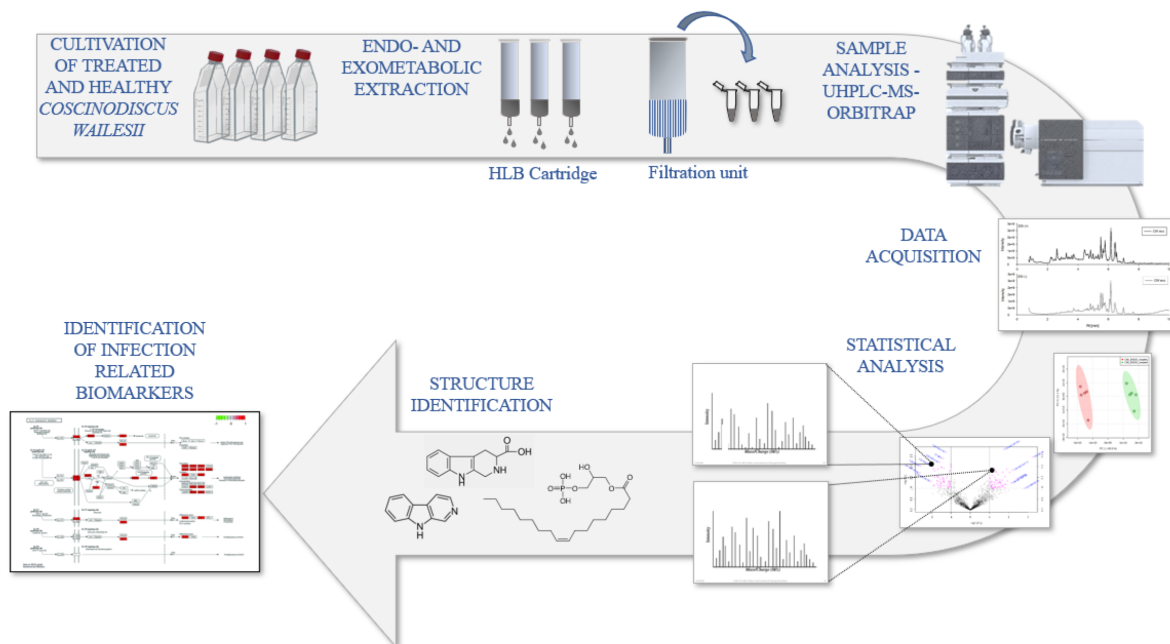

Figure 7 Schematic workflow of the comparative metabolomics pipeline

For the exo-metabolome investigation, solid-phase extraction (SPE) was conducted using HLB cartridges (Waters Oasis, 6cc) cartridges to collect compounds released in the surrounding medium (**Figure 7**). The cartridges were conditioned using 6 ml of MeOH (99.8 %, anhydrous, SIGMA-ALDRICH Chemie GmbH, Munich, Germany) and subsequently adding 6 ml UPLC-grade water. The cartridges were each loaded with 150 ml of exudate medium and for control. One additional cartridge was loaded with 150 ml of f/2 medium to serve as blank. After filtration, the cartridges were washed with 10 ml of water and subsequently eluted with 2 ml of methanol while applying pressure of 600 bar.

The extracts were collected in 4 ml glass vials and the samples were dried using a concentrator (Concentrator plus/Vacufuge plus, Eppendorf, Germany) with the following parameters selected: 3 h V- AL 45° C (removing alcoholic phase), 2:30 h V-AQ 45° C (removing aqueous solutions).

For the endo-metabolomic extraction a filtration was conducted under low vacuum using a filtration unit (Duran/pp, VWR International GmbH, Munich, Germany) equipped with filter plates (24 mm, pore size 4, glass edge, VWR International GmbH, US) and GF/C microfiber filters (25 mm, Whatman plc, Maidstone, UK). After filtration, the glass-filters containing cells were carefully transferred to 2 ml safe-lock Eppendorf tubes and extracted

with 1.8 ml Methanol. The samples were sonicated for 2 min at room temperature and centrifuged for 20 min at 12000 g. The supernatants were transferred into glass vials and dried in a concentrator (Concentrator plus/Vacufuge plus, Eppendorf, Germany). Parameters for drying were 2:30 h V-AL 45°C, 2:30 h V-AQ 45°C. Before preparing the samples for UPLC-MS analysis, normalization based on the cell counts was performed to reach the same cell-density in each vial after dilution (**Table 1**).

Table 1 Cell-counts of *C. wailesii* cultures obtained prior to endo- and exometabolic extractions in presence (treated) or absence (healthy) of parasite . The highest value in cell density equals 100 % (200 µl) of solvent added to the glass vial (**bold** in table). The remaining replicates were normalized according to replicate **3**.

Treatment	**Replicate**	**Cell density (cells ml^{-1})**	**Solvent volume (µl)**
treated	1	54	171
treated	2	58	184
treated	**3**	**63**	**200**
treated	4	56	178
treated	5	55	175
healthy	6	48	152
healthy	7	51	162
healthy	8	57	181
healthy	9	42	133
healthy	10	44	140

The dried samples were diluted with methanol/water (1:1) and 10 µl of each diluted extract was added to prepare a quality control pooled sample (QC).

3.4.2 Parameters for Measurement with UHPLC-HR-MS

20 µl were injected into the UHPLC-HR-MS (UltiMate 3000 UHPLC Dionex) connected to an Accucore C-18 column (100 × 2.1 mm, 2.6 µm) coupled to a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). The second metabolite analysis was performed with higher concentrated samples and the MS acquisition was switched off at 10 min. The metabolite separation method was set following Vallet *et al.*[75] Mass spectrometry was conducted in positive and negative-ion modes with a scan range of 100 to 1500 *m*/*z* at a peak resolution of 140 000 using electrospray ionization mode (ESI). The MS/MS spectra of precursor ions were obtained from analysis of the pooled sample. Selected precursor ions from the first MS screening were selected for performing MS/MS tandem mass spectrometry for obtaining MS/MS spectra with an average higher-energy collisional

dissociation (HCD) of 35eV. To improve signal discrimination and the detection of low abundance ions, parallel reaction monitoring technique (PRM) with an inclusion list was performed. This was applied to spectra with lower intensity than 10^3.

3.5 Data Analysis and statistics

3.5.1 Determination of significant metabolites

Raw data were analysed with Compound DiscovererTM software (version 2.1, Thermo Fisher Scientific, USA) for deconvolution and identification of the metabolites (chemical formula, *m*/*z*, retention time). The software detected chromatographic peaks and the mass of corresponding compounds based on a list of generated theoretical metabolites. Mass tolerance for MS identification was 5 ppm, minimum MS peak intensity was 2E04, and intensity tolerance for isotope search was 50 %. Mass tolerance for fragment identification was 5 ppm and the signal-to-noise ratio threshold was 3.

Relative standard deviation value was set at 50 hence excluding features, which were not represented in all pool samples replicates. This resulting reduced peak matrix was extracted as a .csv file and further processed with the online software MetaboAnalyst 4.0.[83] For displaying statistically significant differences of metabolites between the conditions, t-test and differential analysis yielding in volcano plots were performed, setting the p-value inferior to 0.05. PCA (Principal Component Analysis) and PLS-DA (Partial Least Squares – Discriminant Analysis) were conducted to obtain information about metabolite similarities between extracts of treated and healthy cells. Further statistical analysis was performed using SigmaPlot 12.0 software (Systat Software Inc., London, UK).

3.5.2 Identification by spectral similarity and literature search

MS/MS spectra were further processed with java-based software framework SIRIUS version 4.0 to match compounds in the database PubChem which show spectral similarity. SIRIUS detects molecular formulas by applying isotope pattern analysis and provides information about the fragmentation pattern of metabolite compounds by generating fragmentation trees. The comparison of MS^2 spectra with the database PubChem is implemented by making use of the CSI:FingerID tool integrated into the SIRIUS software.[84]

4. Results and Discussion

4.1 Chemotaxis assay applied to *Lagenisma-Coscinodiscus* model

Capillary assays to investigate chemotactic behavior of motile organisms are commonly used in the field of chemical ecology and have proven successful in testing the response of zoospores from *Phytophthora sojae* to the presence of host extracts containing isoflavone.[85] Two different capillary assay chambers, modified from Abe *et al.*[86], were used to develop a protocol for the assessment of chemotactic behavior of motile, biflagellated zoospores of the parasitic oomycete *Lagenisma coscinodisci*. As a first approach, a capillary assay built with microscopic glass slides, double-sided tape, and cover slides was tested. The examined extracts of healthy and infected *C. granii* could not deliver any conclusive results within the observation time of 10 min. Evaluation of obtained video material revealed a constant airflow surrounding the mouth of the agar-filled capillary. Furthermore, the zoospore suspension inside the assembled chamber would retract, due to evaporation, over the course of 10 min observation, causing again a movement which could negatively affect the outcome of the assay.

Considering these aspects, a second chemotaxis chamber was built from custom made Plexiglas slides with an imbedded circular chamber containing an inlet for the capillary. The chamber holds a fixed volume of zoospore suspension and is assembled by sticking two slides together with silicone grease to prevent air from entering. Additionally, an improvement was achieved utilizing silicone to seal the capillary end. This effectively prevented the agar inside the capillary from drying. Although the chamber was sealed thoroughly with grease, the evaluation of video footage suggested the presence of an airflow directed towards the capillary tip. This airflow would substantially impair the evaluation of chemotactic behavior, so that the obtained pictures and footage was not used for further interpretation. So far, no observation regarding the chemotactic response of *L. coscinodisci* could be accomplished due to the challenges regarding the experimental design of the assay.

Further work in establishing a chemotaxis assay applicable for the *Lagenisma-Coscinodiscus* model is needed. While utilizing two capillary-assay setups for the evaluation of chemotaxis of *L. coscinodisci* towards extracts of *C. granii*, minor setbacks regarding the experimental design of the chemotaxis assay occurred. The transition from the glass-chamber to the Plexiglas device prevented evaporation inside the chamber. However,

with both tested setups an airflow around the capillary mouth occurred during microscopic observations.

Surface tension alongside the wall of the capillary might be responsible for the air convection. Moreover, the latter could be the result of different salinity in the zoospore medium and inside the capillary. The medium of the zoospores is enriched seawater which contains salts and nutrients. The agarose gel inside the capillary is prepared with distilled water. These properties might result in a gradient causing a constant airflow around the capillary. When overlooking these limitations and focusing on the visualization of the zoospores, it was difficult to distinguish those from the background due to a low concentration. For better visualization, it is proposed to get a much higher concentration than 16 000 zoospores ml^{-1} by infecting high densities of cells with the parasite *Lagenisma*.

Another restricting factor in chemotaxis assays can be the concentration of the test compounds. The possible threshold for chemotaxis of *L. coscinodisci* is unknown and can only be approximated by comparing previous work with zoospores of the oomycete kingdom.

Furthermore, it remains unclear whether attraction occurs through the recognition of a chemical gradient. In freshwater diatoms, the attraction of fungal and chytrid parasites were rather unspecific to the host species and that specificity would be determined during adhesion and encystment of the zoospores as compared to the early stages of chemotaxis.[39, 87] This might support the hypothesis that zoospore attachment can also happen upon random encounters and as in the case for *C. wailesii*, the zoospore of *Lagenisma* can still attach but not develop due to intracellular defense mechanisms. For the oomycete *Phytopthora palmivora*, electrical fields generated by the host organism were also proposed to affect zoospore attraction.[65] The use of commercially available microfluidic slides such as the chemotaxis device µ-slide (ibidi, München, Germany) should be considered for further evaluation of chemotactic behavior.

4.2 Chemical Signaling

In the ocean, diatom populations appear in diverse networks, consistently linked by interspecies interactions. Common approaches to study such allelopathic interactions between species are filtrate experiments and dual cultivation of two communicating species. Here, it was investigated, if the resistant diatom *C. wailesii* releases allelochemical compounds upon infection into the culture medium which can be transmitted onto the susceptible *C. granii*. Therefore, co-cultivation experiments were performed to mimic natural conditions and the infection success of *L. coscinodisci* was monitored. In parallel, it was analyzed whether the host resistance of a susceptible species can be induced after co-cultivation. Furthermore, susceptible cells were incubated in the filtrated exudate of *C. wailesii*, infected with the parasite strain and screened for sporangia formation or morphological changes.

4.2.1 Co-Cultivation

The susceptibility of *C. wailesii* to the oomycete *L. coscinodisci* was assessed by co-incubating cells retained in a strainer with infected *C. granii* present in the surrounding medium and the cells were observed daily with microscopy for 10 days. No sporangia of infected cells were seen in treated *C. wailesii.* Few cells displayed a zoospore attached (**Figure 8**). However, the treated *C. wailesii* population was characterized by wide heterogeneity in morphotypes and elevated production of small vesicles. Furthermore, the parasite treated cells depicted a deformed cytoplasmic organization. After daily microscopy for 10 days, all *C. granii* (S) cells were found infected when treated with the parasites and regardless of the addition of seawater medium or *C. wailesii* exudates.

The cytoplasmic deformation of the treated cells may hint towards the induction of resting-cell, as described by Nagai and Imai.[88] They presented the formation of a resting-cell in *C. wailesii* Gran under darkness and anaerobic conditions, where the cytoplasm separates from the cell wall and condenses towards the centre of the cell.[88] Diatoms can transfer to a resting-cell stage to escape unfavorable conditions such as nutrient depletion and alterations in temperature, salinity and light and may return to their vegetative growth in case conditions become favorable again.[88] To determine the existence of resting stage induced by parasitism, further experiments have to be conducted by exposing the resistant strain to different external stressors. Additionally, the cells with a faded cytoplasmic color (homogenously

distributed after treatment with parasite strain LagC7A) resemble the *C. wailesii* cells grown N-depleted medium according to Armbrecht *et al.*[56]

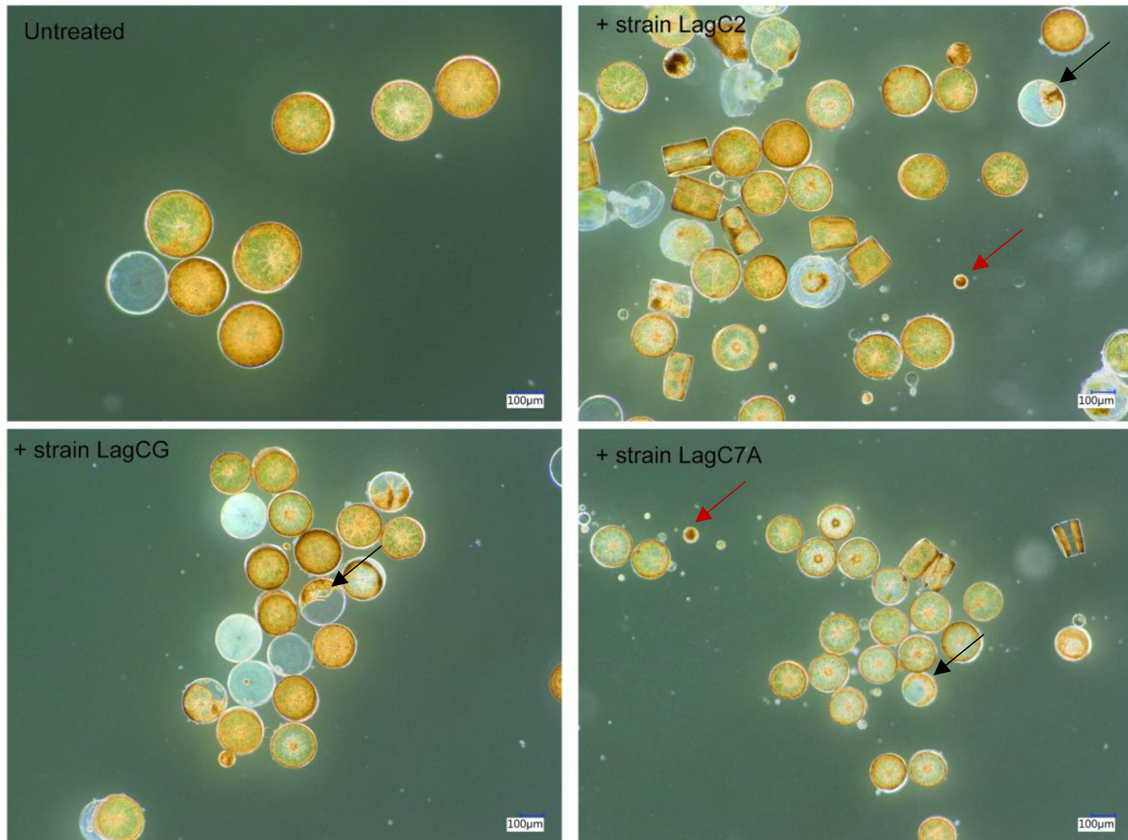

Figure 8 Susceptibility assessment of oomycete *L. coscinodisci* on the diatom *C. wailesii.* The cells co-incubated with the oomycete strains (LagC7A, LagC7B, LagCG) showed various morphotypes and produces many small vesicles (red arrows). The number of dead cells (empty frustules) and cells with deformed cytoplasmic organization (black arrows) was often seen in the oomycete-treated cultures.

The obtained results raise the question, whether external stressors such as nutrient depletion alter the susceptibility to the oomycete. Thus, continuing investigations should be pursued in assessing the effect of N- and P-depletion on the resistance of the diatom by inoculation in different media and addition of the parasite *L. coscinodisci*.

The infection of the susceptible diatom *C. granii* (S), co-cultivated with the resistant species *C. wailesii* (R) was performed and the infection rate was monitored. The setup used for dual-cultivation (a cell strainer inside a petri-dish) enabled the physical separation of the two cultures and constant shaking was used for homogenous distribution of the medium (**Figure**

9). Additionally, a screening for inducible defense in the susceptible species was conducted by pre-incubating both strains (S and R) in dual-culture, transferring the susceptible strain into 6-well plates and subsequently treating it with the parasite (**Figure 9**).

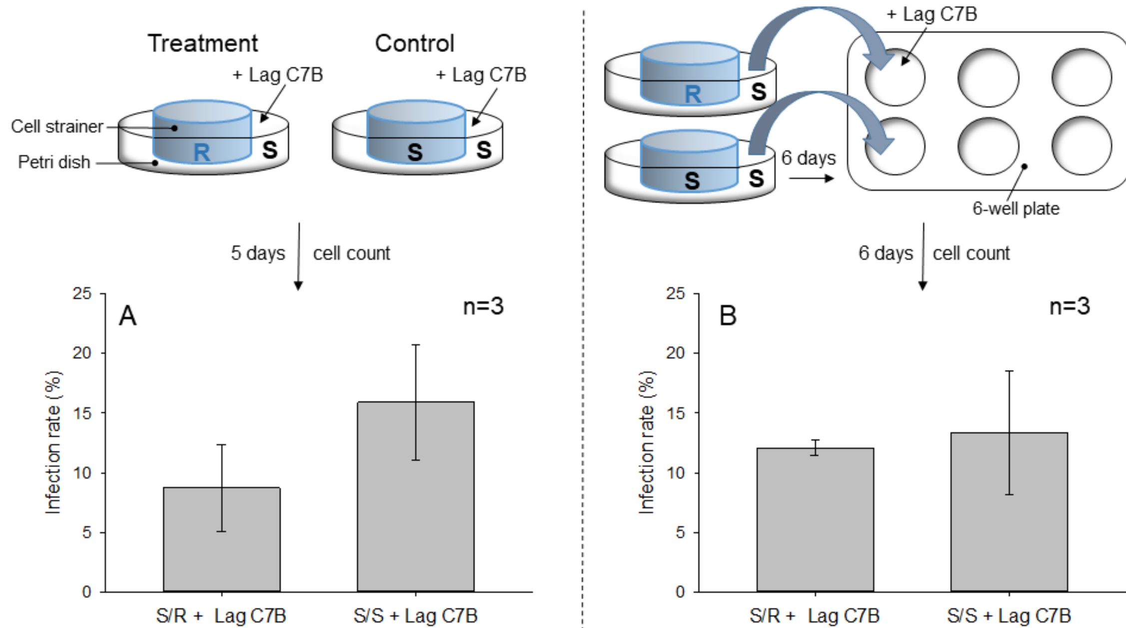

Figure 9 Dual-cultivation experiments **A** Infection rate of co-culture in petri-dish after five days of incubation. A Mann-Whitney Rank Sum Test found no statistical significance ($p = 0.200$). **B** Infection after co-culture. The dataset was normalized for obtaining the infection rate with normalization factor 1.31. T-test delivered no statistical difference (N = 3, $t(4) = -0.417$, $p = 0.698$). Strain S: susceptible *C. granii,* strain R: resistant *C. wailesii*, Lag C7B: parasite strain of *Lagenisma coscinodisci.* Depicted are the means of the infection rates ± SD.

The infection rate in susceptible cells co-cultivated with resistant cells shows no significant difference compared to the control (susceptible cells in both cell-strainer and Petri dish). A similar outcome is observed for the screening of inducible defenses after co-cultivation. The susceptible cells of *C. granii* show no induced resistance after co-cultivation, as suggested by the missing statistical difference of the infection rates between control and treatment.

These results indicate that the dual cultivation of the susceptible strain with the resistant species neither induces cellular resistance of *C. granii*, nor does it impact the performance of the parasite. The experimental outcome points towards the functional role of cellular effectors in mediating the resistance of *C. wailesii* towards *L. coscinodisci*.

When analyzing allelopathic behavior or inducible defense, however, there are notable considerations for the evaluation of the presented results. The concentration of metabolites surrounding a single cell can greatly differ due their genetic and physiological variety as well as dilution effects. Thus, the medium might consist of patches of high and low concentrated chemicals.[7] Furthermore, the range of distance where potential infochemicals might cause an effect are unknown.[7]

When looking at immobile diatoms, in particular, these considerations raise questions about the effectiveness of such defense mechanism, as the place of action cannot be controlled or dilution might extinguish the targeted effect due to undergoing the threshold concentration for inducing an effect. Moreover, the time ranges during which chemicals are produced are not known. According to Vidoudez and Pohnert, the presence of infochemicals depends on the diatoms growth phase and their concentration can be rapidly depleted after expression.[89] An inducible effect should not be excluded, as defense mechanisms can be short- or longterm.[90]

Further experiments need to be conducted with varying parameters such as additional time points of microscopic observations, different stages of growth and varying culture abundance. Still, the setup applied was successfully tested for an initial screening of potential allelopathic effects. Additional experiments are necessary, however, to confirm the observed results by utilizing co-culture devices developed by Pohnert *et al.* The latter will ensure identical growth conditions for both species.[78] Further tripartite interactions of the parasite, the susceptible and resistant species should be monitored, as the susceptibility to allelopathy might be enhanced through parasitic infection, enabling diatoms, naturally occurring in complex networks, to cope with such attacks.[7]

4.2.2 Exudate Treatment

One of our hypotheses was that *C. wailesii* might exude repellents or defense compounds, potentially impeding the swimming of zoospores of *L. coscinodisci*. To study the effect of *C. wailesii* exudates on the infection efficiency, susceptible *C. granii* cells were incubated either in seawater medium or with exudates of *C. wailesii* obtained by filtration and the oomycete was added to the cultures.

Filtrates of a dense monoculture of *C. wailesii* were used for incubating *C. granii* and the effect was monitored by generating growth- and infection rates (**Figure 10**).

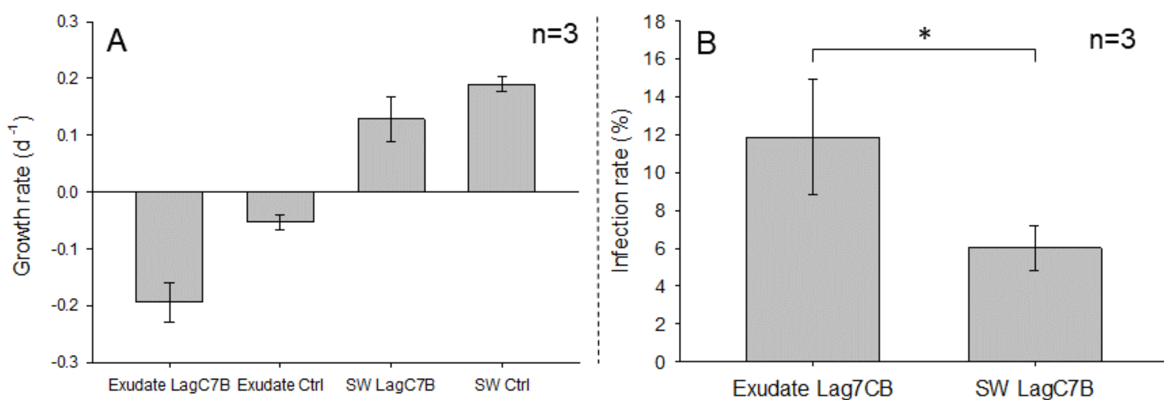

Figure 10 Growth and infection rate of *C. granii* after cultivation with filtrated exudates of *C. wailesii* for six days. **A** One-way ANOVA with posthoc test (Tukeys, N = 3, F = 118.23, df = 3, $p < 0.001$) shows significance in growth between treatments. **B** T-test (N = 3, $t(4) = 3.118$) found statistical difference of infection rate between control and treatment ($p = 0.036$). The averages of the growth rates and infection rates are depicted with the standard deviation.

Cultivating cells of *C. granii* in the exudate medium of *C. wailesii* seems to negatively affect the cell growth while the control treatment with seawater shows a positive effect. The infection rate differs between treatments, although it has to be considered, that a difference in cell density already causes varying infection rates.

It can be concluded that the growth of the susceptible *C. granii* is impeded by inoculation in the filtrate of *C. wailesii*. On the contrary, no valid information was obtained for the allelopathic effect of the exudate on the susceptibility of *C. granii*, since both treatment and control were infected.

4.2.2.1 Treatment of *C. granii* with axenic and non-axenic exudate of *C. wailesii*

Preliminary work within the Pohnert group by Dr. Yung resulted in the identification of bacteria that are present within the medium of three species of *Coscinodiscus*: *C. granii*, *C wailesii* and *C. radiatus*. The diatoms surrounding microbiome was analyzed by 16S rDNA sequencing to identify the bacterial phylotypes associated with *Coscinodiscus* (**Figure 11**).

Diatom-derived transparent exopolymer particles (TEP) are known for recruiting bacteria which can induce cell aggregation in diatoms, reversely favoring sinking rates due to its high molecular weight.[4, 56] Since sinking was suggested to represent a strategy of escaping parasitism[52], special focus was set on isolating and identifying the non-obligate bacteria associated with *Coscinodiscus* spp. (**Table 2**).

Table 2 Identification of taxonomic identity of *Coscinodiscus* associated bacteria

Diatom host species	Isolate Code	Bacteria identity	Score identity %	Reference number
Coscinodiscus granii	*Cg-1*	*Pseudomonas marina*	*100*	FJ457131.1
	Cg-2	*Sulfitobacter marinus*	*99.5*	NR_043936.1
	Cg-3	*Antartobacter heliothermus*	*99.9*	CP022540.1
Coscinodiscus wailesii	*Cw-1*	*Alteromonas stellipolaris*	*98.3*	CP015346.1
	Cw-2	*Maribacter dokdonensis*	*99.8*	NR_043294.1
	Cw-3	*Sulfitobacter spp.*	*99.8*	KT583350.1
	Cw-4	*Antartobacter heliothermus*	*99.8*	CP022540.1
Coscinodiscus radiatus	*Cr-1*	*Pseudoalteromonas spp.*	*99.7*	EU330350.1
	Cr-2	*Antartobacter heliothermus*	*99.9*	CP022540.1

The bacteria strain *Antartobacter heliothermus* was found in all three analyzed species (susceptible *C. granii* and *C. radiatus* and resistant *C. wailesii*). The strain *Alteromonas stellipolaris* appeared to be species-specific to *C. wailesii*. The latter was shown to exhibit antimicrobial as well as quorum quenching activity and to exhibit enhanced exopolysaccharides (EPS) biosynthesis.[91] These findings clearly motivate future investigations on the role of extracellular polysaccharides in the resistance process of *C.*

wailesii. The diatom might display a behavioral response through polysaccharide production, favoring sinking to escape parasitic attacks or to remove infected cells.[52] Additionally, in heterogeneous microalgae cultures, there may exist individuals, which produce high local concentrations of polysaccharides to prevent infection by oomycetes.

Raven *et al.* stated that increased polysaccharide production in microalgae could restrict access to parasites.[52] Further experiments should be conducted to assess the functional role of diatom-derived polysaccharide substances: after acquiring EPS from the culture-medium it could be implemented into an attraction assay of the parasite strain and susceptible diatom. Such procedure could be suitable to answer the question whether these polysaccharides act as a physical barrier to the zoosporic parasites. It must be emphasized, however, that one strategy does not necessarily exclude the other and that an interdisciplinary approach is key for extending our knowledge on microalgae defense strategies.

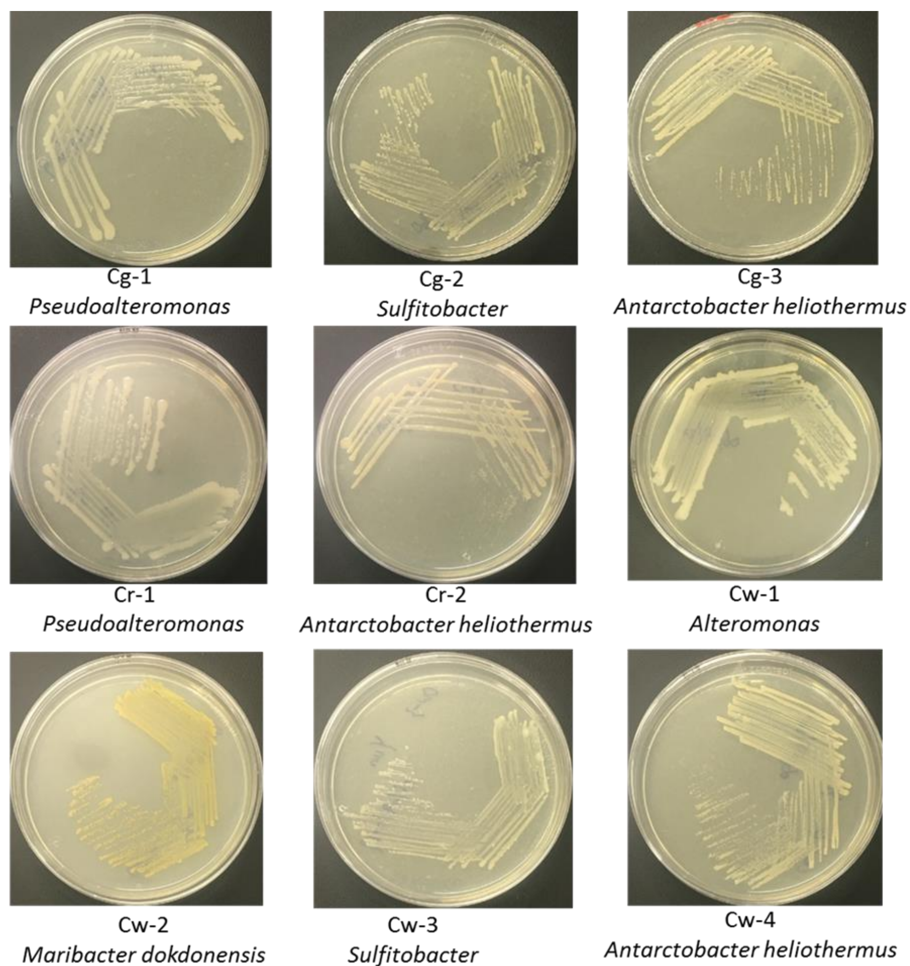

Figure 11 Representative pictures of the bacteria isolates purified on 9 cm diameter agar plates.

Since the effect of bacteria on the susceptibility to the oomycete parasite could not be excluded, further experiments with axenic and non-axenic filtrates of the resistant *C. wailesii* were performed and the effect on the growth of *C. granii* was monitored.

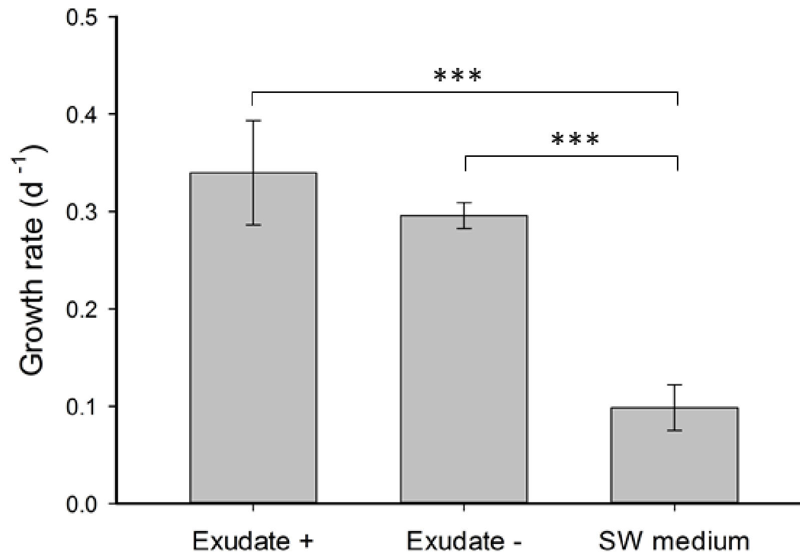

Figure 12 Growth rate of susceptible *C. granii* grown with axenic (-) or xenic (+) filtrates of *C. wailesii*. The controls consisted of cells cultured in seaweater medium without addition of nutrients. No significant difference was found between between treatments (+) and (-). Significant difference between treatments and seawater (SW) control. One-way ANOVA (N = 3, df = 2, $p < 0.001$). Averages of the growth rates with calculated standard deviation are depicted.

The total number of cells and the growth rate were determined after 7 days of incubation. Statistical differences of the growth rate were wound between cells cultured in the seawater medium and cells grown in axenic or xenic exudates (Krustal-Wallis One Way Analysis of Variance on Ranks, N = 3, F = 41, *p*-value < 0.001). Two post hoc tests (Holm-Sidak and Tukey HSD method) revealed significant difference between growth rates of cells incubated in seawater medium compared to cells grown in axenic exudates (df = 0.198, *p*-value < 0.001) and cells grown in xenic conditions (df = 0.242 *p*-value = 0.001).

The obtained results greatly differ from the first performed filtrate experiments, where cultivation in the exudate significantly affected the growth of *C. granii*. It cannot be ruled out, that the effect is attributed to age of the culture and compounds of cell degradation. When comparing both exudate treatments, it must be pointed out, that the cell count of the resistant *C. wailesii* culture used for the initial filtrate studies (**Figure 10**) was 2-fold higher

than in the follow-up experiment. This discrepancy may hint towards a dose dependency of the observed negative allelopathic effect and would support similar findings from Yamasaki *et al.*[79] They presented varying allelopathic effects when performing filtrate experiments using different initial cell densities, with the marine microalgae *Skeletonema costatum* and *Heterosigma akashiwo*.

4.3 Untargeted Metabolomics investigations

The intra- and extracellular response of the giant diatom *Coscinodiscus wailesii* to the endoparasite *Lagenisma coscinodisci* was investigated by performing infection experiments. *C. wailesii* was treated with the oomycete parasite *L. coscinodisci* and after metabolomic extractions, extracts were analyzed using UHPLC-MS/MS. By applying an untargeted metabolomics approach, a screening for low molecular metabolites potentially involved in the resistance of *C. wailesii* was performed. Uni- and multivariate statistical methods were applied to identify promising candidates.

4.3.1 Growth rate of *C. wailesii* treated with *Lagenisma coscinodisci*

A growth rate of *C. wailesii* treated with the parasite strain LagC7B and non-treated was generated from the cell counts prior to extraction. The treatment of *C. wailesii* cultures with the parasite shows a significant increase in the growth as compared to an untreated culture (**Figure 13**). A statistical significance between treated and healthy *C. wailesii* was confirmed by a t-test ($N = 3$, $t(8) = 2.83$, p-value $= 0.022$). Treated cells exhibited a higher growth as a reaction to oomycete infection.

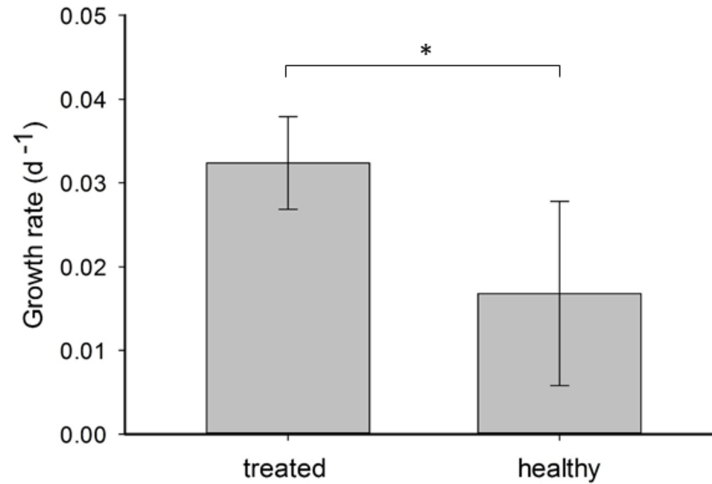

Figure 13 Growth rate of treated and healthy cultures of *C. wailesii* shows a statistical difference according to t-test (N = 3, $t(8) = 2.83$, $p = 0.022$). The mean of the growth rates is depicted along with the calculated standard deviation.

The enhanced growth of *C. wailesii* may indicate towards up-regulation of metabolic processes in presence of the parasite. As according to the Red Queen Hypothesis[59], an increased number of cells might support the assumption of the diatom undergoing sexual reproduction for obtaining defensive traits and maintaining genetic diversity to further withstand parasitic infection.

During microscopic observations of the inoculated cultures, no infection could be observed. However, few cells of *C. wailesii* were detected, where the zoospore of the parasite attached but did not develop into a sporangium.

Since *C. wailesii* became resistant to oomycete infection, preliminary experiments for monitoring the infection already showed parasite-strain dependent attachment of the oomycete (personal communication from Dr. Vallet). These findings support the assumption of the involvement of cellular metabolites in the resistance to parasites. When considering already attached zoospores without infection success, the role of the silica shell in physiological responses should not be disregarded. The membrane pores could be the restricting factor to parasites inserting their feeding tube and therefore the parasite might only go as far as attaching to the host.[52] Considering that host-adhered zoospores were not observed for all *C. wailesii* cells but rather few individuals, their might exist further factors restricting the attachment. Presumably, the host size could influence the selectivity of zoospores, as already discussed by Holfeld *et al.*[92] The cultures used in all experiments are not synchronized and, accordingly, highly heterogeneous in terms of size and genetic

properties. This might explain the differential attachment, but not the universal resistance observed in the whole culture.

4.3.2 Metabolic investigation of *C. wailesii* endometabolome

The first LC-MS measurement of *C. wailesii* cell extracts yielded in the detection of 395 metabolites with a corresponding chemical formula, *m/z* and retention time. The low compound abundancy led to the assumption that most metabolites were below the limit of detection, due to the low concentration of the extracts, leading exclusively in the detection of major metabolites. Therefore, measurements with higher concentrated extracts were performed, resulting in the detection of 1124 metabolites (**Figure 14**). Most ions were detected in positive polarity (ESI +). The reproducibility of the obtained data was validated by comparison of all QC pool samples (see appendix **Figure A 1** - **Figure A 3**).

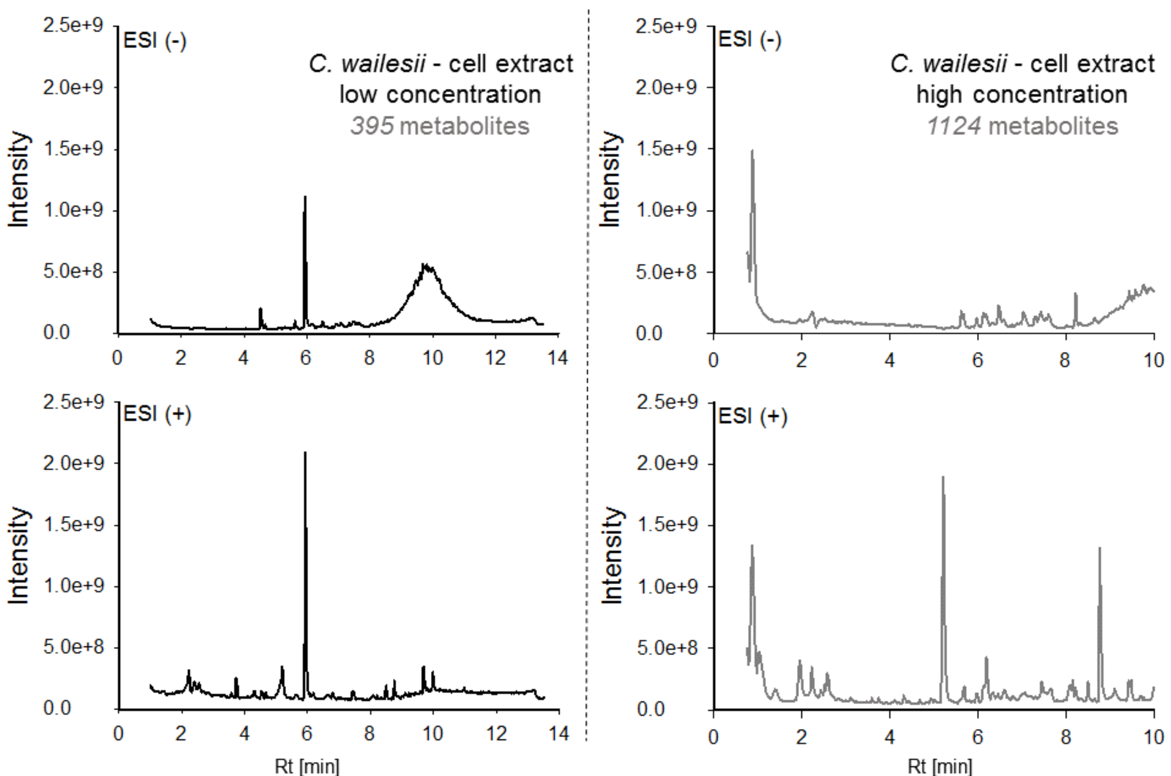

Figure 14 Total ion current chromatograms of cell extracts of *C. wailesii* in positive (ESI +) and negative (ESI -) mode. For measurements of high concentrated extracts, the method was cut from 10 min onwards.

After visualizing the raw data with the Xcalibur software and processing the dataset with Compound Discoverer to generate a data matrix, the statistical analysis was conducted with the online software MetaboAnalyst to determine the metabolic differences in *C. wailesii* untreated or treated with parasite *L. coscinodisci*. Initially, the complete dataset of

1124 metabolites (which were assigned to a chemical formula by Compound Discoverer) was used for statistical analysis with prior logarithmic transformation. A t-test with an adjusted *p*-value of 0.05 delivered no significant features, therefore a further statistical test was applied by generating a volcano plot and conducting a principal component analysis (PCA). A PCA is a statistical method applied to explain the total variance in a dataset by choosing the appropriate linear combinations of correlated data, enabling visualization of these variances. The variances between the groups of treated and healthy cells of the host strain *C. wailesii* are displayed in a PCA plot (**Figure 15**). No discrimination can be made between the healthy and treated cells, as both treatments depict complete overlapping.

To determine the distribution of up- and downregulated metabolites in the different treatments, a volcano plot was obtained. Volcano plots are useful tools for visualizing statistically significant differences in metabolomics datasets. Every point in the volcano plot represents a compound in the endometabolome of *C. wailesii*.

The fold-change value describes the quantitative change of an ion in the treated samples compared to the control. All compounds that possess a fold-change > 2.0 represent an upregulation in the treated cells. The volcano plot (fold-change threshold 2.0, p-value < 0.1) delivered 72 up- or down-regulated compounds in the cell extracts.

With these 72 selected metabolites, an additional data analysis was performed and a PCA was conducted (**Figure 15**). The distribution of both groups using the reduced dataset (p-value < 0.1) shows a clear distinction between treated and healthy cells and 56 % of the total variance can be explained by the first two principal components. The obtained data proposes, that the metabolites produced in the host strain of *C. wailesii* vary depending on the presence or absence of the parasite.

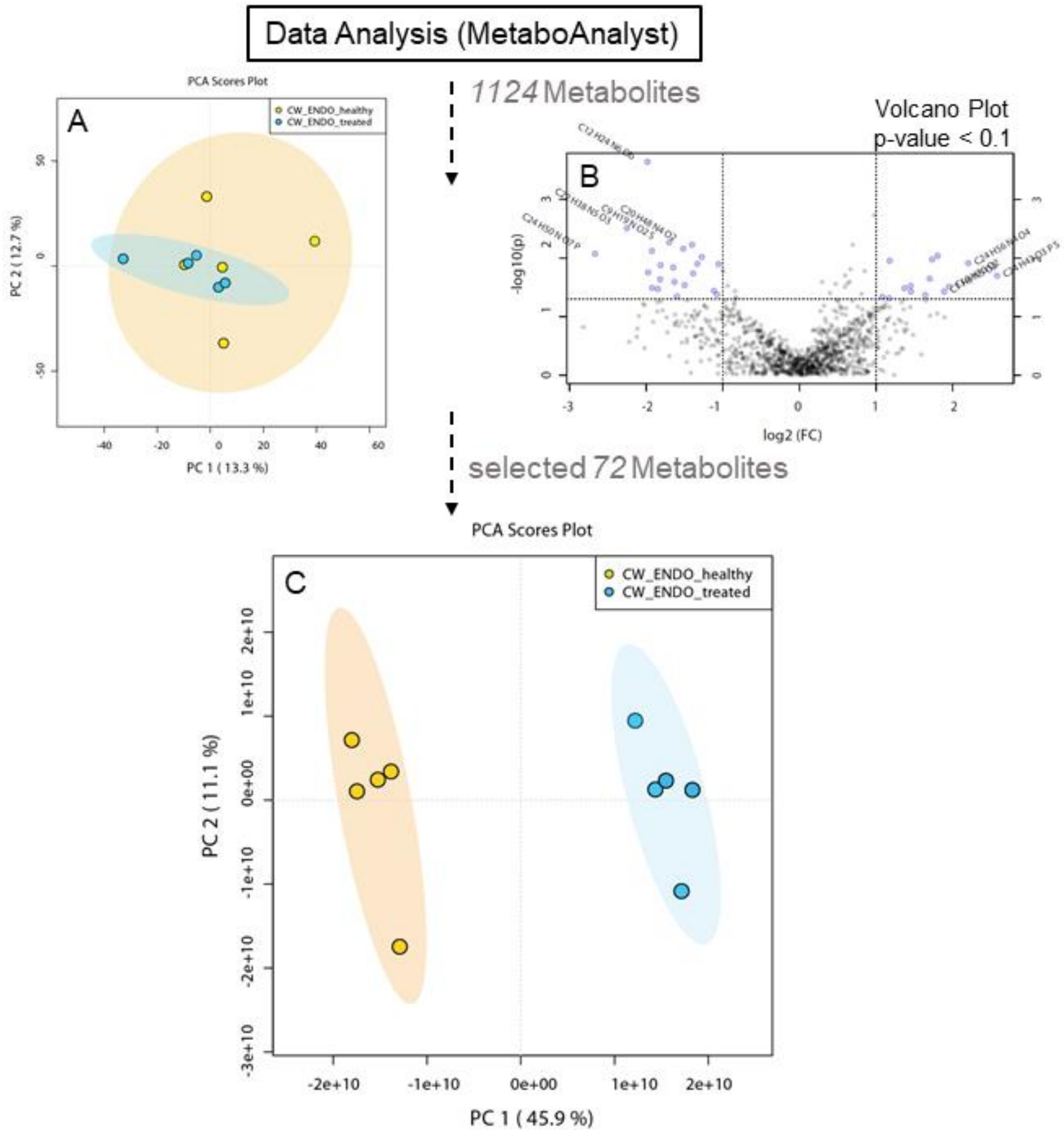

Figure 15 Process of statistical analysis conducted for metabolic profiling **A** PCA scores plot of all metabolites detected in cellular extracts of *C. wailesii* in presence (treated) or absence (healthy) of parasite **B** Volcano plot visualizing up- or downregulation of metabolites in treated cells. Points above the line with p-value < 0.1 and fold-change FC > 2.0, log2(FC) = 1) are considered significant. **C** PCA score plot depicting the discrimination of 72 selected metabolites, based on the compounds selected from the volcano plot analysis (**B**).

For further visualization of metabolite distribution between treated and healthy replicates, a heatmap was generated based on all 1124 compounds (**Figure 16**).

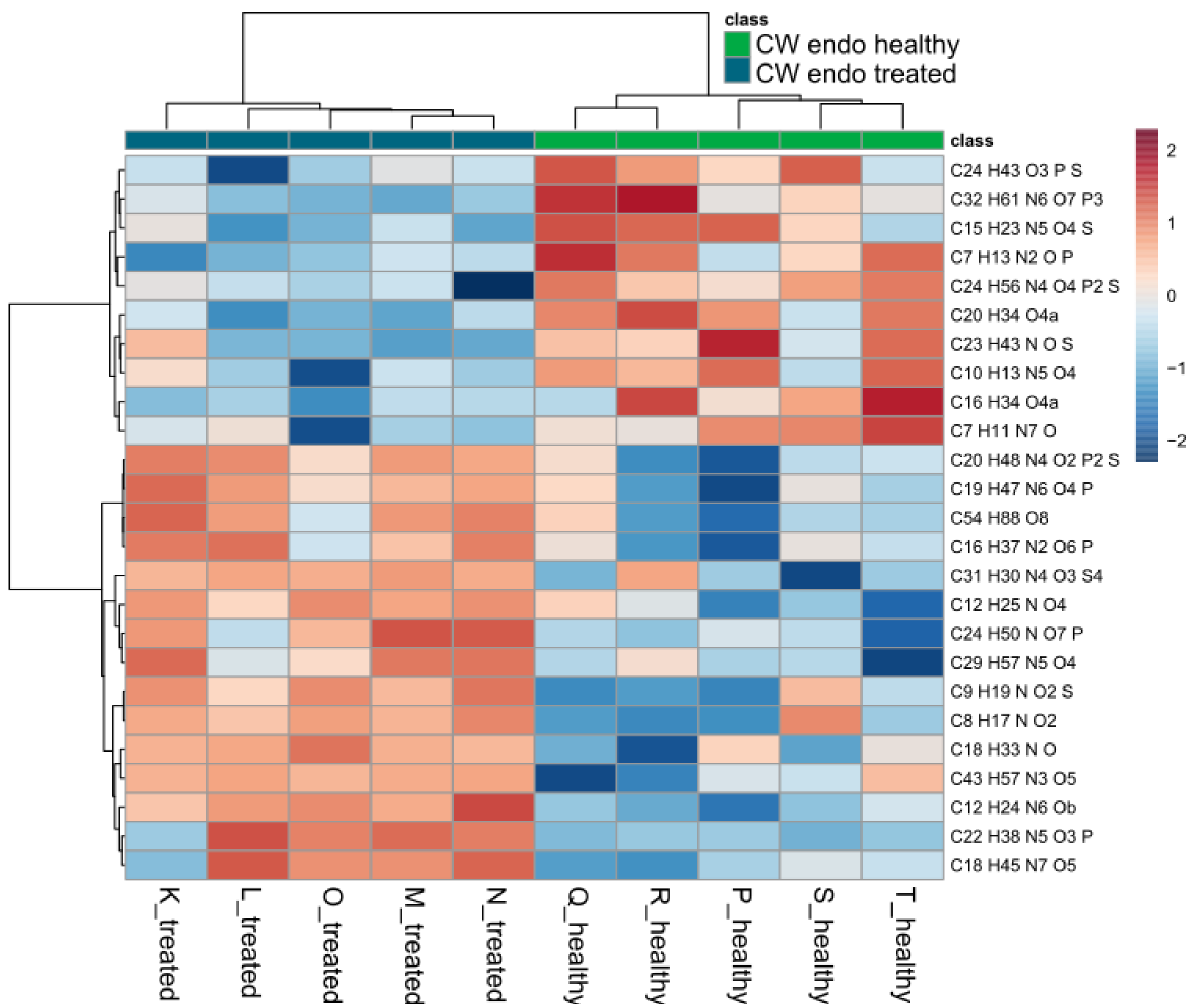

Figure 16 Heatmap of the top 25 metabolites in cellular metabolome of *C. wailesii* in absence (healthy) or presence (treated) of parasite *L. coscinodisci*. Red color represents high concentration and blue color represents low concentration in extracts. Heatmap generated based on data analysis of all 1124 metabolites. Data were log-normalized. CW: *Coscinodiscus wailesii*

Some metabolites elicit a higher concentration in treated cells and support the hypothesis of an induced upregulation upon parasite treatment. After evaluating the general distribution of metabolites in different treatments, a special focus was set on investigating the most significant up- and downregulated compounds. As the criteria for further data processing, only results from the volcano plot with an adjusted p-value < 0.05 were included. The selected 32 significant metabolites (p-value < 0.05 from volcano plot, FC > 2.0) were used for further identification on MS^2 level. MS/MS spectra were chosen for spectral annotation

if they depicted an extracted ion chromatogram (EIC) peak, good separation, and high intensity.

The comparison of MS/MS spectra with public databases in the CSI:FingerID tool led to the identification of compounds (with a similarity score > 90 %) by providing a chemical name and by further database comparison, delivering a compound class or biosynthetic pathway. The selected metabolites were grouped by regulation in parasite-treated cells (**Table 3**). To assess whether the selected compounds are related to host resistance to parasites, further literature search was conducted for each compound with a corresponding chemical name (**in bold** in **Table 3**).

Some precursor ions showed no fragmentation patterns after the initial analysis with MS/MS. Therefore, additional MSMS spectra were obtained and only precursor ions of interest were targeted with the method of parallel reaction monitoring (PRM).

The higher-energy collisional dissociation (HCD) was adjusted to collisional energy in the range of HCD15-HCD45eV (as opposed to the averaged energy HCD35). Notably, depending on the structural properties of the compound of interest, higher energy can enable better fragmentation of the selected precursor ion, however, it can also cause complete dissociation. Thus, it has to be well adjusted for each compound to receive valid results.

The compound, which elicited the highest fold-change in the volcano plot, possessed the chemical formula $C_{24}H_{50}NO_7P$ and its spectral annotation is shown (**Figure 17**). With the exact mass search, an EIC was generated to visualize the corresponding ion trace. This compound was proposed as lysophosphatidylcholine 16:0 (LPC). It exhibits a 6-fold upregulation in the treated cells of *C. wailesii* in comparison to healthy cells. The precursor ion 496.3390 *m/z* shows specific fragmentation at HCD 30 with the characteristic fragments 184.0729 *m/z* and 104.1067 *m/z*.

Table 3 List of metabolites identified with UHPLC-MS/MS in the cell extract of *C. wailesii*. Molecular species were identified as $[M+H]^+$ or $[M-H]^-$ ions. Based on results of volcano-plot (p-value < 0.05, fold-change threshold FC > 2.0). Rt: retention time. Compounds used for further evaluation marked in bold. NA: Compounds with no structural information available from SIRIUS.

Formula	FC	Rt (min)	*m/z* observed	InChIKey	CSI Score (%)	Regulation in treated cells	Compound Class
$C_{24}H_{50}NO_7P$	**6.3**	**7.0**	**496.33896 $[M+H]^+$**	**ASWBNKHCZGQVJV-HSZRJFAPSA-N**	**86**	**up**	**Lysophospho-lipid**
$C_{22}H_{38}N_5O_3P$	4.8	6.2	452.2785 $[M+H]^+$	NA	NA	up	NA
$C_{18}H_{33}NO$	**3.8**	**6.5**	**280.26154 $[M+H]^+$**	**SFIHQZFZMWZOJV-HZJYTTRNSA-N**	**52**	**up**	**Fatty amide**
$C_{10}H_{13}N_5O_3$	**3.8**	**1.2**	**252.10852 $[M+H]^+$**	**OLXZPDWKRNYJJZ-RRKCRQDMSA-N**	**93**	**up**	**Nucleosid**
$C_9H_{19}NO_2S$	3.2	1.2	206.12090 $[M+H]^+$	SOQPWQPBWMJMNQ-UHFFFAOYSA-N	45	up	Carboxylic acid
$C_{10}H_{14}O$	3.1	4.4	151.11136 $[M+H]^+$	SEZLYIWMVRUIKT-UHFFFAOYSA-N	53	up	Monoterpenoid
$C_{43}H_{85}NO_8P_2$	2.8	6.8	806.58069 $[M+H]^+$	NA	NA	up	NA
$C_{20}H_{48}N_4O_2P_2S$	2.6	9.0	469.28473 $[M-H]^-$	NA	NA	up	NA
$C_8H_{17}NO_2$	2.5	0.8	160.13268 $[M+H]^+$	WPFHMRDMNFSOLO-UHFFFAOYSA-O	61	up	Carboxylic acid
$C_8H_{15}NO_3$	2.2	3.0	174.11261 $[M+H]^+$	WXNXCEHXYPACJF-ZETCQYMHSA-N	77	up	Peptide
$C_{27}H_{34}O_8$	2.1	7.6	487.23041 $[M+H]^+$	VEFHDRFIBYWPJH-CUTUUKBJSA-N	45	up	Phenylpropanoid
$C_{10}H_{14}N_2O_{16}S$	0.4	0.9	448.99246 $[M-H]^-$	NA	NA	down	NA
$C_{17}H_{35}OP_3$	0.4	6.3	349.19769 $[M+H]^+$	NA	NA	down	NA
$C_{25}H_{43}N_6O_4PS$	0.4	6.4	553.26855 $[M-H]^-$	NA	NA	down	NA
$C_9H_{18}Cl_3O_4P$	**0.4**	**5.8**	**327.00626 $[M+H]^+$**	**WOURXYYHORRGQO-UHFFFAOYSA-N**	**61**	**down**	**Phosphoric acid**
$C_{15}H_{23}N_5O_4S$	0.3	0.9	370.15430 $[M+H]^+$	LOVSTSWTPWPTCN-UHFFFAOYSA-N	43	down	NA
$C_{32}H_{61}N_6O_7P_3$	0.3	9.1	735.38870 $[M+H]^+$	NA	NA	down	NA
$C_7H_9N_5O_2$	**0.3**	**1.7**	**196.08249 $[M+H]^+$**	**HESAJCAMSAGUBE-UHFFFAOYSA-N**	**69**	**down**	**Purine Alkaloid**
$C_{10}H_{10}O_2$	0.2	7.2	163.07500 $[M+H]^+$	UBJNPHAYKBNFOC-UHFFFAOYSA-N	54	down	Benzoic acid derivate
$C_{24}H_{56}N_4O_4P_2S$	0.2	9.1	557.3385 $[M-H]^-$	NA	NA	down	NA
$C_{24}H_{43}O_3PS$	0.2	7.6	441.25342 $[M-H]^-$	NA	NA	down	NA

Since diatoms are able to synthesize and accumulate lipids that are essential for secondary consumers, they are a potential resource for human diets and may pose an attractive alternative or extension to vegetable obtained lipids.[93, 94] In the terrestrial world, the role of membrane-bound glycerophospholipids as bioactive compounds has been addressed by Drissner *et al.*, emphasizing the function of lysophospholipids as signaling molecules in plants.[95] Studies have shown, that the Lyso-PCs level rises during pathogen infection.[96, 97] Although the presence of LPCs in diatoms was already described[75], no information exists on their involvement in the resistance process to pathogens.

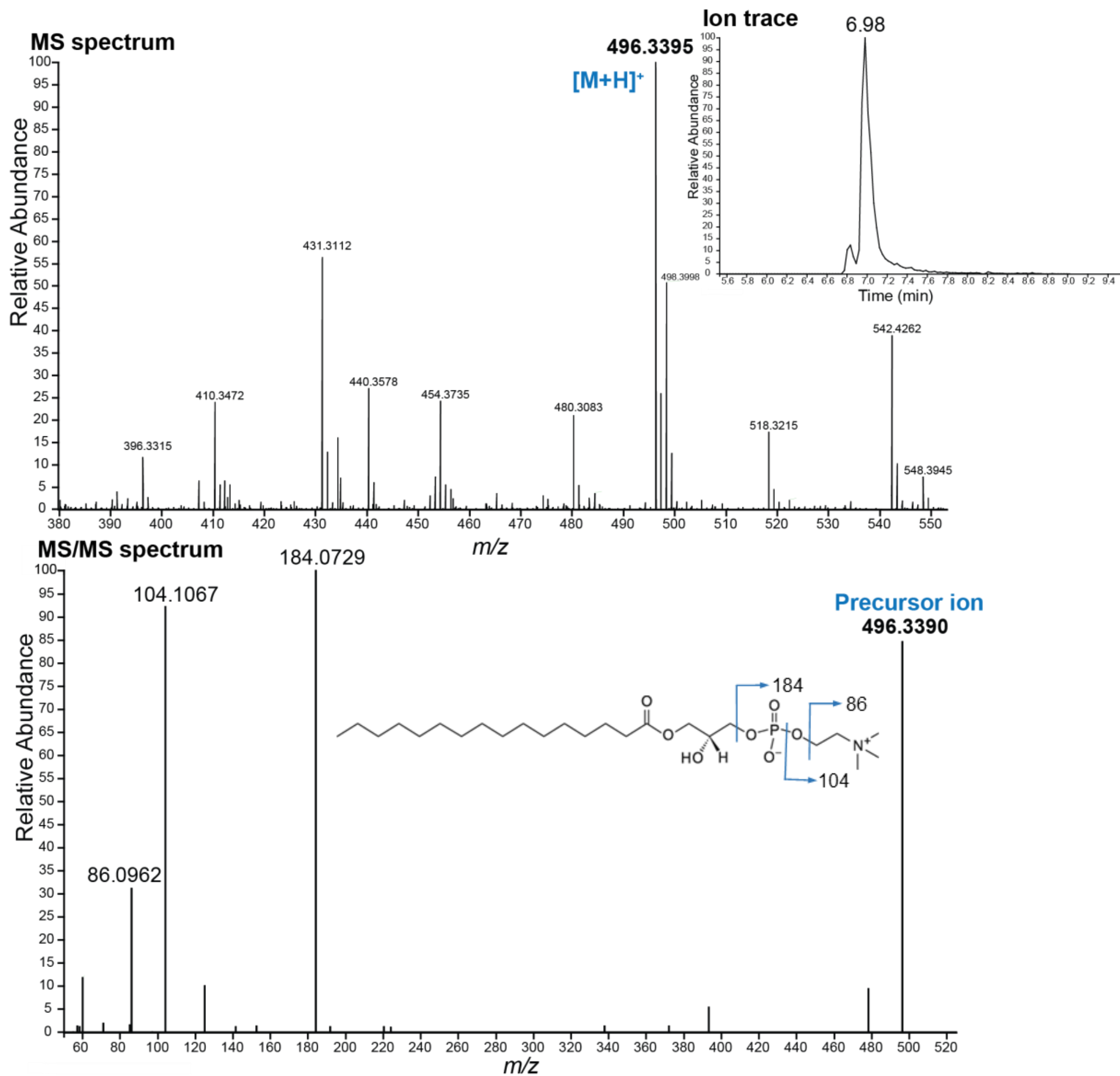

Figure 17 LC-MS/MS chromatogram, extracted ion chromatogram and MSMS spectra obtained from the analysis of cellular extracts of *C. wailesii* in positive ion polarity obtained from precursor ion lysophosphatidylcholine (LPC 16:0, 496.3390 *m/z* $[M+H]^+$). Characteristic fragments according to Kaya *et al.* (2018)[98]

Furthermore, the compound with a molecular mass $C_{18}H_{33}NO$ of 280.26154 *m/z* $[M+H]^+$ corresponding to the fatty amid linoleamide was detected. The metabolites exhibited a concentration of 3,6-fold higher in parasite-treated cells than in healthy cells. Also detected in cellular extracts with an elevated concentration in treated samples was the nucleoside 2'-deoxyadenosine with the chemical formula $C_{10}H_{13}N_5O_3$ and mass 252.10852 *m/z* $[M+H]^+$.

A metabolite that showed upregulation in healthy cells possessed the precursor ion 196.08249 *m/z* $[M+H]^+$ with the corresponding chemical formula $C_7H_9N_5O_2$. Comparison by spectral similarities and chemical databases delivered 2-amino-6-methoxy-9-methyl-7H-purin-8-one with structural properties equal to the amino acid-derived purine alkaloids.

Alkaloids are constituted of nitrogen-rich backbones and serve the secondary metabolism of plants. They can exhibit a bitter taste and are stored in specific tissues to function as constitutive detergents to predators.[99] Nitrogen plays an essential role in metabolic processes such as growth, reproduction and development.[99] The downregulation in treated cells could be explaining two different strategies. Either, the alkaloid might be stored and serves as a carbon and nitrogen source for synthesizing defense compounds. Alternatively, as production and accumulation of alkaloids might be very costly, the algae might reduce the metabolic production of such metabolites upon infection to invest its energy in defense or reproduction.

Recommended steps in validating the identity of the detected metabolites are the analysis of standards and execution of suitable bioassays. After addition of the compounds of interest, further infection experiments with the susceptible *C. granii* should be performed to monitor, whether the resistance can be induced. Regarding the compounds with relation to plant-defense, where no reference standards are purchasable, further structure identification could be conducted by NMR analysis after purification of the compound. All obtained results from the metabolomic analysis of the resistant *C. wailesii* have to be evaluated with regard to the lack of positive control, a susceptible culture of *C. wailesii*. Comparing the metabolome of a susceptible and resistant species from the same genus would deliver a more extensive insight into resistance strategies to infection with *L. coscinodisci*. To investigate the cellular processes involved in diatom resistance, it is proposed to couple metabolomics techniques to the other omics-strategies. Transcriptomics and proteomics studies would provide information about gene expression patterns and metabolic enzymes that modulate the cellular response upon infection. Furthermore, when in possession of a susceptible *C.*

wailesii strain, additional focus could be set on the investigation of physiological defense mechanisms by analyzing structural properties with Scanning Electronic Microscopy (SEM). So far, no information about pore arrangement and shell thickness in the resistant strain exists.

4.3.3 ^{13}C-labeled culture of *C. wailesii*

To confirm that the identified halogenated compound tri(chloropropyl)-phosphate ($C_9H_{18}Cl_3O_4P$, 327.00626 *m/z* $[M+H]^+$) which showed upregulation in healthy cells is indeed a natural product originating from the biosynthesis of *C. wailesii*, ^{13}C-labeling experiments were performed and MS spectra were acquired (**Figure 18**).

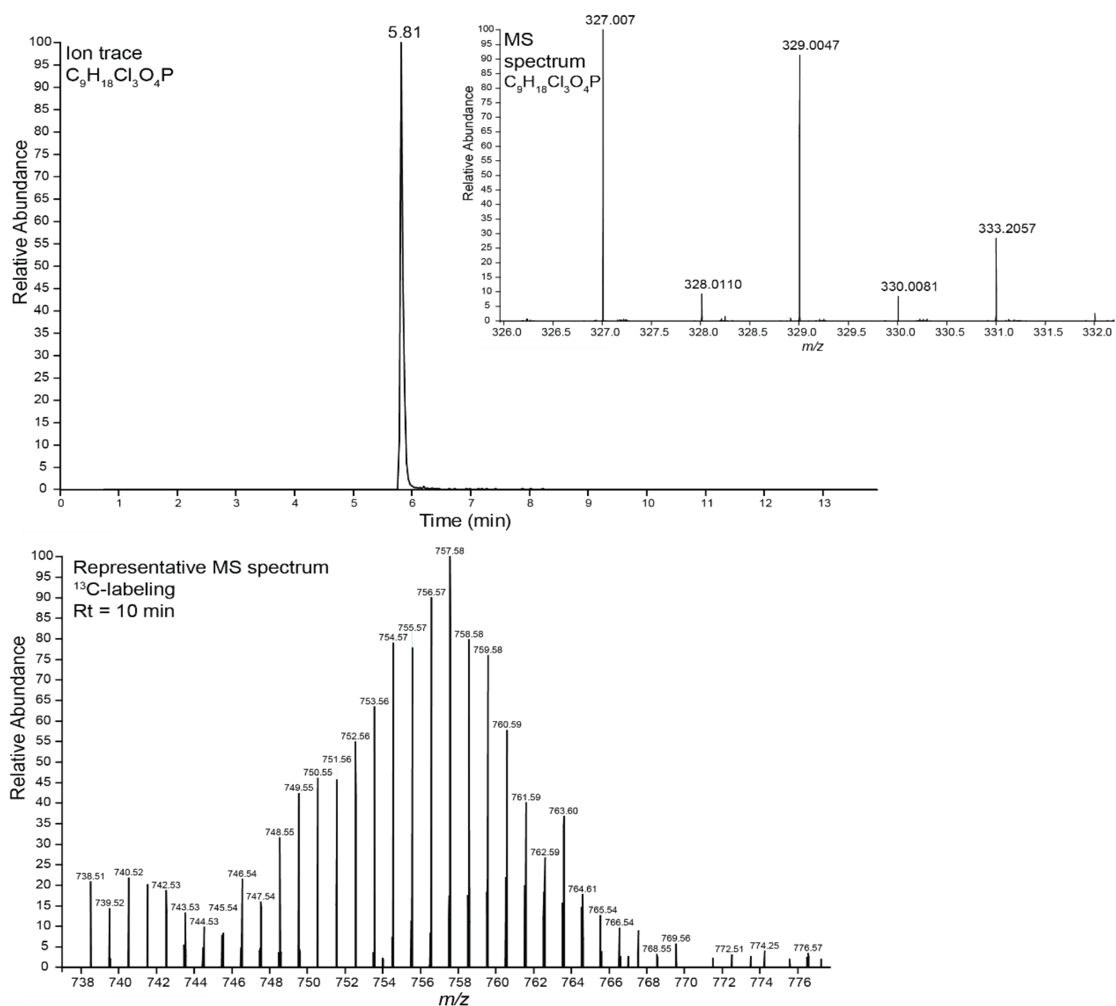

Figure 18 Ion trace and isotope pattern of halogenated compound $C_9H_{18}Cl_3O_4P$ identified in cellular extracts of *C. wailesii*, after incubation in ^{13}C labeled $NaH^{13}CO_3$ medium. Representative ^{13}C-labeled spectrum of a putative lipid (Retention time (Rt) = 10 min) in cellular extract shows successful labeling.

No stable isotope labeling was recognizable for the halogenated metabolite. The success of the ^{13}C-labeling was validated by comparison of the isotopic profiles in the cellular extracts. Thus, the compound might not be of biosynthetic origin but rather a contaminant introduced during the experimental proceedings. The metabolite was, however, also present in the f/2-medium blank, although present in lower abundancy than in cells. Taking these results into account, there might exist the possibility of bioaccumulation of the halogenated compound in *C. wailesii* through uptake from the marine seawater used for culture cultivation. The identified organophosphate is commercially utilized as a flame retardant and such halogenated compounds have been shown to contaminate natural environments.[100] To validate bioaccumulation processes in the diatom, further experiments must be pursued by obtaining analytical standards and conducting uptake experiments through addition of the standard to *C. wailesii* cultures.

4.3.4 Metabolic investigation of *C. wailesii* exometabolome

The results obtained by performing dual-cultivation experiments did not fully support the hypothesis, that the exudate of *C. wailesii* contains molecules that mediate the resistance to the parasite. The main focus was therefore set on the data evaluation of the cellular response in the resistant diatom. However, the obtained data from the LC-MS analysis was screened for notable molecular markers with bioactive properties, hinting towards a functional role in the infection process.

The total number of metabolites detected by the LC-MS analysis did not vary greatly between the low and high concentrated extracts. In the low concentrated extracts, 1489 metabolites (features with corresponding chemical formula, *m/z* and retention time) were detected, whereas the higher concentrated extracts delivered 1577 metabolites.

These results indicate a greater abundance of metabolites in the exudate medium of *C. wailesii* than inside the cell. Considerably, these variations can also be due to different extraction protocols for the endo- and exometabolome.

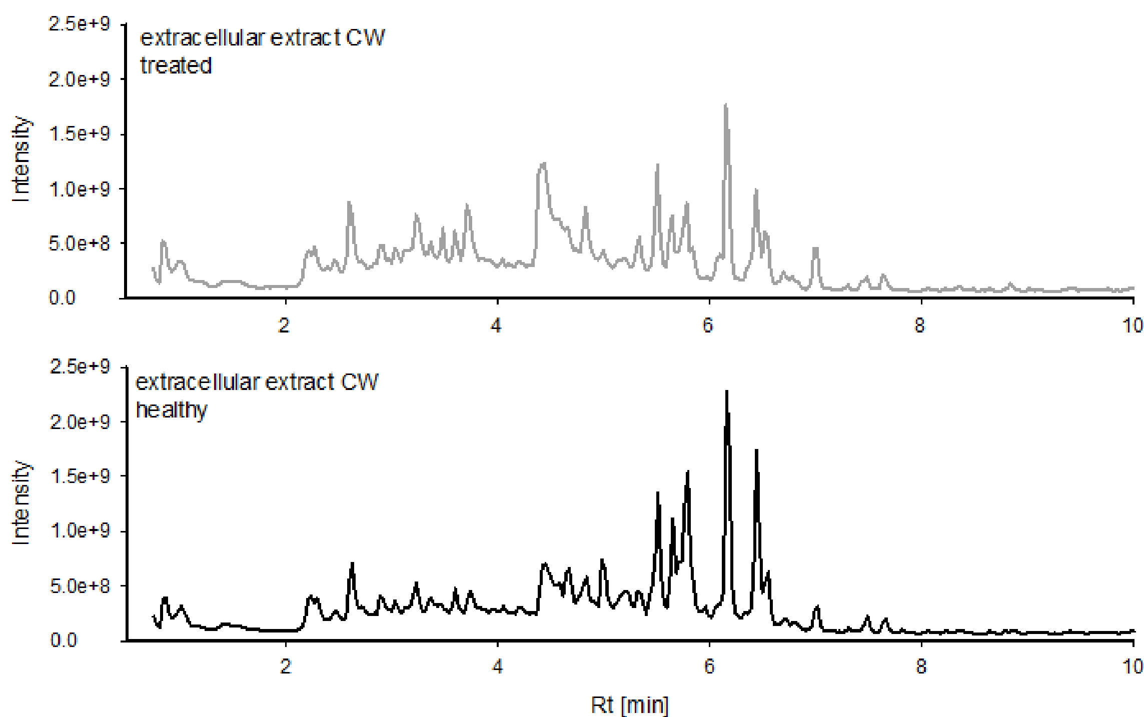

Figure 19 Comparison of TIC chromatograms of treated and healthy extracellular extracts of *C. wailesii*. Shown are intensities only in positive polarization (ESI+), as most metabolites of interest were detected in positive mode. Rt: retention time.

Upon treatment with the parasite *Lagenisma*, the exometabolome of *C. wailesii* is not modified (**Figure 19**). This might hint towards the absence of priming or induction of novel metabolites upon oomycete treatment. When linking statistically significant metabolites to extracellular processes, the evaluation of such data has to be done with careful considerations to the model organism and its surrounding matrix. High dilution effects confine the general compound distribution in aquatic environments. Accordingly, the concentration of bioactive metabolites exuded into the medium might be particularly low, making it difficult to detect minor differences, especially when considering dilution effects.[1]

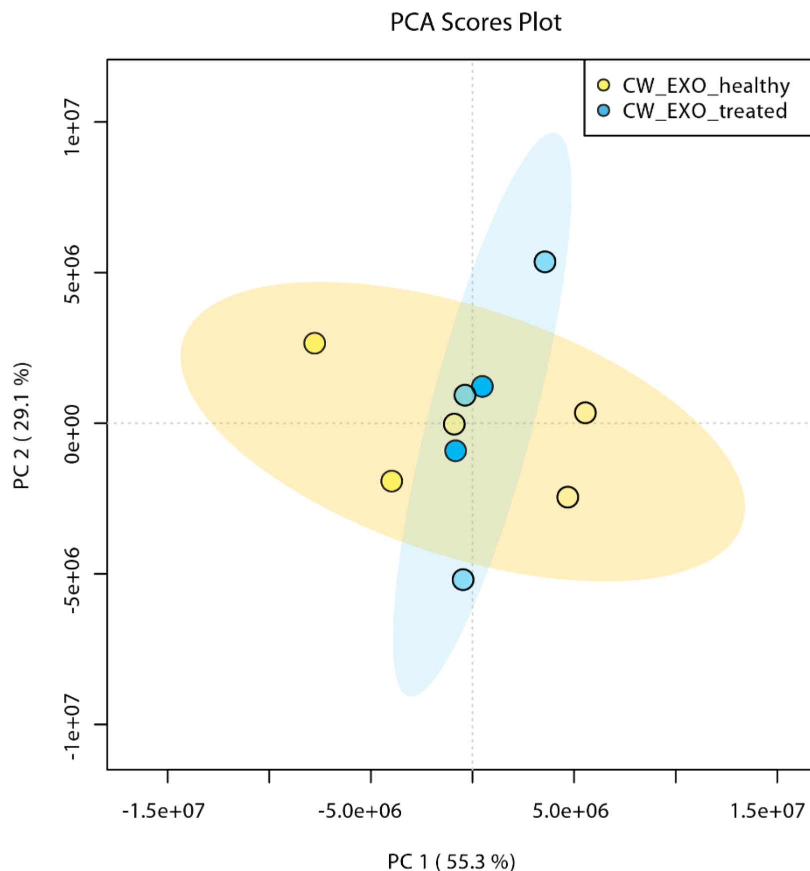

Figure 20 PCA plot of 55 selected metabolites from exudate *C. wailesii* in the presence (treated) and absence (healthy) of parasite. CW: *C. wailesii*. Plot generated according to pipeline depicted in **Figure 16**.

Due to the allelopathic effect observed on *C . granii* treated with *C. wailesii* exudates, the identity of the metabolites in this dataset was still further evaluated. A screening for notable defense-related compounds in the exudate delivered an interesting precursor ion (165.05 *m/z* $[M+H-H_2O]^+$, Rt = 6.17) with the corresponding molecular formula $C_9H_{10}O_4$ (InChIKey: DZAUWHJDUNRCTF-UHFFFAOYSA-N, CSI-score: 50 %).

Comparison with public databases proposed the phenolic acid 3,4-dihydroxycinnamic acid (**Figure 21**). There was no LC-MS/MS reference spectrum available. Thus, the proposed fragmentation pattern was approximated. The metabolite belongs to the chemical class of phenylpropanoids, which are prominent plant secondary metabolites originating from the amino acid phenylalanine.[101] Phenylpropanoids were shown to be synthesized upon root infection by fungal and bacterial pathogens.[101] Interestingly, there exist also few reports on

their appearance in microalgae, as presented in the unicellular algae *C. reinhardtii* by GC-MS analysis.[102, 103]

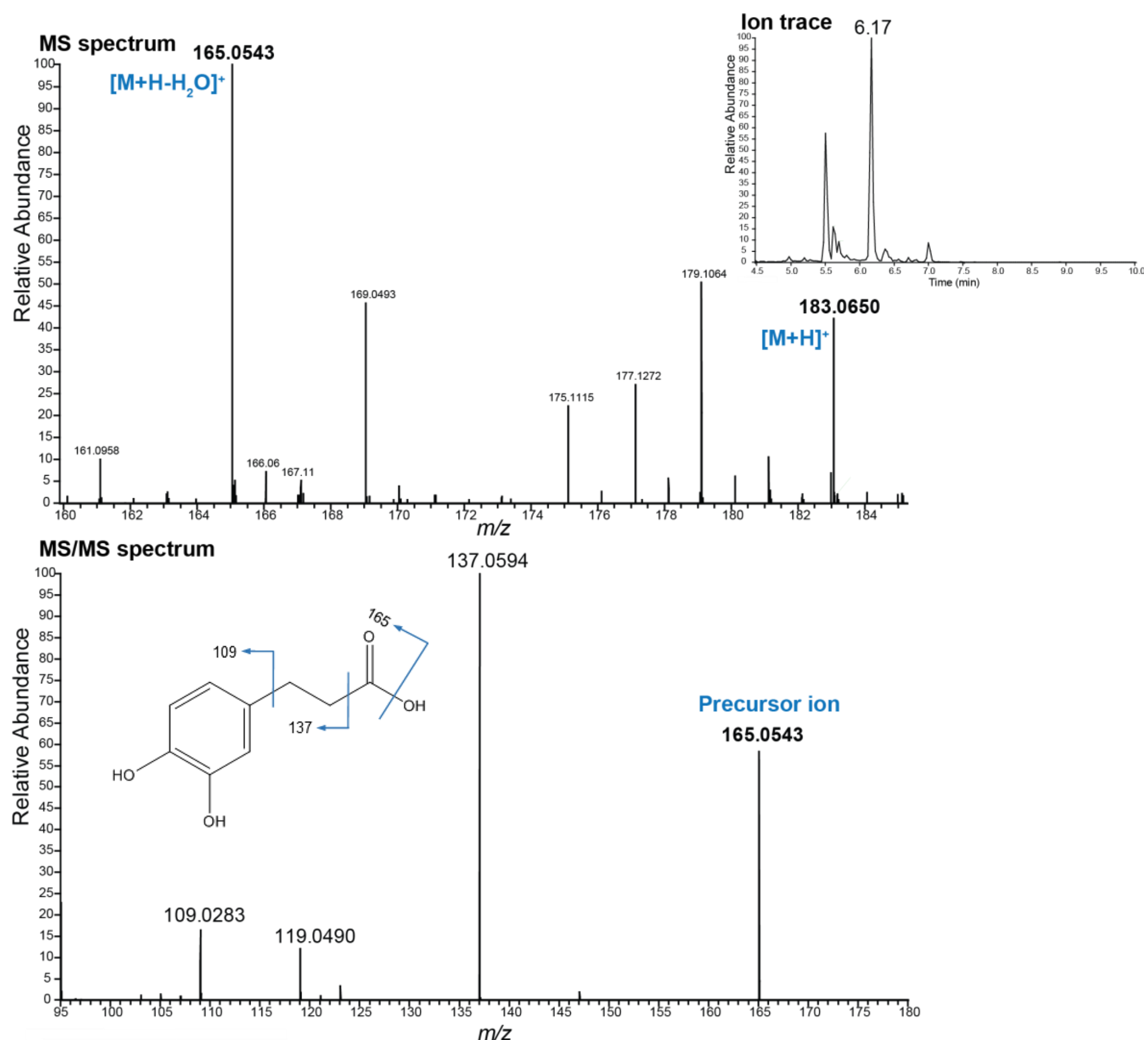

Figure 21 MS fingerprint of 3,4-dihydroxycinnamic acid (as proposed by spectral similarity in CSI:FingerID). LC-MS/MS chromatogram, ion trace (EIC) and MSMS spectra obtained from the analysis of extracellular extracts of *C. wailesii*. Depiction of proposed fragmentation pattern (no reference available).

A further compound exhibiting a 2.6-fold upregulation detected in treated cells with the chemical formula C_7H_6O (Rt = 6.51 min, 106.04169 *m/z*) resembled the volatile organic compound benzaldehyde (InChIKey: HUMNYLRZRPPJDN-UHFFFAOYSA-N, CSI-score: 68 %). Due to its already described biosynthetic origin in the marine diatom *Cocconeis scutellum* upon wounding[104] and in seaweeds[105], as well as its bioactive activity against fungi[106], this compound was found as an interesting target according to the literature.

For all detected metabolites in the exometabolome of *C. wailesii*, further validation is proposed by measuring analytical standards. The influence on the host-susceptibility could be monitored through performing bioassays by addition of the detected compounds of interest to the culture medium. Additionally, perhaps non-polar and volatile organic compounds might be changed upon parasite treatment, requiring further extraction protocols and analysis methods such as GC-MS.

5. Conclusion

The marine diatom *Coscinodiscus* spp. is repeatedly targeted by infection of the parasitoid oomycete *Lagenisma coscinodisci*, which was observed over the years in many different worldwide locations. During the establishment of a viable dual-culture of *L. coscinodisci* with its algal host, the giant diatom *Coscinodiscus wailesii* became resistant to parasite infection after sexual reproduction. On the contrary, *Coscinodiscus granii* and *Coscinodiscus radiatus* isolated from the same community remained susceptible. The aim of this thesis was to shed light on the defense strategies of *C. wailesii* by elucidating the mechanisms by which the diatom acquires resistant traits.

The main hypothesis was that either the diatom exudes low molecular chemical mediators into its surrounding medium, hindering the infection process of the oomycete, or that resistance is gained through reshaping the cellular metabolome. It was further assumed, that diffusible signals, so-called allelochemicals, in the medium of resistant cells could affect the susceptibility of *C. granii* or even induce resistance. To assess the involvement of infochemicals in the infection process, a co-cultivation of susceptible and resistant species was performed and filtrate experiments were conducted. The setup of a co-culture chamber consisted of a cell strainer placed inside a Petri dish, physically separating two species, but allowing signal diffusion. After parasite inoculation, the infection rate showed no significant differences in susceptible cells co-cultivated with resistant cells. Furthermore, no induced resistance was observed after screening for inducible defense by infecting susceptible cells with prior dual-cultivation.

Differing results were obtained for filtrate experiments. Cultivating susceptible cells in the filtrated exudate medium of a dense *C. wailesii* monoculture negatively impacted the growth. On the contrary, no effect was observed when repeating this experiment with a less abundant monoculture. Furthermore, the presence of bacteria in the exudate medium might not affect the diatom growth compared to axenic conditions. These results indicated the involvement of cellular molecules involved in the resistance, and not a diffusible compound(s), although we cannot rule out that metabolites concentration might change the outcome of these findings.

The untargeted metabolic profiling of the intra- and extracellular metabolome of *C. wailesii* was conducted by UHPLC-HR-MSMS to assess the response to parasite inoculation. In the

treated cells, the most elevated compound was the glycerophospholipid lysophosphatidylcholine, often reported in plant defense to pathogenic infection. Increased lipid production in algae can be induced under stress or nitrogen deprivation, but also in *Lagenisma*-infected *C. granii*, which might speak of rather unspecific stress response of *C wailesii* to the parasite presence. One halogenated compound present in parasite-treated *C. wailesii* cells raised questions about its biosynthetic origin. Labeling experiments with ^{13}C-labeled medium could not validate the natural product origin.

On another hand, the phenylpropanoid 3,4-dihydroxycinnamic acid was detected in the exometabolome of treated *C. wailesii* cells. This compound class is related to defense in terrestrial plants. Another metabolite characterizing the exometabolome profiles of treated cells was the volatile benzaldehyde, which was previously reported for its fungicidal properties. The identification of the chemical structure and functional role of these compounds will be pursued.

Taken together, these results point toward a cell-based resistance of the diatom *C. wailesii*, with no implication of repellent or lytic compounds released in the surrounding. Interestingly, a negative allelopathic effect was observed on the species *C. granii*, which might indicate that *C. wailesii* is able to dominate the competing species through the production of small substances. The exo- and endo-metabolomics investigations yielded a number of metabolites with interesting literature related to plant defense, which motivates further study of their identity and function in *Coscinodiscus*-*Lagenisma* ecology.

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Appendix

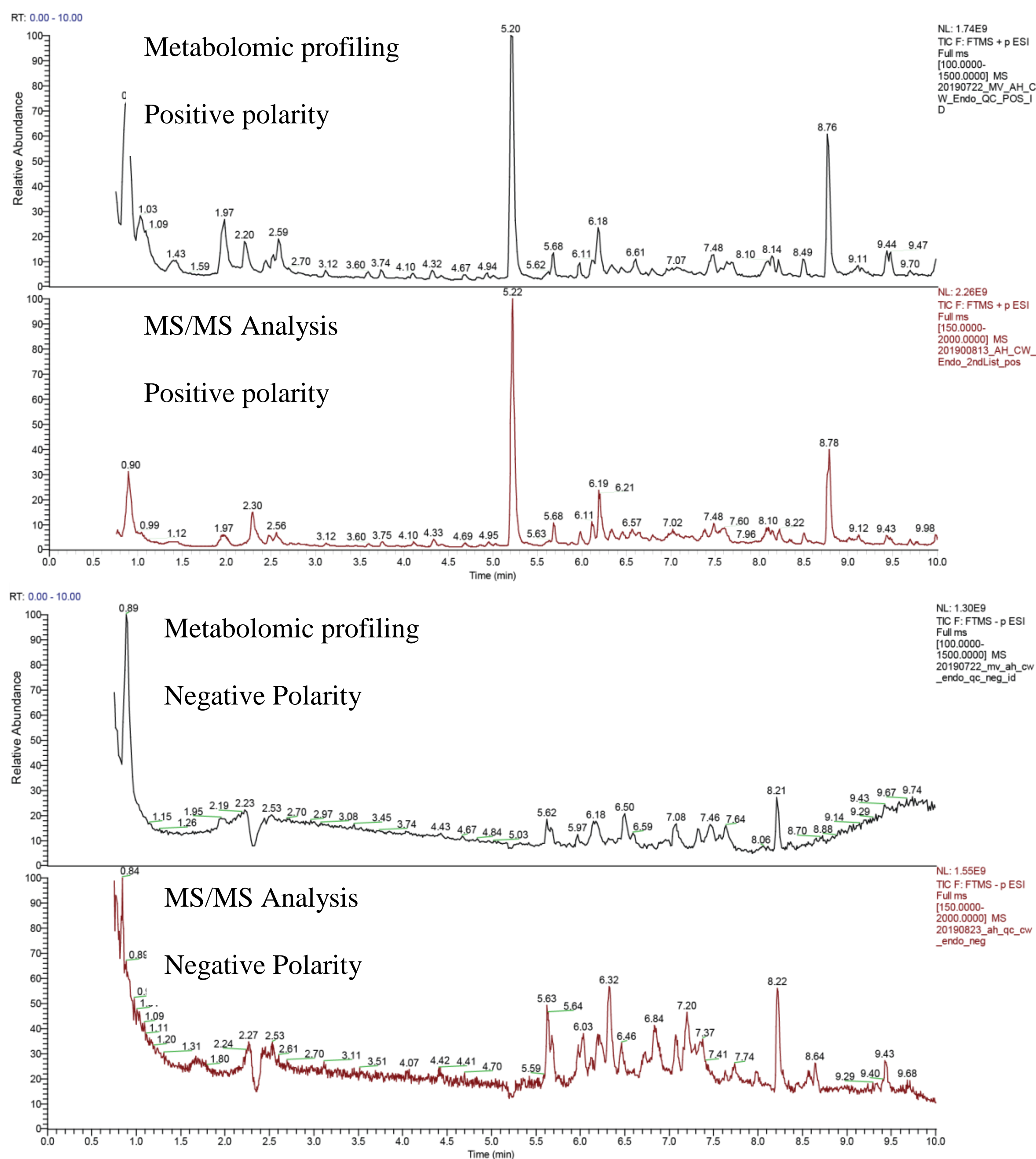

Figure A 1 Depiction of TIC of QC samples from first and last measurements of cellular extracts of *C. wailesii* in positive and negative ionization mode. Measurements of targeted MS/MS analysis with PRM were conducted 3 weeks after metabolomics profiling. Chromatograms show the stability of the compounds within this timespan.

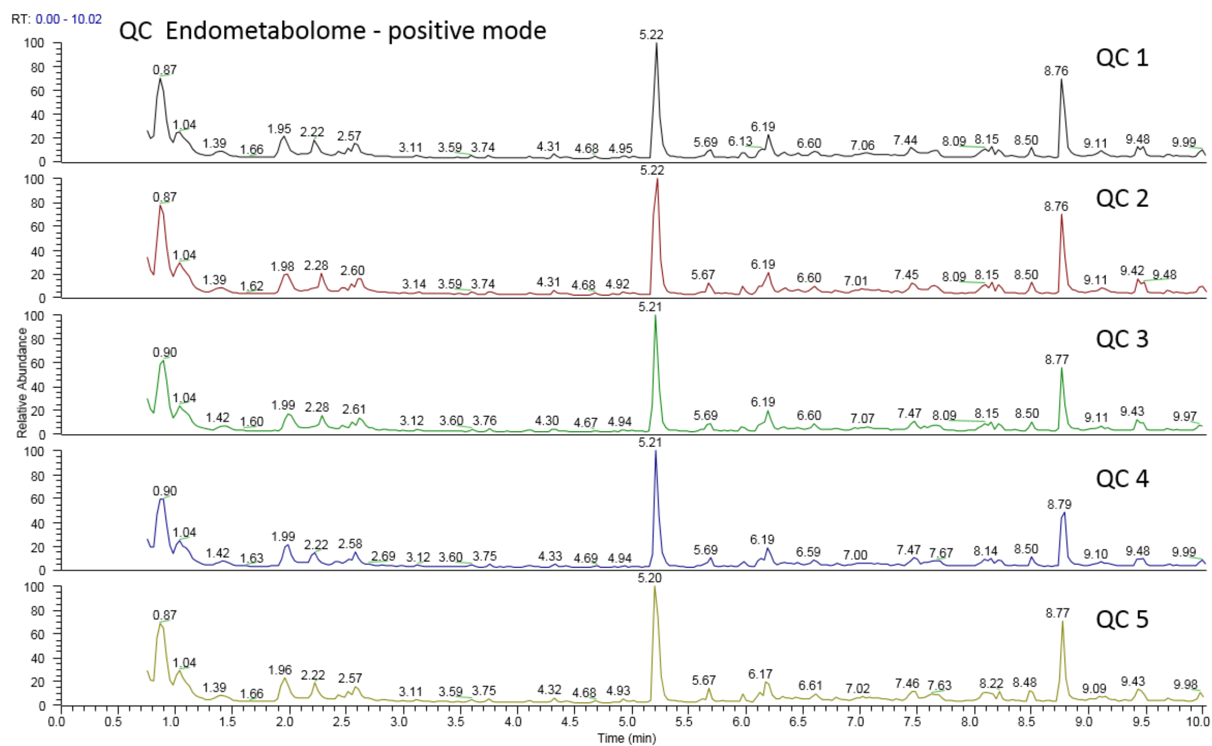

Figure A 2 Depiction of all pool samples (QC) from metabolomic profiling with LC-MS of the cellular extracts of *C. wailesii* in positive polarity.

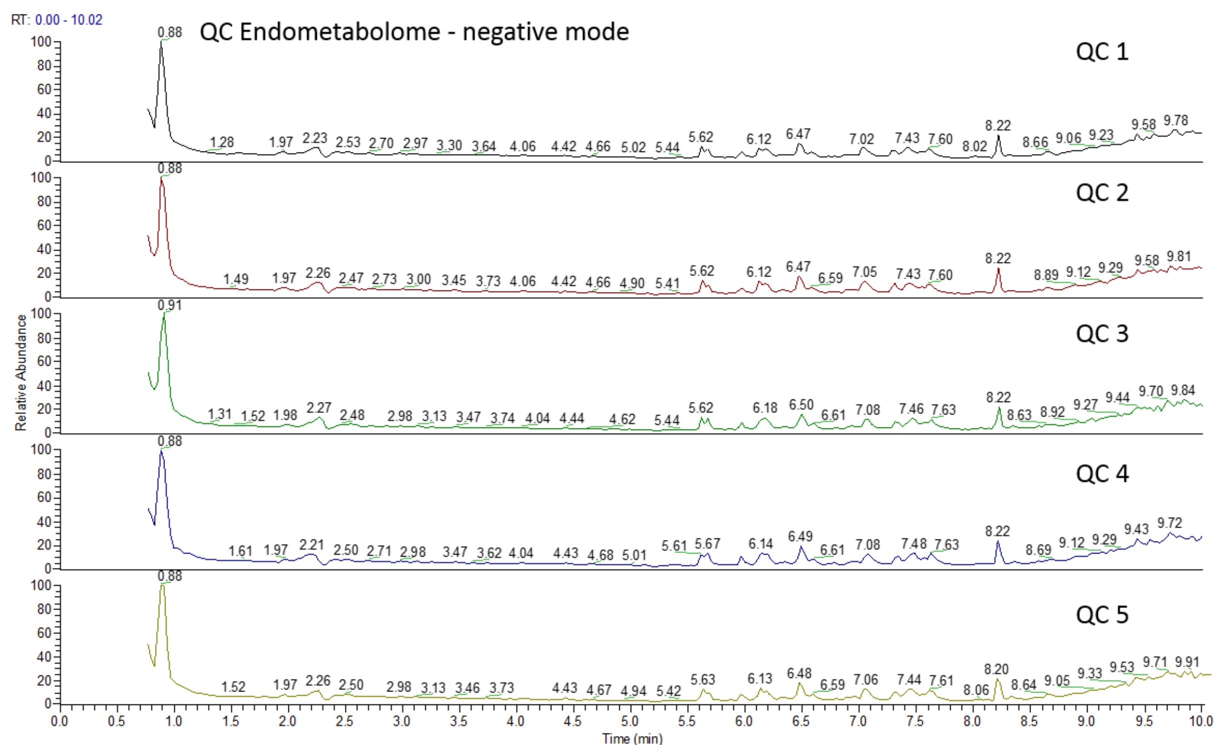

Figure A 3 Depiction of all pool samples (QC) from metabolomic profiling with LC-MS of the cellular extracts of *C. wailesii* in negative polarity.

Table A 1 Sequence order of UHPLC-HR-MS analysis of cellular extracts of *C. wailesii* treated with parasite and untreated and cell counts obtained prior to metabolic extractions.

Sample name	Condition	Cell density (ml $^{-1}$)
blank_solv5		
F2_exo	f2 medium	
AH_CW_Exo_QC1	Pool sample	
AH_CW_Exo_O1	treated	55
AH_CW_Exo_K1	treated	54
blank_solv6a		
AH_CW_Exo_R1	healthy	57
AH_CW_Exo_Q1	healthy	51
AH_CW_Exo_QC2	Pool sample	
blank_solv6		
AH_CW_Exo_T1	healthy	44
AH_CW_Exo_M1	treated	63
AH_CW_Exo_QC5	Pool sample	
blank_solv7		
AH_CW_Exo_P1	healthy	48
AH_CW_Exo_S1	healthy	42
AH_CW_Exo_N1	treated	56
AH_CW_Exo_QC3	Pool sample	
blank_solv8		
AH_CW_Exo_L1	treated	58
AH_CW_Exo_QC4	Pool sample	
blank_solv9		
F2_endo	f2 medium	
AH_CW_Exo_QC_POS_ID	Pool sample	MS/MS Identification positive polarity
AH_CW_Exo_QC_NEG_ID	Pool sample	MS/MS Identification negative polarity
blank_solv10		
AH_CW_Endo_QC1	Pool sample	
AH_CW_Endo_OC	treated	55
AH_CW_Endo_KC	treated	54
blank_solv511		
AH_CW_Endo_LC	treated	58
AH_CW_Endo_QC	healthy	51
AH_CW_Endo_QC2	Pool sample	
blank_solv12		
AH_CW_Endo_TC	healthy	44
AH_CW_Endo_MC	treated	63
AH_CW_Endo_QC5	Pool sample	
blank_solv13		
AH_CW_Endo_PC	healthy	48
AH_CW_Endo_SC	healthy	42
AH_CW_Endo_NC	treated	56
AH_CW_Endo_QC3	Pool sample	

blank_solv14		
AH_CW_Endo_RC	healthy	57
AH_CW_Endo_QC4	Pool sample	
AH_CW_Endo_QC_POS_ID	Pool sample	MS/MS Identification positive polarity
AH_CW_Endo_QC_NEG_ID	Pool sample	MS/MS Identification negative polarity
blank_solv15		

Zusammenfassung

Die marine Kieselalge *Coscinodiscus* spp. wird zunehmend den parasitoiden Oomyzeten *Lagenisma coscinodisci* infiziert. Infektionen konnten im Laufe der Jahre an vielen verschiedenen Orten weltweit beobachtet werden. Während der Etablierung einer langfristig lebensfähigen dualen Kultur von *L. coscinodisci* innerhalb seines Algenwirts entwickelte die riesige Kieselalge *Coscinodiscus wailesii* eine Resistenz gegen die Parasiteninfektion, nach der sexuellen Vermehrung. *Coscinodiscus granii* und *Coscinodiscus radiatus*, die aus derselben Gemeinschaft isoliert wurden, konnten jedoch weiterhin infiziert werden. Das Ziel dieser Arbeit war es, die Verteidigungsstrategien von *C. wailesii* zu untersuchen, um die Mechanismen aufzuklären, mit denen die Kieselalge resistente Eigenschaften erwirbt.

Die Haupthypothese war, dass entweder die Kieselalge niedermolekulare chemische Mediatoren in ihr umgebendes Medium ausscheidet, die den Infektionsprozess des Oomyzeten behindern, oder dass durch die Umgestaltung des zellulären Stoffwechsels *C. wailesii* resistenzen entwickeln konnte. Weiterhin wurde angenommen, dass diffundierbare Signale, sogenannte Allelochemikalien, im Medium resistenter Zellen die Suszeptibilität von *C. granii* beeinträchtigen oder sogar Resistenzen induzieren könnten. Um die Beteiligung von Infochemikalien am Infektionsprozess zu beurteilen, wurde eine duale Kultivierung von anfälligen und resistenten Arten vorgenommen und Filtratversuche durchgeführt. Der Aufbau einer Dual-Kulturkammer bestand aus einem Zellsieb, das in einer Petrischale platziert wurde wodurch eine physische Trennung beider Arten erfolgte, jedoch die Signaldiffusion weiterhin ermöglicht wurde. Die Kultivierung mit der resistenten Alge hatte keinen Einfluss auf die Suszeptibilität der empfindlichen Spezies. Darüber hinaus konnte keine induzierbare Resistenz nach vorheriger Doppelkultivierung festgestellt werden.

Die Filtratversuche ergaben unterschiedliche Ergebnisse. Die Kultivierung empfindlicher Zellen im filtrierten Medium einer abundanten *C. wailesii* Monokultur beeinträchtigte das Wachstum negativ. Bei der Wiederholung dieses Experiments mit einer weniger dichten Monokultur wurde kein Effekt beobachtet. Das Vorhandensein mariner Bakterien im Filtrat der resistenten Alge beeinträchtigte das Wachstum der empfindlichen Alge nicht im Vergleich zu axenischen Bedingungen. Diese Ergebnisse deuteten auf die Beteiligung von zellulären Molekülen an der Resistenzentwicklung von *C. wailesii* hin. Es konnte jedoch nicht ausgeschlossen werden, dass die Konzentration der Metaboliten im Filtrat der Grund für die variierenden Ergebnisse sind.

Die ungerichtete metabolische Analyse des intra- und extrazellulären Metaboloms von *C. wailesii* wurde mittels UHPLC-HR-MSMS durchgeführt, um die Reaktion der Alge auf die Parasitenbehandlung zu untersuchen. In den behandelten Zellen war die am stärksten hochregulierte Verbindung das Glycerophospholipid Lysophosphatidylcholin, das bereits in der Pflanzenabwehr gegen pathogene Infektionen berichtet wurde. Eine erhöhte Lipidproduktion in Algen kann unter Stress oder Stickstoffmangel induziert werden, aber auch in *Lagenisma*-infizierten *C. granii*, was von einer eher unspezifischen Stressreaktion von *C. wailesii* auf die Parasitenpräsenz sprechen könnte. Eine halogenierte Verbindung, die in Parasiten-behandelten *C. wailesii* Zellen vorhanden war, eröffnete die Frage über ihren biosynthetischen Ursprung. Markierungsexperimente mit ^{13}C-markiertem Medium konnten jedoch den natürlichen Ursprung dieses Metaboliten nicht validieren.

Zudem wurde das Phenylpropanoid 3,4-Dihydroxycinnaminsäure im Exometabolom der behandelten *C. wailesii* Zellen nachgewiesen. Diese Verbindungsklasse ist mit der Verteidigung in terrestrischen Pflanzen in Verbindung gebracht. Ein weiterer Metabolit, der innerhalb der Exometabolomprofile der behandelten Zellen identifiziert wurde, war der flüchtige Stoff Benzaldehyd, dessen fungizide Eigenschaften bereits berichtet wurden. Die Identifizierung der chemischen Struktur und der funktionellen Rolle dieser Verbindungen wird angestrebt.

Zusammengenommen deuten diese Ergebnisse auf eine zellbasierte Resistenz der Kieselalge *C. wailesii* hin, ohne dass Abwehrstoffe oder lytische Verbindungen in der Umgebung freigesetzt werden. Interessanterweise wurde ein negativer allelopathischer Effekt auf die Art *C. granii* beobachtet, was darauf hindeuten könnte, dass *C. wailesii* in der Lage ist, die konkurrierenden Arten durch die Produktion von Signalmolekülen zu dominieren. Die Exo- und Endometabolom-Untersuchungen ergaben eine Reihe von Metaboliten mit interessanter Literatur zur Pflanzenabwehr, was zu weiteren Untersuchungen ihrer Identität und Funktion in der *Coscinodiscus-Lagenisma*-Ökologie motiviert.

Declaration of Authorship

I hereby declare that I have written this Masterthesis independently and that I have not used any sources other than those mentioned.

Selbständigkeitserklärung

Ich versichere hiermit, dass ich die vorliegende Masterarbeit selbständig verfasst und keine als die angegebenen Hilfsmittel und Quellen benutzt habe.

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Ort, Datum, Unterschrift