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Characterization and pathway elucidation of halogenated metabolites in *Nitzschia* cf. *pellucida*

Masterarbeit zur Erlangung des akademischen Grades
Master of Science (M. Sc.) im Studiengang Chemische Biologie

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List of Abbreviations

2D-TBA	3,5-D_2-2,4,6-Tribromoaniline
u	unit
ASW	Artificial seawater
BrCN	Cyanogen bromide
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethylsulfoxid
EI	Electron ionization
EIC	Extracted ion chromatograms
EPS	Expolisaccharide matrix
EtOAc	Ethyl Acetate
EtOH	Ethanol
FR	Flame retardant
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HCN	Hydrogen Cyanide
HESI	Heated electrospray ionization
HFBA	Heptafluoro butyric acid
HOBr	Hypobromite
HPLC	Highperformance liquid chromatography
HRMS	High-resolution mass spectometry
HS	Headspace

MeOH	Methanol
mQ-water	Milli-Q water
MS	Mass spectometry
rcf	Relative centrifugal force
SPE	Solid phase extraction
SPME	Solid phase micro extractions
TBA	2,4,6-Tribromoaniline
t_R	Retention time
UPLC	Ultraperformance Liquid Chromatography
V-BrPO	Vanadium bromoperoxidases

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1. Introduction

All living organisms, including plants, microorganisms, mammals and algae are communicating through the production of signal molecules, the infochemicals. These compounds can have either a positive or a negative effect on the receiver. [8] Although both effects exist, studies tend to investigate the negative effect, especially the inhibitory activity on another organism. [9] This prevalence is due to the competition for resources between two different species (interspecific interactions) or within one species (intra-specific interactions). Allelopathy is the biological phenomenon by which an organism produces one or more chemicals that influence the germination, growth, survival, and reproduction of other organisms. The term allelopathy was first introduced in 1937 by the Austrian botanist Molisch to define the inhibition observed between plants that compete the same territory resource. [10] More recently, the term was defined more precisely as the inhibitory or simulative effects of one plant on another plant by releasing chemical compounds into the environment, wherein microorganisms are included in the group of the plants. [11]

Contrary to the terrestrial world, allelopathy in aquatic ecosystems is less well understood, and in particular the molecular mechanisms remain unclarified in most cases. In prokaryotic algae, the cyanobacteria, several chemicals have been effectively identified. A well-known compound is Fischerellin A, the most active allelochemical compound of the cyanobacterium *Fischerella muscicola*. It is a potent photosystem-II inhibitor of other cyanobacteria and photoautotrophic organisms described first in 1991 by Gross *et al.* [12, 13]

Algae possess two lifestyles in aquatic ecosystems: Pelagic algae, living and floating in the water column, or benthic algae living fixated on sea and river floors, sediments, rocks, other organisms, like corals, seaweeds, seashore or mammals and even inorganic men made substrata, like boat hulls or platform foundations. [14] Hence the benthic organisms can establish long term association by forming dense and diverse community in a biofilm. Almost all biotic and abiotic surfaces in marine waters are colonized by biofilms. This close coexistence of many different organisms offers numerous advantages, and mainly protection for all partners of the community, while providing nutrients and other chemicals for the thriving of some species. The cells adhere to each other in close units and are usually protected by an exopolymeric

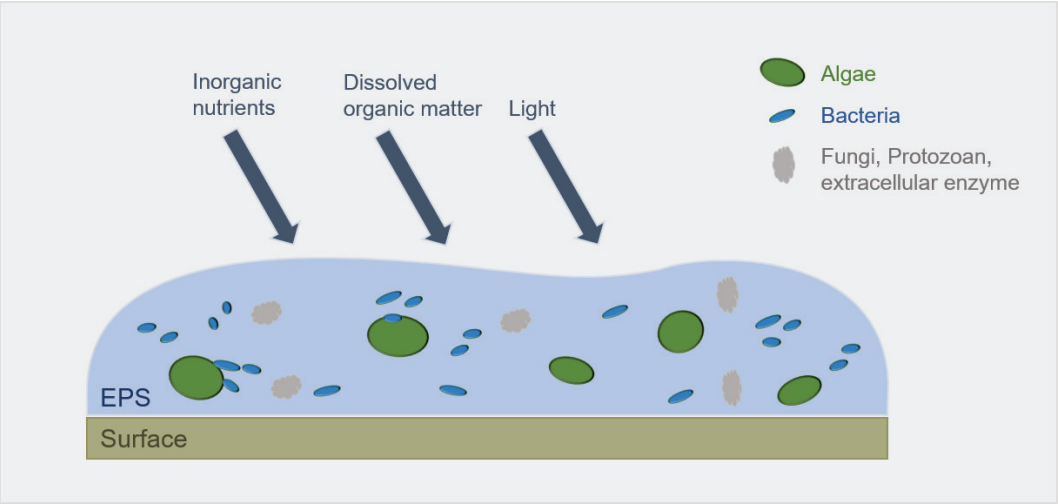


Figure 1.1.: An idealized scheme of a biofilm community adapted from Sabater et al. [1] EPS stands for exopolymeric substance.

substance (EPS) as shown in Figure 1.1. [1] This shields the organisms from temperature and pH changes, UV light and short-term changes in nutrient and salt content. [15, 16] Microorganisms also benefit from interactions with other species and the exchange of metabolites. Even autotrophs may find it energetically and resource-wise more advantageous to absorb organic compounds present in the biofilm instead of producing them themselves. [17, 18, 19] Some organisms even have taken the step into becoming heterotrophic and therefore completely dependent on the biofilm community. The group of Kindaichi *et al.* described the effective interaction of heterotrophic bacteria in an otherwise autotrophic biofilm. [20]

Although providing certain advantages, living in a biofilm can also bring some cost and disadvantages. As many organisms share a tightly restricted area, the competition for space, light and nutrients is major. [21] To prevent oneself from suffering a deficiency, some microorganisms produce allelochemicals that inhibit the growth of other organisms around them. Looking at only the three largest biofilm-forming algae groups, cyanobacteria, diatoms and green algae, a very large diversity is already evident in the chemical structure of these inhibitory compounds. [22] There are amongst others known allelochemicals from the chemical groups of polyunsaturated aldehydes, polyphenolic compounds, halogenated compounds and fatty acids. [23, 24, 25, 26]

One of the main inhabitants of the biofilm are the diatoms, unicellular eukaryotic algae which possess a siliceous exoskeleton (frustule) composed of two valves. Diatoms colonize ubiquitously the aquatic habitats, including freshwater, marine ecosystems,

from high and low temperatures and adapted to different pH-values. [27, 28] The photosynthetic power of the phytoplankton is comparable with the one of all terrestrial rainforests combined . [29] Diatoms are the fifth most abundant group of marine eukaryotes and play therefore an important role in the carbon cycle and the carbon dioxide fixation. [30] Among the benthic diatoms found in biofilms is recessed the genus *Nitzschia*, which can produce domoic acid, a neurotoxin that may accumulate in the food chains and cause the human sickness amnesic shellfish poisoning. [31] Several *Nitzschia* species are also reported for their haloperoxidase activity along with the production of volatile halogenated compounds. [32] These natural products have been first investigated in the benthic diatom *Nitzschia* cf. *pellucida* and the production of cyanogen bromide (BrCN) has been identified and associated to light-dependent production. [25] Hence, the production of the structurally simple volatile is characterized by short bursts just after sunrise. It induced the bleaching of other algae species, e.g. *Navicula arenaria, Cylindrotheca closterium* and *Entomoneis paludosa*. The diatom *N.* cf. *pellucida* has the potential to produce a microscale chemical territoria in daily so-called 'cleaning' events, where algal competitors are hence inhibited or killed. Therefore, this process was termed the 'molecular toothbrush'. With this method, *N.* cf. *pellucida* gains better access to nutrients and light and, on the other hand, prevents exposure to the toxin over a longer period of time. Nitzschia cf. pellucida was found resistant to to BrCN to a concentration of 30 µM. [25] *Nitzschia* cf. *pellucida* possess a haloperoxidase activity involved in the deactivation of quorum sensing molecules of bacteria, the *N*-β-ketoacylated homoserine lactones. The disruption of the quorum sensing molecules is dependent on presence and activation of hydrogen peroxide. [33]

Nitzschia cf. *pellucida* is here investigated and a new halogenated metabolite, 2,4,6-Tribromoaniline (later only referred to as tribromoaniline or TBA), has been identified in the preparation of this work. [34] TBA was only known as a synthetic product to date and not as a natural product. Synthetically, it is very easy to reach by triple bromination of aniline, which has been known for over 100 years. [35] Due to the simple and inexpensive synthesis, it is not surprising that TBA has found its application in various areas. For instance, this compound is used as an internal standard in gas chromatography mass spectometry (GC/MS) measurements of halocarbon compounds. [36, 37] The main field of application is as a flame retardant (FR) and these substances are added to highly flammable materials to increase the ignition point, thus suppressing combustion. In general, most FRs belong to the group of polybrominated aromatics. Two thirds of these are polybrominated diphenyl ethers and tetrabromobisphenol A. Tribromoaniline is part of the remaining third and is also partly formed during the synthesis of the first two substance groups. Since its properties exhibit the same flame-suppressing effect, it is usually not removed before use and can therefore be found in many plastics and other polymeric materials.

[38, 39, 40] TBA was also found to be a degradation product of certain dyes, which are mainly used in cosmetics. The dyes are polybrominated fluorescein compounds and Weisz *et al.* suspected that TBA is formed during the manufacturing process. [41]

In several studies the bioaccumulation of polybrominated flame retardants was shown. Thus, these compounds could be detected in a variety of marine organisms studied. FR were found in mussels, fish and even mammals such as seals and whales. [42, 43, 44] In particular, TBA could be detected in American eels. [45] It could also be found in the analysis of water at concentrations up to 0.7 ng/L in samples from the Western Scheldt estuary. [38] Toxicity tests of TBA were performed by Lu *et al.* on the green alga *Scenedesmus obliquus* and the bacterium *Vibrio fischeri* and an effective concentration (EC_{50}) at around 4 mol/L was determined. [46, 47]

In this context and in light of the recent identification of TBA in *Nitzschia* cf. *pellucida* cultures, one objective of the master thesis was to determine if the compound is synthetic and a potential contaminant, or a natural product biosynthesized by the benthic diatoms. The biosynthetic origin was investigated in a stable isotopic labelling experiment. Thereby ^{15}N-labeled TBA could be found in cultures grown in $Na^{15}NO_3$-enriched medium, as I showed in previous work.

1.1. Enzymatic formation of halogenated compounds

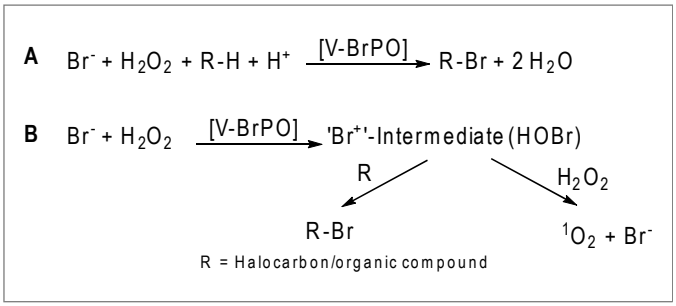


Figure 1.2.: Overall reaction scheme of vanadium bromoperoxidases (V-BrPO) according to Butler *et al.* [2]

When it comes to the biosynthesis pathways of the halogenated metabolites in *N.* cf. *pellucida*, little knowledge has been obtained so far. In fact, halogenated metabolites are not usual and common natural products. But they have been already described in seaweed, corals, sponges, marine bacteria and fungi. [48, 49] The type of halogens present in natural products depends above all on their availability in the specified

environment where the organism lives. For example, terrestrial organisms tend to produce chlorinated compounds, while marine organisms tend to produce iodised, brominated and chlorinated compounds. There are various enzymatic mechanisms for halogenation which produce different active halogen species, as in the case of bromine, bromine radicals ($Br^{\bullet}$), bromide (X^{-}) or bromonium ions (Br^{+}). The latter is mainly produced by haloperoxidases, but is not present as a free cation, but as hypobromite (HOBr). Vanadium-dependent haloperoxidases are the main source of halogenated hydrocarbons in marine algae. [50, 51] Vanadium bromoperoxidases (V-BrPO) can oxidase bromide and iodide catalysed by hydrogen peroxide (H_2O_2). The overall reaction is shown in Figure 1.2 **A**. Butler *et al.* stated the mechanism as shown in Figure 1.2 **B**. First Bromide is oxidized with H_2O_2 in a two-electron transition step to the hypobromite as an 'Br^{+}'-intermediate. Depending on the reaction conditions it can now either react with the halocarbon or with another H_2O_2 and form singlet oxygen. [2, 52, 6] In 1996 Moore *et al.* already found, several diatom cultures of the species *Nitzschia* sp. are using vanadium-bromoperoxidases to produce halogenated methane. [32]

1.2. Biosynthetic pathway of cyanide

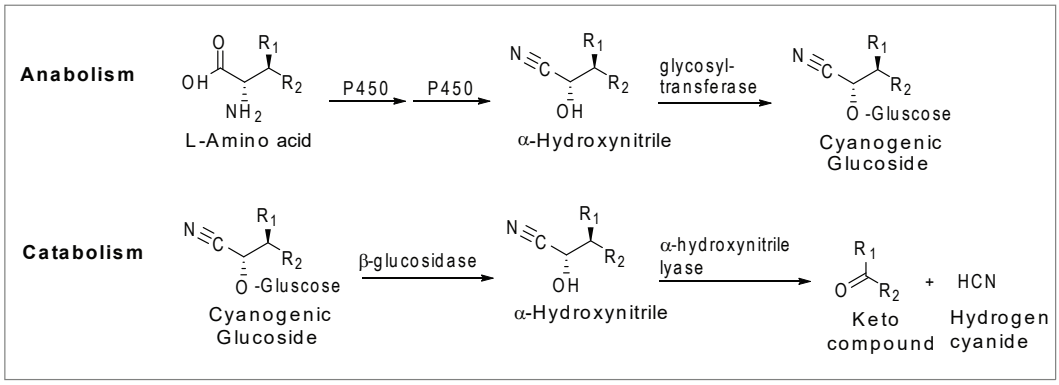


Figure 1.3.: Cyanogenesis in plants through anabolism and catabolism of cyanogenic glucosides according to Zagrobelny *et al.* [3]

Apart from the bromine the second part of the cyanogen bromide is the cyanide. Cyanide itself is a potent toxin and is known to be produced by bacteria, algae and plants. But the biosynthetic pathways differ fundamentally especially for plants. Plants use cyanogenic glucosides as precursors and split off the cyanide through an enzymatic catabolism reaction. [53] The cyanogenic glucosides are produced in the anabolism reaction starting from amino acids. The main mechanistic steps and the involved enzymes are shown Figure 1.3. [3] The potential amino acids precursors

of cyanogenic glucosides are *L*-valine, *L*-isoleucine, *L*-leucine, *L*-phenylalanine, *L*-tyrosine and the non-protein amino acid 2-(20-cyclopentenyl)glycine. α-Hydroxynitrile is synthesised in two steps and catalysed by a cytochrome P450. Then the cyanogenic glucoside is reached through glycosylation catalysed by a glycosyl-transferase. At this stage, cyanogenesis is only activated in response to predators or grazers to produce defensive chemicals containing the cyanide. The first step of the catabolism is the enzymatic hydrolysis by a β-glucosidase back to the α-hydroxynitrile. The compound is then dissociated into a keto compound and hydrogen cyanide by catalysis of an α-hydroxynitrile lyase. [3, 54, 53] The dissociation proceeds spontaneously without enzymatic catalysation if the pH-value is above 6. [55]

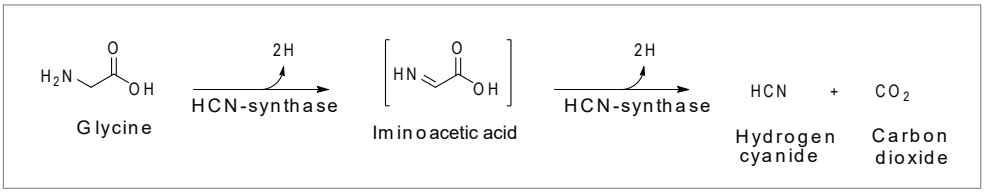


Figure 1.4.: Cyanogenesis in bacteria from glycine to hydrogen cyanide (HCN) catalysed through the HCN synthase according to Laville *et al.* [4]

The best-known bacterial pathway towards cyanide is with glycine as the precursor. This was already found by Lorck in *Pseudomonas aeruginosa*. [56] Michaels *et al.* showed on *Chromobacterium violaceum* that alanine and especially methionine can increase the yield of cyanide. [57] One potential pathway that was stated by Wissing in 1973 and further investigated by Laville *et al.* in 1998 is shown in Figure 1.4. They postulate that the enzyme HCN synthase transforms glycine in two oxidation steps directly to hydrogen cyanide and carbon dioxide. In each step two hydrogen atoms are split off and in between the non-stable imino acetic acid occurs. [58, 4, 59]

The occurrence of cyanide in algae has hardly been described so far. Only in one species, the green alga *Chlorella vulgaris*, cyanide has been found as a metabolite. Gewitz *et al.* also detected *L*-histidine as a potential precursor. [60, 61] Later, *D*-histidine was found a more effective precursor and other naturally occurring aromatic amino acids like tyrosine, phenylalanine and tryptophan can also serve as cyanide precursor. [62, 63] However, no details were given on the exact mechanism of cyanidin biosynthesis in *Chlorella*. Even less is known about the possible cyanogenesis in diatoms. A comparison of the biosynthetic pathways described above shows that they all derive from amino acids. However, the pathways to cyanide show clear differences. Likewise, there is no information how the cyanogen bromide is eventually formed.

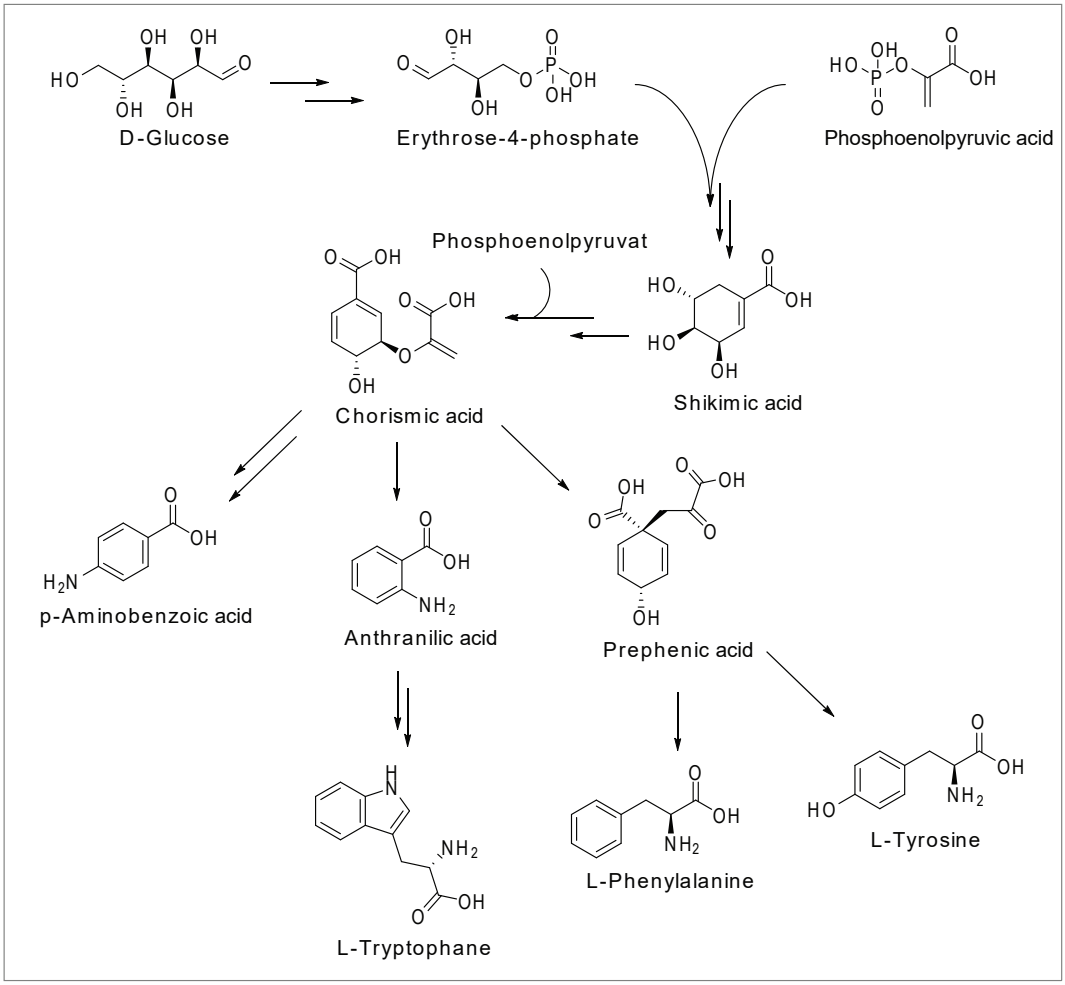


Figure 1.5.: Shortened scheme of the shikimate pathway in diatoms adapted by Bromke und Dewick. [5, 6]

1.3. Biosynthetic pathway of aromatic amines

Looking at the possible biosynthetic pathways for tribromoaniline, it is more likely that the bromine atoms were introduced into the molecule after the amino function. Even if enzymatic catalysis simplifies or even makes possible certain reactions, the basic chemical reactivities have to be considered. It can be assumed that bromination, similar to BrCN, takes place via haloperoxidases. However, the biosynthesis of aniline is much more uncertain. Aniline itself is not really common as a natural product, yet it is ubiquitous in nature. It is released into the environment together with other aromatic amine pollutants through a variety of anthropogenic sources. Especially in the last century, the pollutants were brought into nature from pesticides and industrial wastewater. [64, 65, 66] In the meantime, a large number of

organisms, mainly bacteria like *Pseudomonas* sp. and *Delftia* sp., have been found which can absorb aniline and degrade the compound further through different aerobic and anaerobic pathways. [67, 68, 69, 70, 71]

Nevertheless, little is known about the biosynthesis of anilines. When thinking about the biosynthesis pathway of aromatic metabolites, especially the shikimate pathway comes into mind. Through this pathway also the aromatic amino acids *L*-tyrosine, *L*-phenylalanine and *L*-tryptophane are synthesised. [6] But also, many other aromatic metabolites and alkaloids have their origin in this pathway. In 2006 Richards *et al.* detected genes in the diatom model *Thalassiosira pseudonana* related to the shikimate pathway, which leads to the conclusion that this biosynthetic pathway is also present in diatoms. [72] Using the sequencing data of *T. pseudonana* and other diatoms some biosynthetic pathways and metabolic networks in diatoms were reconstructed including the shikimate pathway. [73, 74]

In Figure 1.5 a shortened scheme of the biosynthesis of the aromatic amino acids in diatoms through the shikimate pathway adapted by Bromke and Dewick is shown. [5, 6] Starting from D-glucose with the intermediate erythrose-4-phosphate through addition of phosphoenolpyruvate shikimic acid is reached in several enzymatic catalysed steps. With addition of another phosphoenolpyruvate in some more steps chorismic acid is reached. The chorismic acid is then the precursor for some parting ways to the aromatic amino acids. *L*-Tryptophane is reached over anthranilic acid, while *L*-phenylalanine and *L*-tyrosine are synthesised with prephenic acid as an intermediate compound. The biosynthesis of tribromoaniline can be based on several of these substances. In addition to anthranilic acid, *p*-aminobenzoic acid can also be obtained from chorismic acid in several enzyme catalysed steps. These two compounds already have an amino function on the benzene ring and are therefore in some way substituted anilines. [5, 75, 76] Pharkya applied a patent in 2011 describing a non-naturally occurring microbial organism producing aniline through a pathway quite similar to the one described above. It was using chorismic acid as a precursor and either anthranilic acid or *p*-aminobenzoic acid as an intermediate. [77]

1.4. Stable isotope labelling

In this master thesis, the biosynthetic pathways of TBA and BrCN were thoroughly investigated with a stable isotope labelling method. This is a powerful tool in the field of chemical biology, whereby the stable isotopes of the most common elements in biomolecules, hydrogen, nitrogen, oxygen and carbon, are used to monitor processes and pathways in living organisms. Known precursors of the investigated metabolite

or even the direct nutrient sources are exchanged to their stable isotope analogue. Thereby one or more atoms can be replaced by a stable isotope. Especially in combination with high-resolution mass spectrometry (HRMS), a technique for the determination of molecular formulas, a good assignment of the labelling can be achieved. Based on the resulting isotope pattern of the metabolite in the mass spectrum of the extract with and without labelling, conclusions can then be drawn about the biosynthetic pathways. [78, 79, 80] In parallel to the chemical investigations, functional bioassays were conducted to address the potential allelopathic role of TBA on other members of the benthic community, e.g. marine bacteria, benthic diatoms and grazers.

2. Objective of the thesis

Allelopatic metabolites play an important role in shaping and structuring the species community in biofilms. Some key-player species can dominate others through the production of bioactive molecules. *Nitzschia* cf. *pellucida* is a benthic diatom producing two halogenated metabolites, cyanogen bromide and tribromoaniline. These compounds may act as mediators of allelopathic interactions. The detrimental effect of BrCN against two other algal species has been previously unravelled [25], but no role has yet been elucidated for the newly isolated and described tribromoaniline.

The objective of this thesis is to investigate the biosynthetic origin of these metabolites and to unravel their ecological role. In the first section of this thesis,I will investigate the biosynthesis of cyanide in BrCN and narrow down the potential amino acid precursors. Therefore, I will separate a $^{13}C^{15}N$-labelled amino acid mix and perform feeding experiments with solid phase micro extractions (SPME) to confirm which amino acid is involved.

Tribromoaniline will be characterised with stable isotope labelling experiments with ^{13}C- and ^{15}N-labelling. For a first glance into the biosynthesis, feeding experiments will be conducted by labelling phenylalanine and tyrosine. The intra- and extracellular concentrations of TBA will I determine with extraction of the endometabolome and solid phase extraction (SPE). Throughout time series, changes in concentration at different stages of growth and accumulation of TBA will monitored. In addition, I will invetigate the release of TBA into the medium in a shorter period of time. The extracts will be analysed using gas chromatography coupled with high resolution mass spectrometry (GC/HRMS). To get an insight into the allelopathic effect of tribromoaniline, this compound will be tested in functional bioassays with different organisms that can be encountered in the biofilms and its surroundings. Hence, the grazer *Littorina obtusata*, various marine bacteria isolated from diatoms and the benthic diatom *Cylindrotheca closterium* will be exposed to TBA. I will monitor the growth and observe potential phenotypic effect.

3. Material and methods

The water used for all experiments and the cultivation is mQ-Water: milli-Q water (mQ-water, purified with a Milli-Q Integral Water Purification System for Ultrapure Water; JWT GmbH, Germany). The ethyl acetate (EtOAc, GC gradient grade), the methanol (MeOH, UPLC gradient grade) and the absolute ethanol (EtOH, HPLC gradient grade) were purchased by VWR chemicals (United States of America). The dimethylsulfoxide (DMSO) was purchased by Carl Roth GmbH & Co. KG (Germany).

3.1. Cultivation of the diatom *Nitzschia* cf. *pellucida*

Nitzschia cf. *pellucida* cultures (strain DCG 0303) were obtained from the diatom culture collection of the Belgian Coordinated Collection of Microorganisms and cultivated with artificial seawater (ASW) medium prepared according to Maier et al. [81] The cultures were incubated in TC-bottles (T25 and T75, Sarstedt AG & Co, Germany) and grown under two different light and temperature conditions due to different experimental buildings. For the time series and the labelling experiments, the diatoms were maintained under a 12 h photoperiod illuminated at a 40-50 µmol·m^{-2}·s^{-1} irradiance coupled with a thermoregulation cycle (16 °C / 12 °C day/night). For solid phase micro extractions (SPME), the pathway elucidation experiments and the experiment about the 48 h production were maintained under a 12 h photoperiod illuminated at a 50-60 µmol·m^{-2}·s^{-1} irradiance at a temperature of 18 °C. All experiments were done with cells in the late exponential growing phase (150,000 - 200,000 cells/mL) if not otherwise described and carried out under sterile conditions at room temperature. The cell count was determined using a Fuchs-Rosenthal hemocytometer (Laboroptik, United Kingdom) after scraping the diatoms from the ground of the flask and homogenization of the cultures.

3.2. Biosynthetic origin of cyanogen bromide (BrCN)

3.2.1. Amino acid separation

The amino acid separation was performed on an Agilent 1100 highperformance liquid chromatograpy (HPLC) with an Agilent 1260 Infinity Fraction collector. For the separation, an Agilent Zorbax SB-C18 column (length 250 mm, diameter 4.6 mm, particle size 5 µm) was used with a flow rate of 1 mL/min. An isocratic solvent mix of water and methanol (3:1) with 0.3% of heptafluorobutyric acid (HFBA) was used as mobile phase. For the Quantification ultraperformance liquid chromatography mass spectrometry UPLC/MS measurements of the were done without further dilution.

The UPLC/MS measurements were performed on a Dionex UltiMate 3000 (Thermo Scientific, United States of America) with a Q Exactive plus Orbitrap mass spectrometer (Thermo Scientific, United States of America) with heated electrospray ionization (HESI). For the separation, an Accucore C18 column (length 150 mm, diameter 2.1 mm, particle size 2.6 µm) was used under a flowrate of 0.25 mL/min. The composition and gradient of the mobile phase is shown in Table 3.1. The MS measurement run from 0.8 min to 9.5 min probe run time with a scan range from m/z 60 to 900 and a resolution of 140,000 in the positive mode. For quantification, the peak areas were integrated with the Xcalibur Quan Browser from Thermo Scientific (United States of America). The masses of the hydrogen adduct used are shown in Table A.1.3 in the appendix (3 ppm tolerance). The fractions were dried with a Christ AVC 2-25 CO plus vacuum centrifuge (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) at 57 relative centrifugal force (rcf) at room temperature.

Table 3.1.: Solvent gradient of the UPLC measurement. Solvent **A**: H_2O + 2% Acetonitrile + 0.1% formic acid, Solvent **B**: 2% Acetonitrile + 0.1% formic acid

time [min]	solvent gradient
0 - 5	100% A
5 - 7	100% A to 100% B
7 - 9	100% B
9 - 9.1	100% B to 100% A
9.1 - 10	100% A

A cell free $^{13}C^{15}N$-amino acid mixture (Sigma Aldrich, United States of America) has been diluted in the ratio 1:10 with mQ-water to get the concentrations that are shown

in Table A.1.1 in the appendix. 50 µL of the diluted mix were injected in six runs and the same method was applied for each run. The fractions of the different runs were check separately for their contents in amino acids and then combined during the drying process as there were no major differences between the runs. The end concentrations were determined through UPLC/MS measurement from the combined constricted fractions.

3.2.2. SPME measurment of BrCN

To detect BrCN in the headspace culture of the diatom cultures, the method of Janna Kuchinka [7] was further optimized. Following conditions were applied after the optimization. Cultures of *N.* cf. *pellucida* were grown in T25 culture flasks and extracted by SPME in the medium directly from the flask at the end of the exponential growth phase . The substances present in the medium were enriched on a SPME fiber (black fiber containing carboxes® and polydimethylsiloxane; Supelco, United States of America) for three hours starting at the beginning of the light phase. After three hours, the substances enriched on the fiber were analysed immediately using gas chromatography high resolution mass spectrometry (GC/HRMS).

GC/HRMS measurements were performed on a GC TRACE 1310 UPLC (Thermo Scientific, Waltham, USA) QExactive GC Orbitrap mass spectrometer with electron ionization (EI) from Thermo Scientific (Waltham, USA). The separation was conducted on a Phenomex ZB MS column (length 40 m, diameter 0.25 mm, film 0.25 µm, phase 5% phenyl, Phenomex) was used operated with a SSL injector (split ratio 10, split flow 10 mL/min, temperature 260 °C), with a carrier flow rate of 1 mL/min. The temperature gradient of the GC oven is shown in Figure 3.1, the rate between minute 3 and 6.75 was 8 °C/min, 100 °C/min between minute 11.75 and 13.55 and 70 °C between minute 15.55 and 16.55. The MS measurement started at 5.5 min probe run time with a scan range from m/z 50 to 450 and a resolution of 60,000 in the positive mode. The transfer line temperature was set to 250 °C and the ion source temperature to 300 °C.

The same set up served for feeding experiment to determine the incorporation by labelled aminoacids (separated as described above) added 15 hours before the extraction. The amounts of amino acids are summarized in Table A.1.4 in the appendix.

BrCN was quantified based on the extracted ion chromatograms (EICs) of the two isotopes peaks and their proton adducts, with a mass tolerance of 3 ppm. The theoretical exact masses are summarized in Table A.1.5 in the appendix.

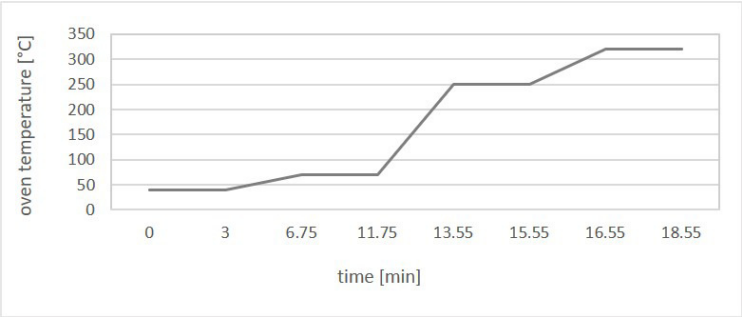


Figure 3.1.: Temperature gradient of the GC oven for the analysis of BrCN.

3.3. Characterization of tribromoaniline

3.3.1. Intracellular and extracellular extraction

The cells were removed from the wall of the bottle with a Cell Scraper (Sarstedt AG & Co, Germany) and a sample was taken for the determination of the cell density . The cells were collected on a glass microfiber filter (GF/C, diameter 25 mm; Whatman, United Kingdom) and the medium was collected in a round flask. The microfiber filter was placed in an 2 mL Eppendorf tube and the cells were re-suspended in 1.8 mL ethyl acetate . The samples were sonicated for 5 minutes and then centrifuged (16,100 rcf, 15 min). 1.4 mL of the supernatant were removed and centrifuged again (16,100 rcf, 15 min). The extracts were completely dried by nitrogen flow and stored at -20 °C. For the GC/MS measurement one aliquot of the extracts was dissolved in 30 µL ethyl acetate.

The collected supernatant was extracted with solid phase extraction (Oasis HLB cartridge (3cc, 60 mg; Waters, United States of America) to obtain the exometabolome. The cartridge was conditioned with 2 mL ethyl acetate, 2 mL methanol and 2 mL mQ-water. Then the cartridge was loaded with the medium and washed with 10 mL mQ-water. The elution was performed with 2 mL of ethyl acetate. The extracts were split into two aliquots and completely dried by nitrogen flow and stored at -20 °C. For the GC/HRMS measurement one aliquot of the extracts was dissolved in 30 µL ethyl acetate.

GC/HRMS measurements were performed on a GC TRACE 1310 UPLC (Thermo Scientific, Waltham, USA) QExactive GC Orbitrap mass spectrometer with electron ionization (EI) from Thermo Scientific (Waltham, USA). For the separation a Phenomex ZB MS column (length 40 m, diameter 0.25 mm, film 0.25 µm, phase 5%

phenyl, Phenomex) was used operated with a SSL injector (split ratio 10, split flow 10 mL/min, temperature 250 °C) with a carrier flowrate of 1 mL/min. The temperature gradient of the GC oven is shown in Figure 3.2, the rate between minute 2 and 5 was 20 °C/min, 5 °C/min between minute 6 and 32 and 10 °C between minute 32 and 39. The MS measurement started at 5.5 min probe run time with a scan range from *m/z* 50 to 600 and a resolution of 120,000 in the positive mode. The transfer line temperature was set to 250 °C and the ion source temperature to 300 °C.

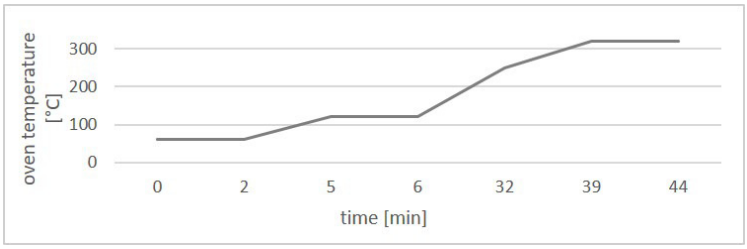


Figure 3.2.: Temperature gradient of the GC oven for the quantification of TBA.

TBA was quantified based on the EICs of all four bromide isotope peaks with a mass tolerance of 3 ppm. The theoretical exact masses are summarized in Table A.2.6 in the appendix.

3.3.2. Stable isotope labelling experiments

To perform labelling experiment, the culture medium was prepared but the main source of nitrogen or carbon ($NaHCO_3$ for the carbon labelling and $NaNO_3$ for the Nitrogen labelling) was excluded. After autoclaving, the $NaH^{13}CO_3$ or $Na^{15}NO_3$ was sterically added to the medium in the same amount as the unlabelled in the normal medium. To replace the medium, a culture of *N.* cf. *pellucida* in the late exponential growing phase (150,000 – 200,000 cell/mL) was centrifuged (4000 rcf, 15 min) and the cells were washed twice with fresh, labelled medium. Then some of the washed cells were added to fresh labelled medium to start new cultures (2000 – 5000 cells/mL). The cultures in the $NaH^{13}CO_3$-enriched medium were stored with upstanding culture flasks to reduce the headspace and with that the uptake of CO_2 as carbon source.

The intracellular and extracellular extractions were performed as described at sections 3.3.1 with cultures in the stationary growing phase (150,000 – 250,000 cells/mL) in 40 mL Medium (^{15}N-Labelling) or 80 mL Medium (^{13}C-Labelling) with three replicates each.

3.3.3. Quantification experiments

The time series were performed with five biological replicates and one ASW-medium blank at every extraction with 80 mL medium. For each time series the cultures and medium blanks were started at the same time and extracted at day 2, 5, and 8 (first time series) or day 8, 15, 22 and 29 (second time series). Both intra- and extracellular extractions were performed. The cultures were extracted as described at sections 3.3.1 with 3,5-D_2-2,4,6-tribromoaniline (2D-TBA) as an internal standard. For intracellular extraction, 1.8 mL EtOAc at the resuspension of the cells at a concentration of 0.02 µg/mL (total amount per culture: 36 ng). For the extracellular extraction, 50 µL EtOAc with 2D-TBA (0.8 µg/mL) were added directly before the solid phase extraction (total amount per culture: 40 ng).

For the biosynthetic pathway elucidation of TBA cultures of *N.* cf. *pellucida* (80 mL), Tyrosine-D_4-phenyl and Phenylalanine-$^{13}C_6$-phenyl were added at the early stationary growing phase (incubation day 15). The cells were extracted after 70 h and 7 days of incubation. The experiments were performed in biological triplicates. One ASW-medium blank for each amino acid was performed in similar conditions. The cultures were extracted as described at sections 3.3.1 with 2D-TBA as an internal standard. For intracellular extraction, 1.8 mL EtOAc at the resuspension of the cells at a concentration of 0.02 µg/mL (total amount per culture: 36 ng).

$$V = \frac{\pi}{4} \cdot a \cdot b \cdot c \tag{3.1}$$

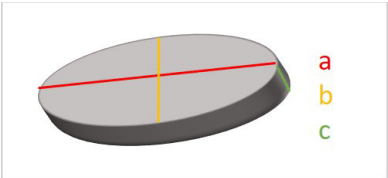


Figure 3.3.: Definition of the variables for the cell volume determination.

For the calculation of the intracellular concentration, the diatom cell volume was determined according to Hillebrand et al. [82] The shape of a *N.* cf. *pellucida* cell was assumed as a prism on an elliptic base and the Volume V was with formula 3.1. The definition of the variables a, b and c is shown in Figure 3.3. The cell dimensions were determined with an inverted microscope from Leica (Leica Camera, Germany) at 40 x magnification in at least 20 measurements for each variable. The volume was determined with the mean for each variable.

For the stability test a solution of tribromoaniline (5 µg/mL) and methyl stearate (5 µg/mL) in hexane was prepared and split into aliquots. The aliquots were stored at room temperature and were measured with GC/HRMS as described at sections 3.3.1 but with a different temperature gradient. The temperature gradient of the GC oven is shown in Figure 3.4. The rate between minute 3 and 6.75 was 8 °C/min and 50 °C between minute 6.75 and 11.75. All the other parameters are similar. The theoretical exact mass of methyl stearate used for the quantification is 298.28663 u.

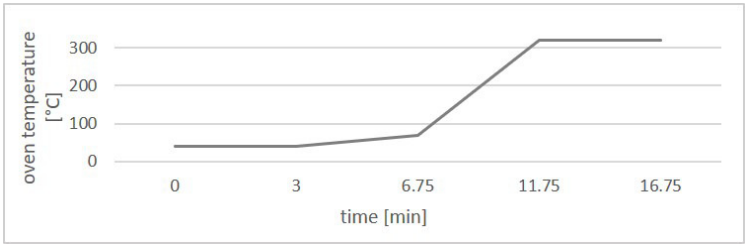


Figure 3.4.: Temperature gradient of the GC oven for the stability test of TBA.

3.3.4. Bioassay with grazer *Littorina obtusata*

The bioassay with the seasnail *Littorina obtusata* was performed as described by Rempt et al. [83] *Littorina obtusata* was sampled at maximal low tide in Roscoff Bay (France) on 06/06/2018 within 50 m area. All the snails were grazing on the seaweed *Ascophyllum nodosum*. The snails were kept with the algae and seawater for 5 days in a cooling chamber and transferred to Jena within one day. The effect of TBA was assessed by palatability assay on agar plates containing the compound (final concentration 0.1 mg/mL, dissolved in methanol). Methanol served as negative control (0.1%) and CuSO4 (1 mg/mL) as a positive control. One snail per plate was placed on the agar and left there for grazing for 7 min. After this time, the plates were turned around and the snails which felt down were counted. The snails which remained stuck on the agar after this time were considered not repelled.

3.3.5. Bioassay with bacteria

The antibiotic effect of TBA was tested with a disc diffusion assay described by Ericsson et al. [84]. *Roseovarius* sp., *Maribacter* sp. and *Marinobacter* sp. strains were grown in half Marine Broth at room temperature for 3 days. These bacteria were previously isolated from the benthic diatom *Seminavis robusta*. [85] Agar plates with half marine broth were prepared and filter discs were prepared under sterile conditions in a Petri dish. The compounds were dissolved in mQ-water and ethanol

in the required concentrations (see table 3.2), sterile filtrated and applied to the filter discs, which were then completely dried. Each culture was diluted to an optical density of 0.5 and 500 µL were spread evenly throughout an agar plate. Then the filter discs loaded with the compounds were placed upon the agar and slightly pressed. The cultures were grown for two days at 20 °C and the area around the disc was daily observed for inhibition area. At day 2, the inhibition zone around the discs was measured and indicate potential antibiotic effect. This was done in triplicates with the antibiotic gentamycin as a positive control.

Table 3.2.: Concentrations and solvents for the bacterial assay.

compound	solvent	diluted concentration	amount on disc
TBA	ethanol	0.1 mg/mL	2 µg
TBA	ethanol	0.5 mg/mL	10 µg
TBA	ethanol	2.5 mg/mL	50 µg
Gentamycine	mQ-water	0.5 mg/mL	10 µg

3.3.6. Activity against the diatom *Cylindrotheca closterium*

Cylindrotheca closterium cultures (strain CA 1.15) were cultivated in F/2. The cultures were incubated in TC-bottles (T25, Sarstedt AG & Co, Germany) and grown under a 12 h photoperiod illuminated at a 15-20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance at a temperature of 18 °C. During the bioassays the cultures were grown in 12-well plates (Sarstedt AG & Co, Germany). In the first bioassay the cell count was determined through cell counting on an inverted microscope. For the second and third bioassay the cell density was determined through a fluorometrical analysis of chlorophyll a with a Mithras LB 940 Fluorometer (Berthold Technologies, Germany) with an excitation filter of 430 nm. [86]

In the first bioassay the cultures were started in two 12-well plates with 2.5 mL of F/2 medium. Four different treatments were carried out in triplicates. In the Blank nothing was added, in the DMSO blank 20 µL DMSO was added and for the wells with TBA 20 µL DMSO with the required amount of TBA was added. The concentrations tested in this bioassay were 10 µM and 100 µM. In the first plate, the additions were given directly after the cultures were started. In the second place they were added three days after that, when the cultures already were in the exponential growing phase.

For the second bioassay, parameters were optimized. This time the DMSO and the DMSO with the TBA was added to the medium before the cultures were started and mixed well by shaking. One plate was done in this bioassay with four treatments

and three replicates: Blank, DMSO blank and TBA in the concentrations 5 µM and 50 µM. Each culture was grown in 1.5 mL F/2 medium and with all addition the volume of the DMSO was 30 µL. The growth was observed through the determination of the chlorophyll a fluorescence. In the third bioassay, the experimental set-up was very similar to that of the second assay. Eight treatments were performed with nine replicates: Blank, DMSO blank, TBA in the concentrations 5 µM, 20 µM, 40 µM and 50 µM and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the concentrations 5 µM and 50 µM as a positive control.

4. Results and discussion

In the following, two halogenated metabolites of *Nitzschia* cf. *pellucida*, cyanogen bromide and tribromoaniline were examined in closer detail. The biosynthetic pathways of the metabolites will be investigated through stable isotope labelling. Further the allelopathic role of tribromoaniline will be oberseved with other marine organisms from the same habitat.

4.1. Biosynthetic origin of cyanogen bromide (BrCN)

The benthic diatom *N.* cf. *pellucida* produces cyanogen bromide as already described in previous studies. [25] Cyanide compounds, especially those as small as BrCN, are very rare in nature. In the following experiments, the biosynthesic pathway and possible precursors of this unusual allopathic compound and in particular the cyano group were examined.

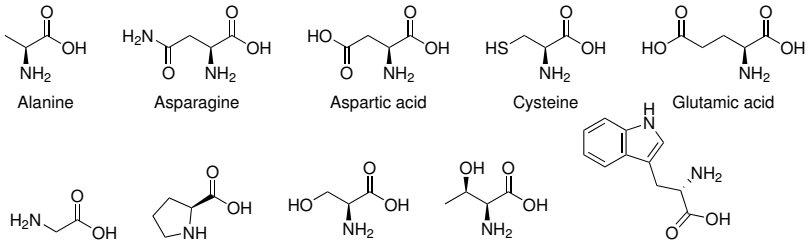
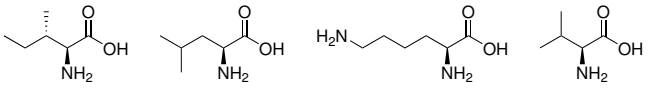
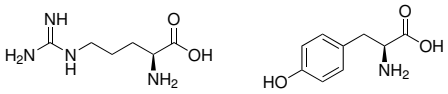
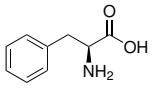
4.1.1. Amino acid separation

In previous studies, after feeding a $^{13}C^{15}N$-labelled amino acid mix, the occurrence of labelled BrCN had been observed. [7] In order to better identify the amino acids involved in the biosynthesis of the cyanide group, this mix was separated into the individual amino acids. In previous work, I already developed a method to separate an amino acid mix semi preparatively with the help of an ion-pair reagent as an eluent in for HPLC. The amino acid mix was separated using a semi-preparative HPLC on a C18 column. The ion pair reagent used was heptaflourobutyric acid, which forms an ion pair with the amino acids. This increases the hydrophobic character of the amino acids and thus also improves the interaction with the hydrophobic column material. [87] The content of the individual fractions was identified and quantified via UPLC/MS.

Using this method, the amino acid mix could be separated into seven fractions. The amino acids, which are present in larger amounts in the fractions are depicted in

Table 4.1. These amino acids were quantified by calibration, the resulting data can be found in the appendix in figure A.1.1 to A.1.5. The absolute concentrations of the amino acids in the fractions and data used for the determination can be seen in Table A.1.2 and Table A.1.3 in the appendix.

Table 4.1.: Amino acids contained in the fractions with a concentration above 25 µM. All amino acids within the fractions and the exact concentrations are shown in table A.1.2 in the appendix.

Fraction	Amino acids with a concentration over 25 µM
Fraction A 0 - 7 min	 Alanine, Asparagine, Aspartic acid, Cysteine, Glutamic acid, Glycine, Proline, Serine, Threonine, Tryptophan
Fraction B 7 - 8.5 min	-
Fraction C 8.5 - 11.5 min	 Isoleucine, Leucine, Lysine, Valine
Fraction D 11.5 - 15 min	 Arginine, Tyrosine
Fraction E 15 - 27 min	-
Fraction F 27 - 39 min	 Phenylalanine
Fraction G 39 - 50 min	-

The separation proceeded mostly as expected according to the reported analytical method. One conspicuous feature, however, is the tryptophan, which was expected in the last faction. However, this compound was identified in the first faction. Most likely this is due to a too short time of separation. The replictes were carried out directly one after the other and the equilibration time might have been to short. The tryptophan from the previous run was likely transferred to the first fraction of

the following run. Although the column was flushed, the carry over could not be tested due to the lack of a suitable detector. The semi-preparative HPLC itself is only equipped with a UV detector, which does not allow any clear conclusions to be drawn about the remaining amino acids. The fractions were then subsequently analyzed using UPLC/MS.

With the amino acid mix separated into fractions containing only a few different amino acids, further labelling experiments are possible. In the case of feeding experiments with the labelled amino acid mix, if a labelling of a natural substance was detected, the amino acids active in biosynthesis were narrowed down in subsequent experiments with the obtained fractions.

4.1.2. Stable isotope labelling of cyanogen bromide

In her master thesis, Janna Kuchinka already did some investigations into the biosynthetic origin of BrCN with stable isotope labelling experiments with amino acids. [7] She could already show that in feeding experiments with a $^{13}C^{15}N$-labeled amino acid mix, a labelling of BrCN took place. Further experiments showed that $^{13}C^{15}N$-glycine could be used as a precursor. However, there was also some evidence that other amino acids are better accepted precursors for BrCN. In order to verify these findings, the results were replicated. The fractions obtained by amino acid separation from this study were tested in feeding experiments to further confirm the amino acids involved in the cyanide biosynthesis.

The halogenated metabolite was concentrated through headspace solid phase microextraction (HS/SPME) and analysed by gas chromatography coupled with high-resolution mass spectrometry (GC/HRMS). For the extraction, a SPME fiber made of Carboxen and Polydimethylsolixane was used, which allows the adsorption of compounds in the medium polarity range. For an unambiguous assignment, a standard of BrCN was measured by SPME and GC/HRMS before the experiments were started. The retention time (t_R) at which BrCN eluts is 2.43 min. The mass spectrometric investigations were performed in an orbitrap mass analyser. It was shown that in addition to the expected peaks for ^{79}BrCN and ^{81}BrCN, which result from the natural isotope distribution of bromine, two further peaks occur. These peaks are proton adducts of BrCN, which form directly before measurement in the C-trap of the orbitrap mass analyser. [34] In the following, the proton adducts HBrCN and BrCN are taken together and summed up as BrCN. The masses were also grouped together during quantification.

When replicating the feeding experiments, the same approach was initially chosen

as described by Janna Kuchinka. A *N.* cf. *pellucida* culture was transferred into a glass vial during the exponential growth phase and 15 hours from the beginning of illumination the amino acids were fed. The concentrations used were ranging from 4 mM to 109 mM. The sampling with the SPME fiber was done over a period of three hours after light and was analysed immediately afterwards. In order to establish the experimental setup, it was first repeated without additional feeding of the amino acids. No BrCN could be detected. Before starting the feeding experiments, thus the experimental setup had to be optimized.

After changing various parameters, BrCN could again be detected by SPME. Above all, the transfe of cultures from the culture flask into a glass vial was omitted and the measurement was carried out directly in the culture flask. Substances that are additionally detected, such as plasticizers and styrene, hardly interfere with the targeted analysis of BrCN. In addition, the BrCN was extracted from the liquid phase instead of the headspace. BrCN is present as a solid-state at room temperature, which only makes it a moderately volatile compound. It can therefore be assumed that the concentration in the medium is significantly higher, which has also been shown in the experiments.

The feeding experiment started with the $^{13}C^{15}N$-labelled amino acid mix and $^{13}C^{15}N$-labelled glycine to replicate the existing results. The concentrations, which were present in the cultures after addition, can be seen in Table A.1.4 in the appendix.

Unfortunately, the feeding of the labelled amino acids led to results that were no longer reproducable. In some experiments, BrCN and also labelled BrCN could be detected. In most cases, however, even BrCN was no longer detectable. Even under identical conditions the BrCN concentration fluctuated strongly. Of the measurements in which the labelling of BrCN was recognizable, the proportions of the labelled BrCN are clearly distinguishable from the natural isotope ratio only in one measurement. In this measurement, the fraction A of the separation (section 4.1.1), which contains alanine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine, threonine and tryptophane, was added. Nevertheless, the proportion of labelled BrCN is significantly lower than that obtained by Janna Kuchinka with the addition of the $^{13}C^{15}N$-labelled amino acid mixes (see Figure 4.1). It also shows that the proportion of $^{13}C^{15}N$-labelled BrCN after addition of fraction A is significantly lower than obtained with the $^{13}C^{15}N$-labelled amino acid mixes. Even though it cannot be ruled out that this is due to the different concentrations, it leads to the suspicion that amino acids that are not present in fraction A, are also involved in the biosynthesis of cyanide. Since the concentrations of the added amino acids are not identical compared to previous studies, further conclusions are not possible without additional results.

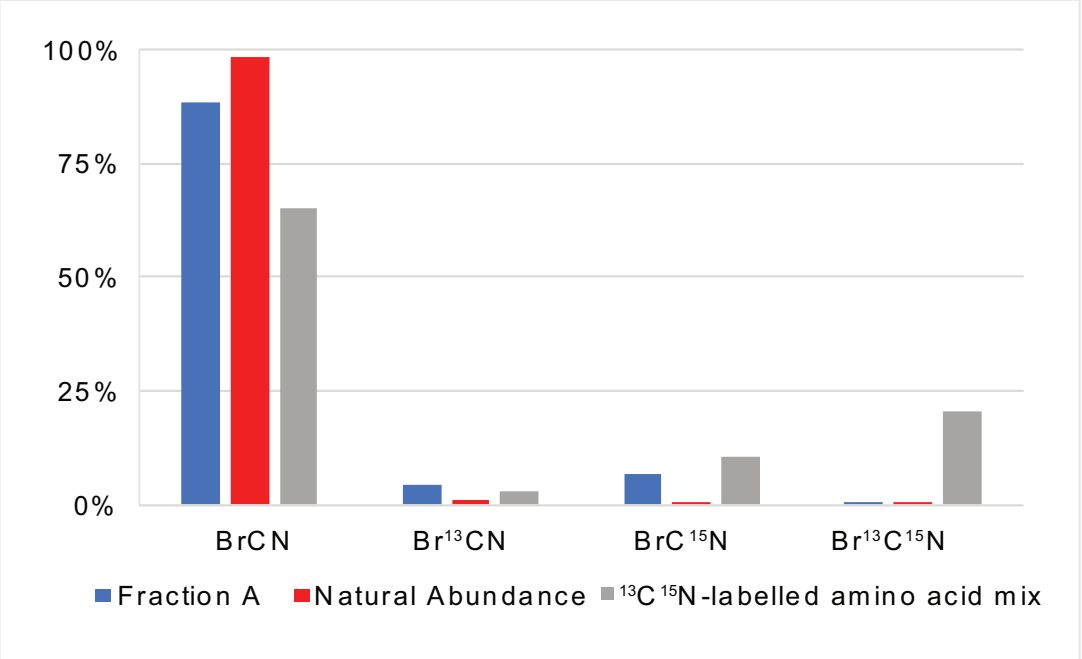


Figure 4.1.: The ratios of BrCN to $Br^{13}CN$, $BrC^{15}N$ and $Br^{13}C^{15}N$ of the feeding experiment with fraction A compared to natural abundance and labelling in the feeding experiment of Janna Kuchinka with the $^{13}C^{15}N$-labeled amino acid mix. [7]

Before further comparative feeding experiments with the fractions obtained in the separation, the experimental setup must be further improved. Since the amount of separated amino acids in the fractions is very limited, further experiments are only advisable if the detection of BrCN is stable even with additional feeding.

4.2. Characterization of tribromoaniline

During the metabolic survey of the benthic diatom *Nitzschia* cf. *pellucida* the halogenated compound tribromoaniline has been identified for the first time as a natural product. In the following, the biosynthesis and functional role of this unusual halogenated compound was investigated.

4.2.1. Identification through stable isotope labelling studies

In order to verify the biosynthetic origin of TBA, stable isotope labelling experiments were performed. Therby, the released TBA from the cell was investigated by solid phase extraction (SPE) of the medium. In cultures grown in $Na^{15}NO_3$-enriched medium, ^{15}N-labelled TBA is present in the endometabolome, as I have shown in previous work. As a further step, TBA in the medium was investigated.

Cultures grown in $NaH^{13}CO_3$-enriched medium were extracted to investigate the intramolecular degree of labelling. This appeared to be more difficult as the TBA could not be detected in some samples, neither in a labelled nor in an unlabelled form. In cultures extracted after 15 days, TBA could however be found i. e. intracellularly. Both unlabelled and with three, four, five or six ^{13}C-atoms. After 23 days all levels of labelling from unlabelled to fully labelled were present in the endometabolome of the cultures (see figure 4.2 and figure A.1.6 in the appendix). In the distribution it is noticeable that the fully labelled TBA is most frequently found. As the number of ^{13}C atoms decreases, the amount of TBA continues to decrease. This is mostly due to already existing unlabelled precursors or was already present at the beginning of growth in the $NaH^{13}CO_3$-enriched medium. Taking the ratio of ^{12}C to ^{13}C atoms, the degree of labelling is 92%. This clearly confirms that TBA is produced through biosynthesis by *N.* cf. *pellucida*.

During the evaluation of the SPE samples, TBA was also found in the medium blank. Different tests were carried out to determine the source of the contamination. A blank of the cartridge was extracted to determine contamination during SPE. TBA could not be clearly detected. TBA could be detected in the blanks of the $Na^{15}NO_3$- and $NaH^{13}CO_3$-enriched ASW-medium, but only in the unlabelled form. This shows that TBA does not enter the medium blanks of the SPE through cross-contamination but enters the medium from other sources during the cultivation and extraction process. However, since the quantities are significantly lower than those of the extracts of *N.* cf. *pellucida*, the method can still be used with inclusion of the blank into quantitative consideration.

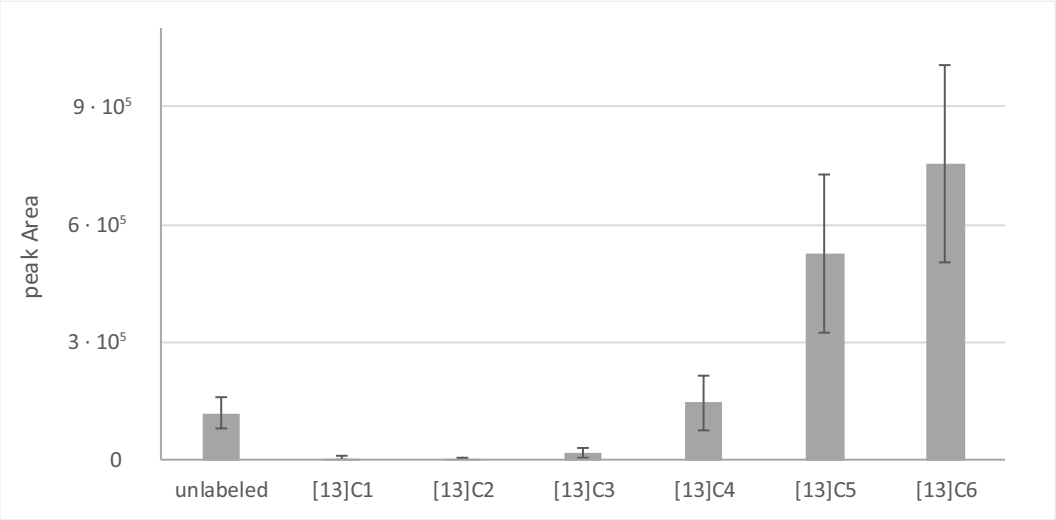


Figure 4.2.: Distribution of the labelling in TBA in extracts of *N.* cf. *pellucida* cultures grown in $NaH^{13}CO_3$-enriched medium after 23 days.

4.2.2. Pathway elucidation of the biosynthesis

Since previous experiments have shown that tribromoaniline is produced through biosynthesis by *N.* cf. *pellucida*, the biosynthesis pathway should now be investigated. For aromatic metabolites in diatoms the biosynthetic pathway is probably the shikimate pathway with shikmic acid, chorismic acid and the aromatic amino acids as the main intermediate products (see figure 1.5 in the introduction). [5, 6, 88] To determine whether TBA is formed from amino acids, feeding experiments with labelled tyrosine and phenylalanine were performed.

For this feeding experiments with tyrosine-D_4-phenyl and phenylalanine-$^{13}C_6$-phenyl were performed and the cells were extracted with ethyl acetate. Previous work in the group showed that the diatom could take up tyrosine and phenylalanine provided in the medium and therefore be ready for biosynthesis. [7] The concentrations used are in the range studied there to ensure that the amino acids can be absorbed by the organisms. The cultures were extracted at two points in time after addition of the amino acids, after 70 hours and after 7 days. This covers a larger time range and it can be safely assumed that the amino acids could be absorbed and metabolized by the cells. If TBA had been successfully labelled, the comparison of the results of the two points in time would have provided information on the time frame of biosynthesis.

The extracts from the feeding experiments did not show any difference in regards to TBA, no matter which amino acid was fed and after which time the extraction was

done. In all samples TBA was present in an amount as expected from the control. Labelled TBA, however, could not be detected, neither with deuterium, nor ^{13}C-labelled. Since the two amino acids can be taken up by the cells and were therefore available for biosynthesis, this result speaks strongly for the fact that the biosynthesis of TBA does not proceed via the aromatic amino acids. It can therefore be assumed that the biosynthesis does not proceed via the aromatic amino acids.

However, the biosynthesis might take place *via* shikmic acid and possibly chorismic acid as intermediate products, as this is the main synthesis pathway for aromatic metabolites in diatoms. [6, 5] However, in order to be able to narrow down more precisely the pathway, further feeding experiments with these compounds must be carried out. Shikmic acid and chorismic acid would initially be appropriate for this purpose, but it will be necessary to check in preliminary experiments whether these compounds can be absorbed by the cells.

4.2.3. Quantification of tribromoaniline

Previous extractions have shown a high variation in the quantity of TBA at different growth phases. To get a clearer picture of the TBA concentration, it was quantified through different approaches.

Since the concentration of TBA seemed to vary depending on the growing phase of *N.* cf. *pellucida*, this chance of concentration should be determined in a time series. Hereby 20 cultures of *N.* cf. *pellucida* were started at the same time and extracted at three-day intervals with five replicates. The intracellular TBA was extracted as well as TBA from the medium. Both Samples were quantified with 2D-Tribromoaniline as internal standard.

At the first extractions after three and six days, the cultures were only in the middle of the exponential growing phase and the cell density was rather low. For this reason, the amount of TBA was too low to quantify. At day eight there was a quantifiable amount of TBA, but it was still quite low. In the following time series, this was taking as the time point for the first extraction.

The second time series was done with the same settings but with more time in between the extractions. Four extractions were done every seven days until the late stationary growing phase (day 14). In addition to the amount of TBA per cell, the intracellular concentration of TBA was determined. The cell volume was determined according to a method by Hillebrand *et al.*, whereby the shape of *N.* cf. *pellucida* was supposed as a prism on an elliptic base. [82] Even if this is just an extremely simplified

approach and only determines approximately the inner volume of the frustule, the order of magnitude of the concentration of TBA can be determined.

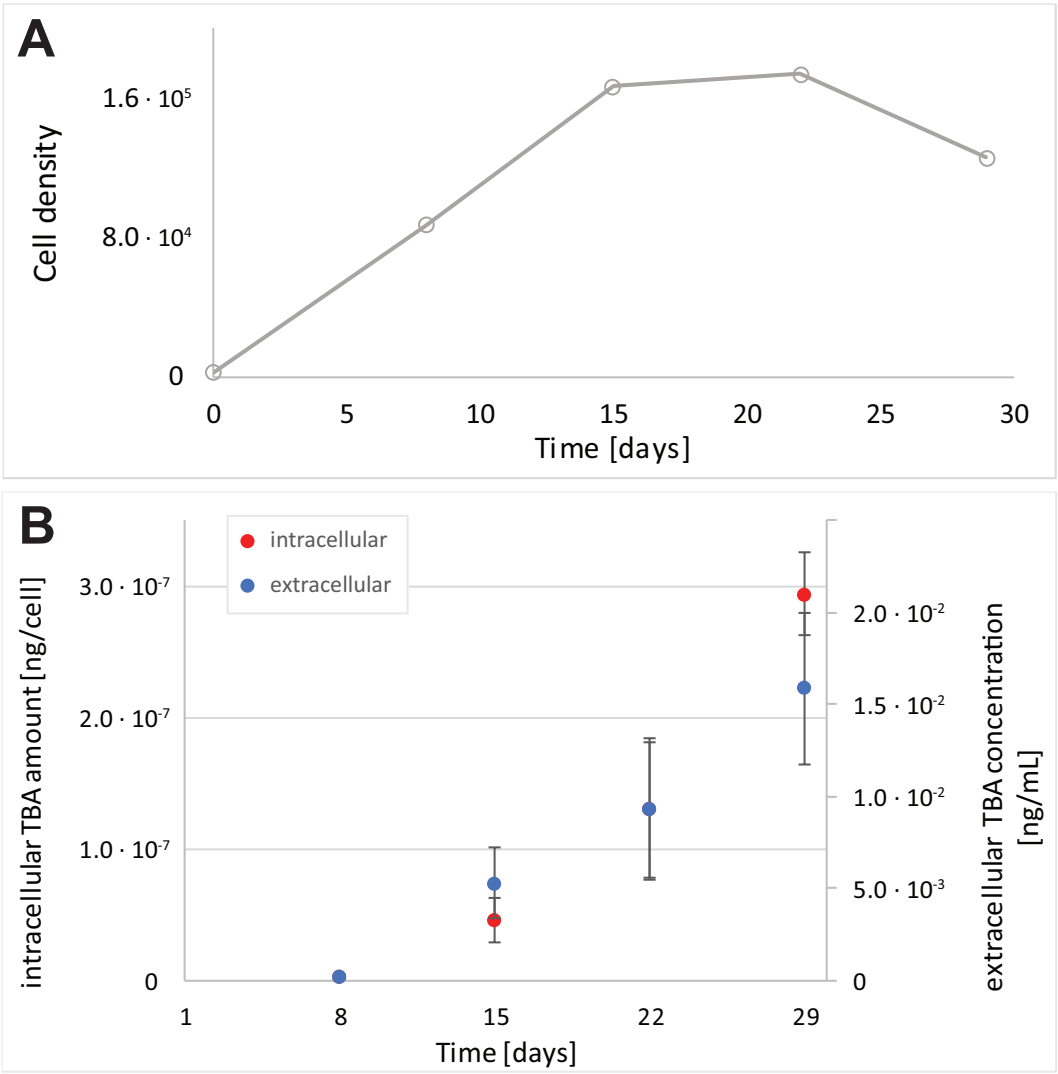


Figure 4.3.: **A**: Growth curve of *Nitzschia* cf. *pellucida* in the second time series. **B**: Intracellular and extracellular TBA concentrations in the second time series.

The cell-normalized TBA concentration increased steadily both intracellularly and in the medium from one extraction to the next. Especially in the stationary growth phase, the concentration increases more rapidly. Hereby at the end of the time series it reached an TBA amount per cell of 30 fg. This corresponds approximately to an intracellular concentration of roughly 400 ng/mL which is in the µM-range. The extracellular concentration is significantly smaller with 0.02 ng/mL in the range of 50 pM. At any time, the intracellular concentration is significantly higher than the concentration in the medium. This shows that after the production of TBA, the substance is not immediately released into the environment, but is initially enriched

in the cell. In this way an immediate dilution, which takes place very quickly in the sea due to the water flow, can also be prevented. Especially at the beginning of the growth phase, when the concentration and cell density are still very low, it is important not to release the TBA. A release far below an effective concentration leads to a waste of biosynthesic resources, energy and precursors. [89]

When evaluating the extracellular concentration in the medium, it should be considered that *N.* cf. *pellucida* is not a planktonic, but a benthic diatom, which is located in a biofilm. So in the marine environment the amount of TBA would be diluted enormously outside the biofilm. For quantification, the diatoms were homogenized with the medium. Any differences in concentration between the biofilm and the surrounding medium are therefore no longer detectable. Various studies have already shown, hydrophobic substances in particular, including tribromoaniline, accumulate in biofilms. [90, 91, 92] It can therefore be assumed that the determined concentration in the medium does not correspond to the extracellular concentration in the biofilm, but rather that the concentration in the biofilm is higher. However, the exact concentration in the biofilm cannot easily be measured.

In order to determine whether tribromoaniline accumulates or if biosynthetic production increases even more than expected, the stability of TBA was investigated. Four identical samples with TBA and methyl stearate as internal standards were prepared and stored at room temperature. Over a period of 17 days, the concentration was determined by GC/HRMS measurement (see Figure A.2.7 in the appendix). During this period, the concentration of TBA decreased only slightly. It can therefore be assumed that TBA released by *N.* cf. *pellucida* cultures is also stable and that an accumulation of the substance can therefore take place.

In order to obtain a more accurate picture of the release of TBA, the change in concentration of TBA over a shorter period of time was studied. Six cultures at the beginning of the stationary growth phase were converted into fresh medium with several washing steps. For three cultures, the medium was extracted immediately afterwards, for the remaining three after 48 hours (see Figure 4.4). Although the average concentration after 48 h is higher than in the start extractions, there is no significant difference (t-test, P=0198).

There can be various reasons why the difference in concentration is quite small. It is possible that the time period was chosen too short, so that the change in concentration is still within the variation of the biological replicas. Extracting cultures at a later stage in the stationary growth phase is also a possibility. However, it should be noted that the TBA production is presumably dependent on the growth phase of the cultures, as the time series has shown. When comparing the initial concentration

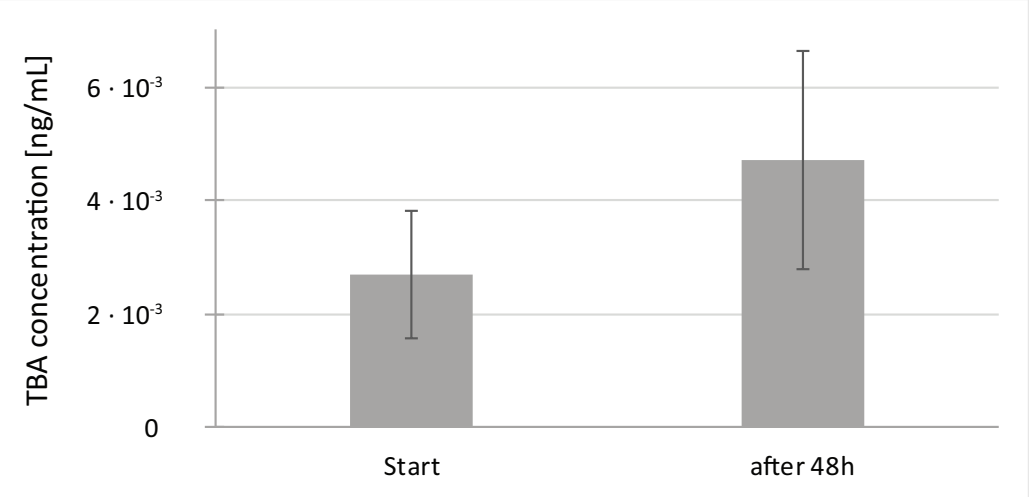


Figure 4.4.: Extracellular amount of TBA directly after the washing steps and after 48 hours. There is no significant difference between the start extraction and the extraction after 48 h (P=0.198).

with the concentration of TBA in the medium in the same growth phase of the time series, it is noticeable that the concentration was only reduced by about half by the washing steps. This could mean that the washing steps were not sufficient enough to remove the entire TBA from the medium. However, it can also mean that *N.* cf. *pellucida* immediately releases large quantities of TBA when it is transferred into a fresh medium.

In further optimization two different cultures, half of one culture should be extracted at each time. Thus, instead of the difference in two concentrations, the direct concentration change could be considered. Even if the previous results were normalized to the cell density, it would significantly increase the precision.

4.2.4. Investigation of the allopathic role of tribromoaniline

As a benthic diatom, *N.* cf. *pellucida* naturally occurs in the biofilm. Little is known about the allopathic role of TBA in this environment. To get a clearer picture, the effect of TBA on different organisms occurring in a similar natural habitat was investigated.

As a biofilm grazer the sea snail *Littorina obtusata* was tested with a plate bioassay described by Rempt *et al.* in 2012. [83] Therefore, agar plates with different concentrations of TBA as well as a blank plate as negative control and a plate with $CuSO_4$ as positive control were prepared. The snails were placed upon the agar. After a

short time, in which the snails could graze, the plates were turned around and the individuals that remained attached at the plates were counted. Due to not optimal health conditions, not enough individuals of *Littorina obtusata* could be tested for a statistically reliable result. Within the obtained results, there was no clear noticeable difference between the negative control and the plates containing TBA. This is an indication that TBA has no effect on the grazer *Littorina obtusata* even in the tested maximum concentration of 0.1 mg/mL.

Since there are also bacteria living with in the biofilm, it is possible that there is an allelopathic effect of TBA on these organisms. With a disc-diffusion bioassay, the effect of TBA was assessed on three species isolated from the benthic diatom *Seminavis robusta*. The species were *Rosevarius* sp., *Maribacter* sp. and *Marinobacter* sp. This was done with a disc diffusion assay on agar plates. Filter discs were loaded with a defined amount of substance and then placed on a freshly spread bacterial culture. After three days of growth, a growth-inhibiting effect shows an area with reduced bacterial growth around the corresponding disc. This was compared with gentamycin as a positive control.

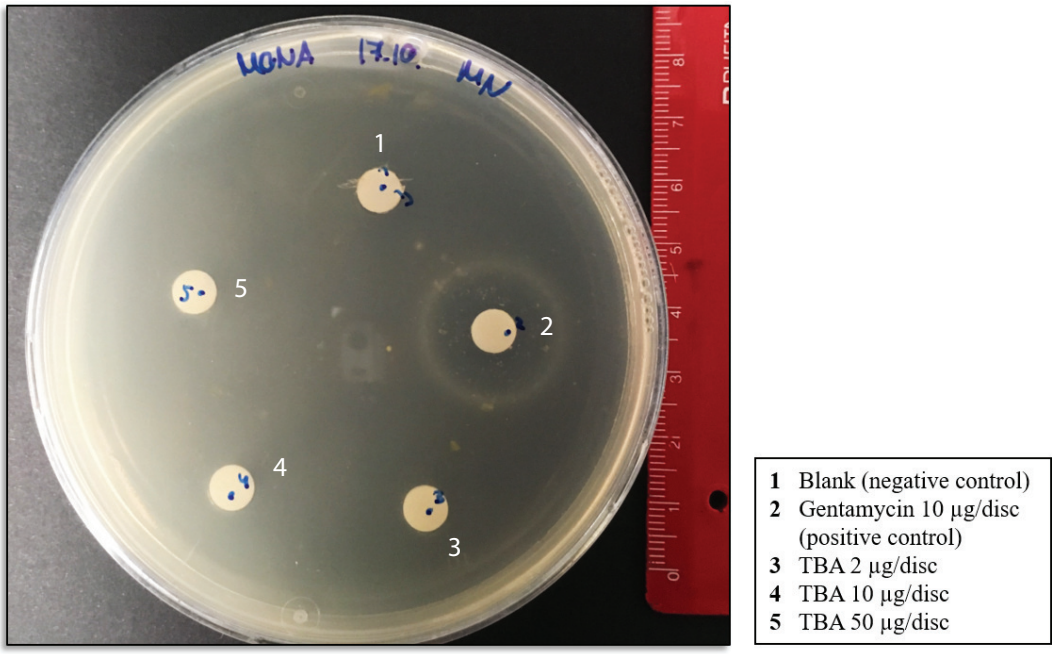


Figure 4.5.: Disc diffusion assay on agar plate after three days with *Marinobacter* sp. An inhibitory zone could only be seen at the positive control gentamycin (2).

For the tested concentrations no effect could be detected. There was a clear inhibition zone for gentamycin, as shown with *Marinobacter* sp. in figure 4.5. But for the discs with TBA no inhibition zone could be detected with either of the bacteria. With the same assay concentrations up to 400 µg per disc were tested and no effect was

noticeable. [93] The results indicate that TBA has no effect on the growth of the bacterial strains studied. However, an influence on other bacterial strains or an influence other than growth inhibition can not be precluded.

The effect on other diatoms was tested with the benthic diatom *Cylindrotheca closterium*. Several experiments were conducted to optimize a method and determine valid results on the effect of TBA on the diatom. For the first attend TBA was diluted in DMSO and added to the culture directly after starting it and in other cultures three days later. At the concentrations to be tested, the solubility of TBA in the medium defines the upper limit. The concentrations 100 µM and 10 µM were tested. Since the maximum internal concentration in *N.* cf. *pellucida* can reach significantly higher concentrations than this value, the results may have biological relevance. The growth was monitored via counting on an inverted microscope. Since the TBA with the DMSO was subsequently added to the cultures, complete mixing is not possible. The concentration of the cultures with 100 µM TBA was therefore in some places higher than the solubility of TBA in water, which led to crystal formation. In these cultures, it was therefore no longer possible to determine the exact concentration. In addition, it cannot be ruled out that further nutrients were removed from the medium by the crystal formation and that this also affects growth.

The cultures were not homogenized before the counting to not disturb the benthic cultures. Since there were only taken three pictures per culture and then counted per hand, the precision of the results and the time required are not normalized. In all following experiments the growth was determined by fluorescence measurements of the chlorophyll a concentration. Although this method does not allow the determination of absolute cell density, the relative growth can still be determined with the chlorophyll a concentration. Since solubility was a problem in the first approach, the TBA dissolved in DMSO was first added to the medium in the second approach and completely homogenized and dissolved before the diatom *C. closterium* was added. Furthermore, the TBA concentration range tested was slightly reduced to 5 µM and 50 µM.

Already in the first bioassay, a change in morphology could be observed at the concentration of 10 µM TBA, although there was no clear inhibition of growth (see figure 4.6 **A**) The cultures were already in the exponential growing phase when the TBA was added and the picture was taken a few hours afterwards. There are two different morphotypes of cells visible. This could be due to the fact that the cells are in different cell division phases and are thus weakened to different degrees by TBA. These changes could also be observed in other bioassays, where the pictures were taken after 8 days after the starting of the cultures. In figure 4.6 **B-F** the *C. closterium* cultures at different TBA concentrations are shown. These cultures were

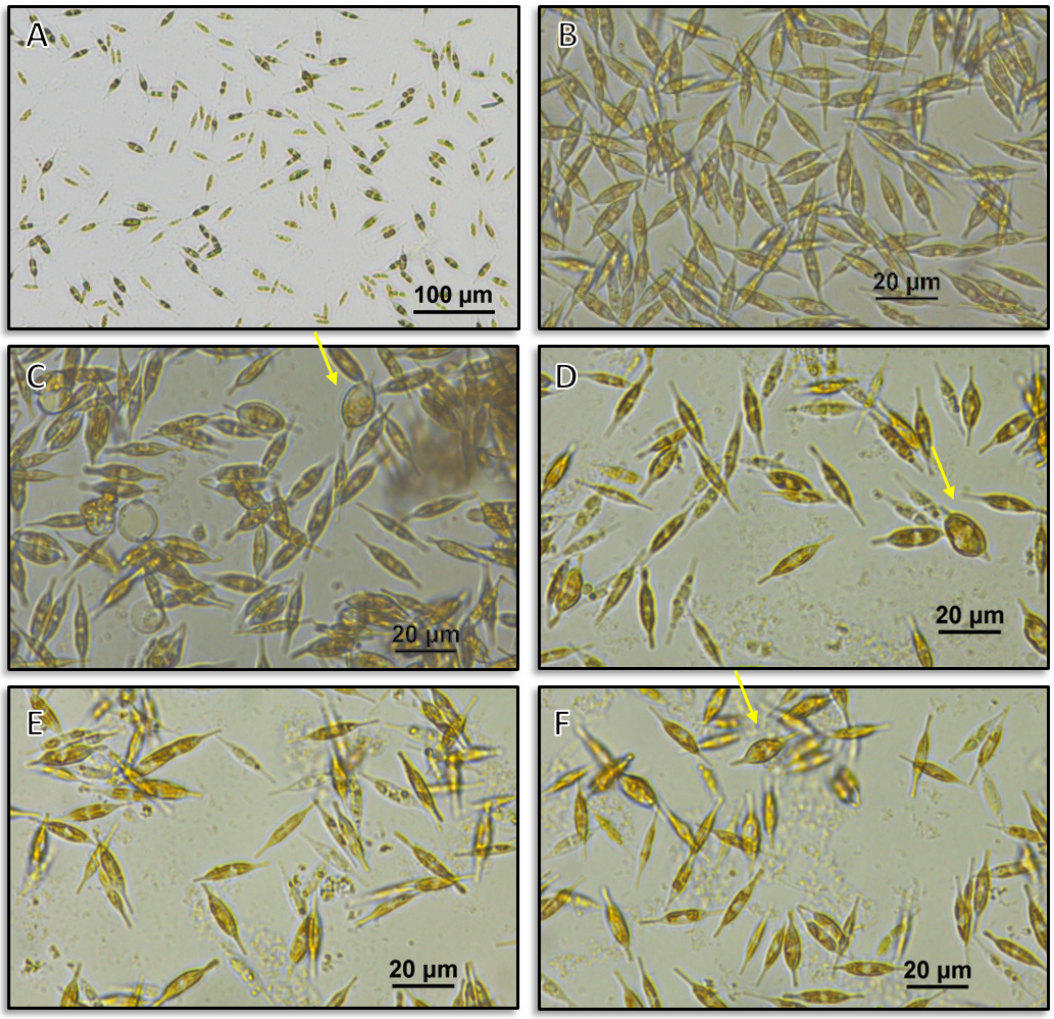


Figure 4.6.: *Cylindrotheca closterium* cultures growing in medium with different TBA concentrations in the first (**A**) and third (**B-F**) bioassay. TBA concentrations: **A** 10 µM, **B**: none (negative control), **C**: 5 µM, **D**: 20 µM, **E**: 40 µM, **F**: 50µM.

exposed to the TBA for a longer time. In all four cultures growing with TBA in the medium, some cells changed drastically in their morphology. Especially in the lower concentrations some of the cells seem to 'blow up' (see yellow arrows). Also, with a rising concentration of TBA, the inorganic compounds contained in the medium are getting more. It can be assumed that this is from dead cells. Therefore, it can be concluded that this is a reaction to the presents TBA. [94]

As shown in figure 4.7, the growth curve of the cultures with a concentration of 5 µM TBA does not differ from the control. With a concentration of 50 µM on the other hand, the growth is inhibited quite a lot. The results show that TBA at a concentration of 50 µM definitely has an influence on the growth of *C. closterium*. Since this effect does not appear to be present at a concentration of 5 µM, the limit

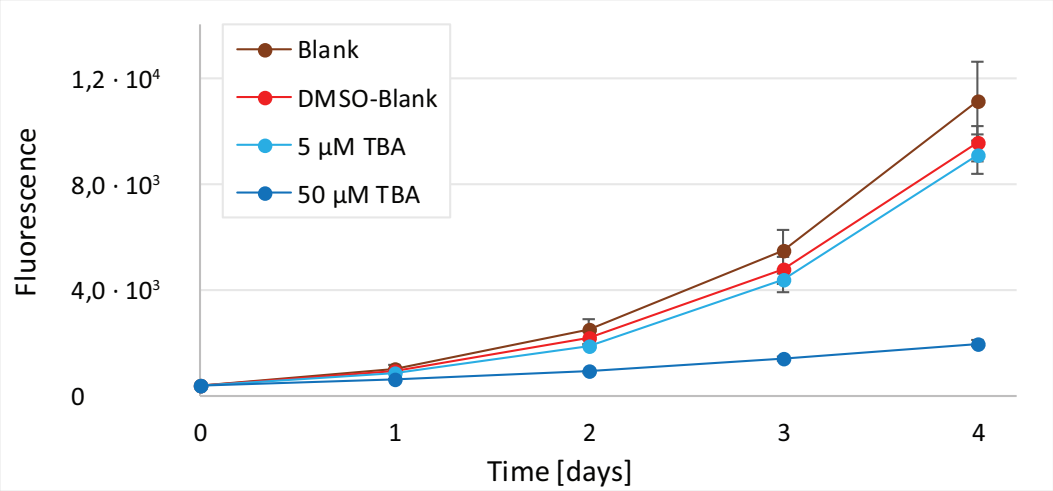


Figure 4.7.: Growing curve of the *Cylindrotheca closterium* cultures in the second bioassay (n=3).

of the effect concentration is in the intermediate range of 20 µM and 40 µM.

In order to validate the results and to further narrow down the cut-off range, another assay with a similar setup was performed. This time three biological and three technical replicates each were performed. Besides 5 µM and 50 µM, the TBA concentrations 20 µM and 40 µM were tested. As a positive control 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a known algaecide, was tested in in the concentrations 5 µM and 50 µM. [95]

The growth curves of the third bioassay looked similar to the ones of the second bioassay (see figure A.2.8 in the appendix). In order to obtain a statistically evaluable result, the growth rate µ was determined for all conditions. This was done with equation 4.1 with F_t being the mean fluorescence at the time t. [86, 96, 97] The results are plotted in figure 4.8.

$$\mu = t^{-1} \cdot ln\frac{F_t}{F_0} \tag{4.1}$$

As expected, the growth factor of both concentrations of the positive control with DCMU is negative, which means that the cultures were dying. As for the samples with TBA, for the concentrations 5 µM and 20 µM there is no significant difference to the negative blank. For 40 µM and 50 µM on the other hand there is a significant difference in the growth compared to the negative control and each other. This result shows that there is a replicable effect of TBA on the growth of the diatom *Cylindrotheca closterium*. The concentration above which a significant effect can be

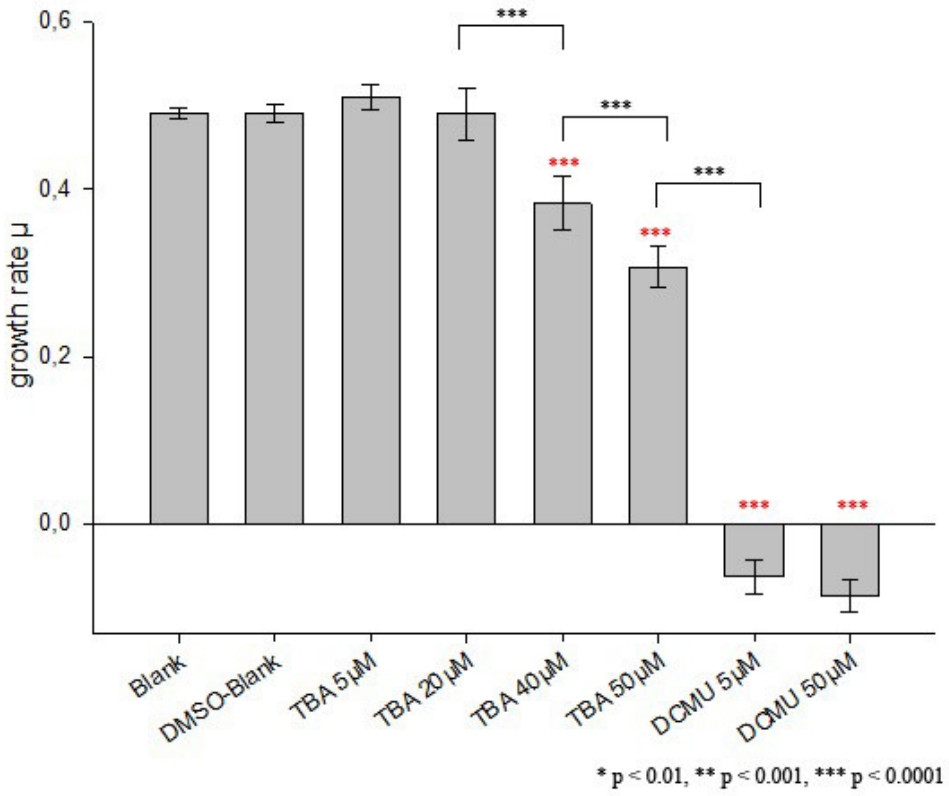


Figure 4.8.: Growth factor of the *C. closterium* cultures in the third bioassay grown in different conditions (n=9). The stars show the results of an ANOVA analysis, the red stars compare to the blank as negative control.

observed is between 20 µM and 40 µM. The effective concentration is only slightly higher than the internal concentration in the micro molar range, which could be measured in the stationary growth phase of *N.* cf. *pellucida*. An allelopathic effect of TBA on another benthic diatom, which are found in the same habitat, was proven. The TBA production of *N.* cf. *pellucida* and thus the concentration increases in the late phase of the exponential growth phase. This is the time when it is important to inhibit the growth of competing organisms in the same habitat in order to be able to spread further. [98, 99] Whether this effect is also observed with other species of benthic algae will be tested in further experiments.

4.3. Conclusion and outlook

The main aspect of this thesis was the investigation of two halogenated metabolites of *N.* cf. *pellucida*. Cyanogen bromide is already well known. The allelopathic and toxic effects for BrCN on surrounding organisms in the biofilm has been studied. The biosynthesis of cyanide in this organism was investigated during this master thesis. Earlier works had already shown that in feeding experiments with a $^{13}C^{15}N$-labelled amino acid mix a labelling of BrCN takes place. Thus, it was assumed that the biosynthesis requires one or more amino acids as precursors. These results were replicated and refined to further narrow down the involved amino acids. For this purpose, the labelled amino acid mix was separated preparatively into fractions with subgroups of amino acids. Feeding experiments were conducted with the separated amino acids to determine the potential precursor(s).

For the detection of BrCN, an established method using SPME and GC/HRMS was employed. However, no BrCN could be detected during the replication of the reported conditions. By optimizing the extraction method this could be overcome and BrCN could be detected from cultures. Feeding experiments show great fluctuations with regard to the detection and presence of BrCN and labelled BrCN. In the time of this thesis, no stable labelling could be achieved. A proper BrCN or labelled BrCN detection was observed for incubation with only few fractions. In order to be able to understand the biosynthesis of cyanide more precisely, the SPME must be improved. As substrate quantity is a limiting factor, the fractions of the amino acid separation can be tested in the feeding experiments only after the already existing results are steadily replicated. Experimental conditions that could be further improved are the concentration, the time from feeding to extraction and also the extraction time itself.

The second metabolite, which was investigated in more detail in this thesis, is tribromoaniline. This compound was not known as a natural substance up to now. For this reason, no information is available on the natural occurrence and ecological significance of this compound. As a synthetic compound, TBA is mainly used as flame retardant. This is probably also the reason why TBA could be partly detected in the blanks, thus the negative controls. Stable isotope labelling experiments with ^{15}N and ^{13}C combined with GC/HRMS analysis, however, clearly confirmed the biosynthetic origin. A clear differentiation could be made between the concentration of synthetic TBA, which was introduced into the extracts as an impurity, and concentration of natural TBA of *N.* cf. *pellucida*. ^{15}N-labelling clearly showed that the vast majority of TBA in the medium was of natural origin.

The biosynthesis of TBA was investigated further. In the first step the aromatic amino acids were tested as potential precursors of the possible biosynthesic pathways. In feeding experiments, ^{13}C-labelled phenylalanine and *D*-labelled tyrosine were added to cultures of *N.* cf. *pellucida* and the endometabolome was then extracted and analysed through GC/HRMS. No labelling of TBA could be observed, although the diatom could take up these amino acids from the medium. Biosynthesis via the aromatic amino acids is not the only possible biosynthesis pathway. Shikimic acid and chorismic acid, the precursors of the aromatic amino acids, are promising intermediates in the biosynthesis of TBA as well. Whether these two are involved in biosynthesis will have to be determined in further experiments. However, it must also be determined beforehand whether these compounds can be taken up by the cells. If this is the case, feeding experiments similar to those with phenylalanine and tyrosine will be carried out.

The intracellular and extracellular concentration of TBA was determined in various experiments. Therefore, the endometabolome and the medium were extracted and analysed by GC/HRMS. The change of the TBA concentration over time was determined during two time series. In the first experiment, the time span was too short and the extraction was too early, so that the concentration was mostly below the limit of detection. For the second time series, extraction of late-exponentially growing cells yielded positive results. Both intracellular and extracellular TBA concentrations increased significantly over time. The intracellular concentration was significantly higher than the concentration in the medium. At the end, concentrations in the micromolar range could be determined in cells and in the picomolar range for the surrounding environment.

Another experiment was conducted to investigate the short-term accumulation of TBA in the medium. It was investigated how much TBA is released into the medium within 48 hours. Unfortunately, no significant difference was found between the concentration at the beginning of the experiment and after 48 hours. In order to further characterize the short-term TBA release, some improvements have to be made to the experimental setup. The time span should be slightly increased so that the difference can become clearer. Another measure would be to reduce the variation between biological replicates. Instead of extracting different cultures at the two points in time as before, it would be useful to extract half of a culture each. Thus it is possible to determine the direct concentration change within a culture.

Since tribromoaniline as a metabolite is also a potential allelochemical, the allelopathic effect on different organisms also associated with marine biofilm was investigated. *Littorina obtusata* as biofilm grazer, multiple marine bacteria and *Cylindrotheca closterium* as competing benthic diatom were investigated for their suscep-

tibility towards TBA. For the bioassays with snails and bacteria, no effect could be observed. TBA displayed however a strong activity against *C. closterium*. Hence, TBA induced morphological cell alterations and inhibited the growth, even at a concentrations of 5 µM, which is only slightly above the intracellular concentration. The cellular alteration may be the result of a stress reaction. At a concentration of 40 µM and above there was also a significant reduction in growth. In the future, TBA will be tested against diverse algal species in the near future.

In summary, biosynthesis, concentration and activity of halogenated metabolites in *Nitzschia* cf. *pellucida* could give advanced insights into the chemical ecology of unicellular microalgae. While the experimental setup for BrCN needs to be further optimized, many new findings on TBA have been gained. Thus, the biosynthetic origin was clearly proven and the concentration of TBA in different growth phases of *N.* cf. *pellucida* was determined. The allelopathic effect of this compound was determined in a bioassay.

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E. Lindquist, M. Lommer, V. Martin-Jezequel, P. J. Lopez, S. Lucas, M. Mangogna, K. McGinnis, L. K. Medlin, A. Montsant, M. P. Oudot-Le Secq, C. Napoli, M. Obornik, M. S. Parker, J. L. Petit, B. M. Porcel, N. Poulsen, M. Robison, L. Rychlewski, T. A. Rynearson, J. Schmutz, H. Shapiro, M. Siaut, M. Stanley, M. R. Sussman, A. R. Taylor, A. Vardi, P. von Dassow, W. Vyverman, A. Willis, L. S. Wyrwicz, D. S. Rokhsar, J. Weissenbach, E. V. Armbrust, B. R. Green, Y. Van de Peer, and I. V. Grigoriev. The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature*, **456**(7219):239–44, 2008.

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A. Appendix

A.1. BrCN

Table A.1.1.: Concentrations of the $^{13}C^{15}N$-labelled amino acid mix used for the separation.

	concentration		concentration
alanine	7.3 mM	**leucine**	5.1 mM
arginine	2.4 mM	**lysine**	2.1 mM
asparagine	1.9 mM	**methionine**	1.0 mM
aspartic acid	5.8 mM	**phenylalanine**	1.9 mM
cysteine	2.0 mM	**proline**	2.1 mM
glutamine	2.0 mM	**serine**	3.1 mM
glutamic acid	4.7 mM	**threonine**	3.2 mM
glycine	5.0 mM	**tryptophan**	2.0 mM
histidine	0.5 mM	**tyrosine**	1.5 mM
isoleucine	2.4 mM	**valine**	3.2 mM

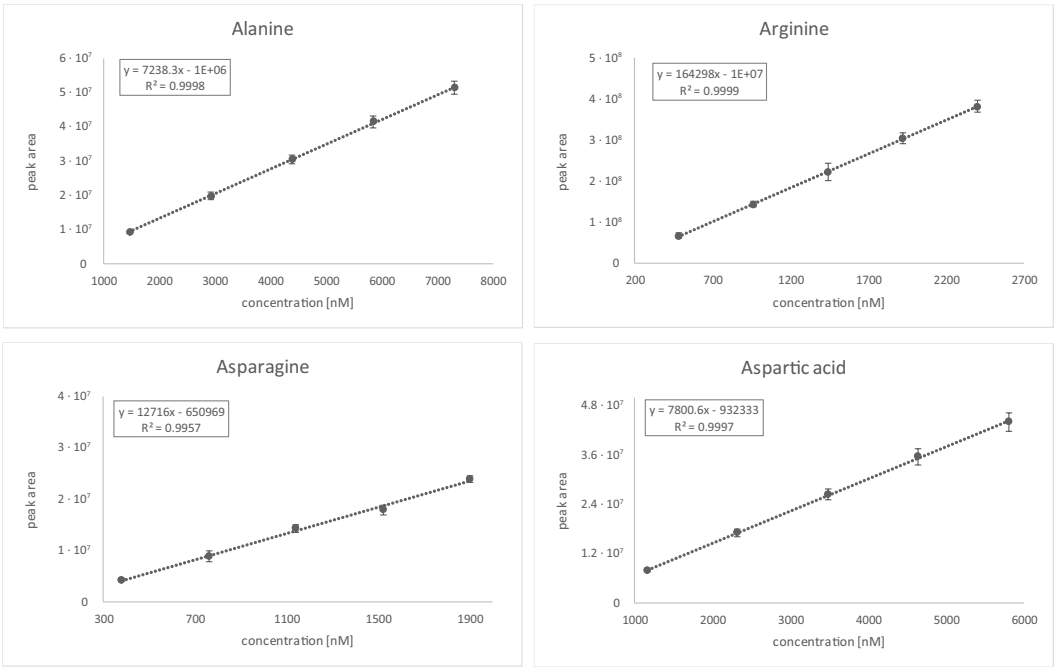

Figure A.1.1.: Calibration curves of the amino acids alanine, arginine, asparagine and aspartic acid.

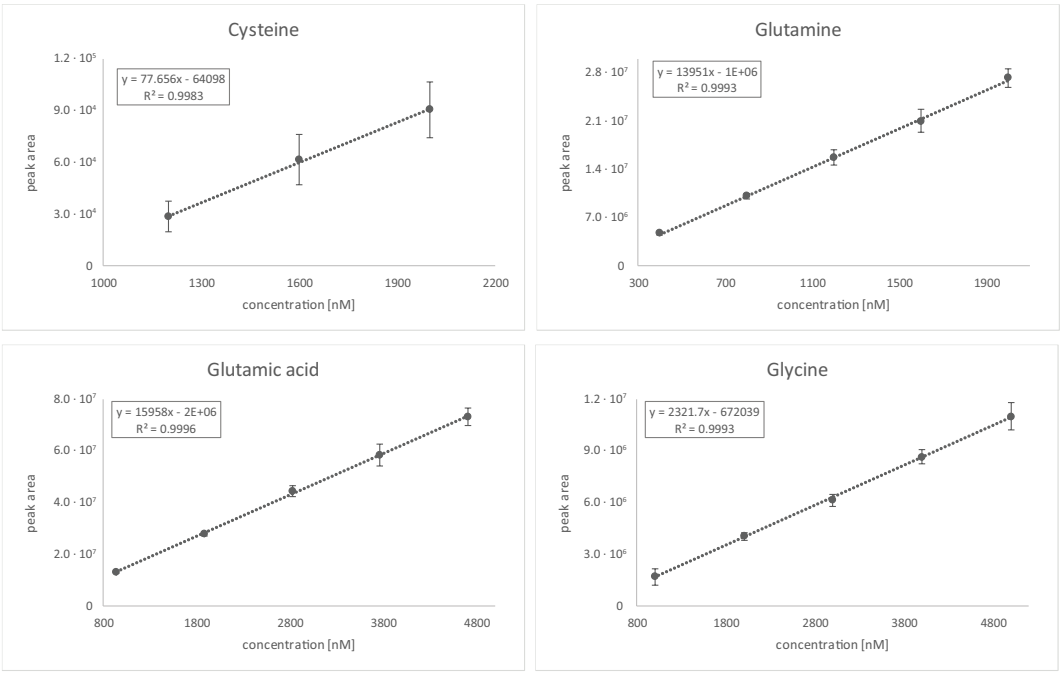

Figure A.1.2.: Calibration curves of the amino acids cysteine*, glutamine, glutamic acid and glycine. (*) For cysteine, the linear range of the calibration curve is at the limit of detection. Therefore, no good calibration line can be determined. However, the calibration curve shown was used for the rough determination of the concentration.

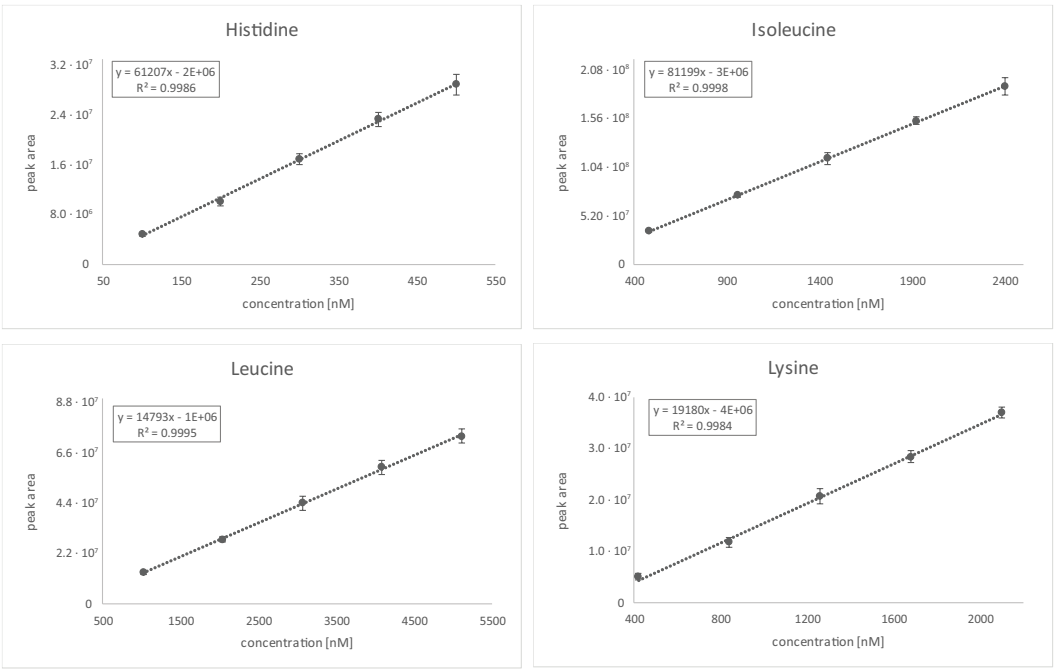

Figure A.1.3.: Calibration curves of the amino acids histidine, isoleucine, leucine and lysine.

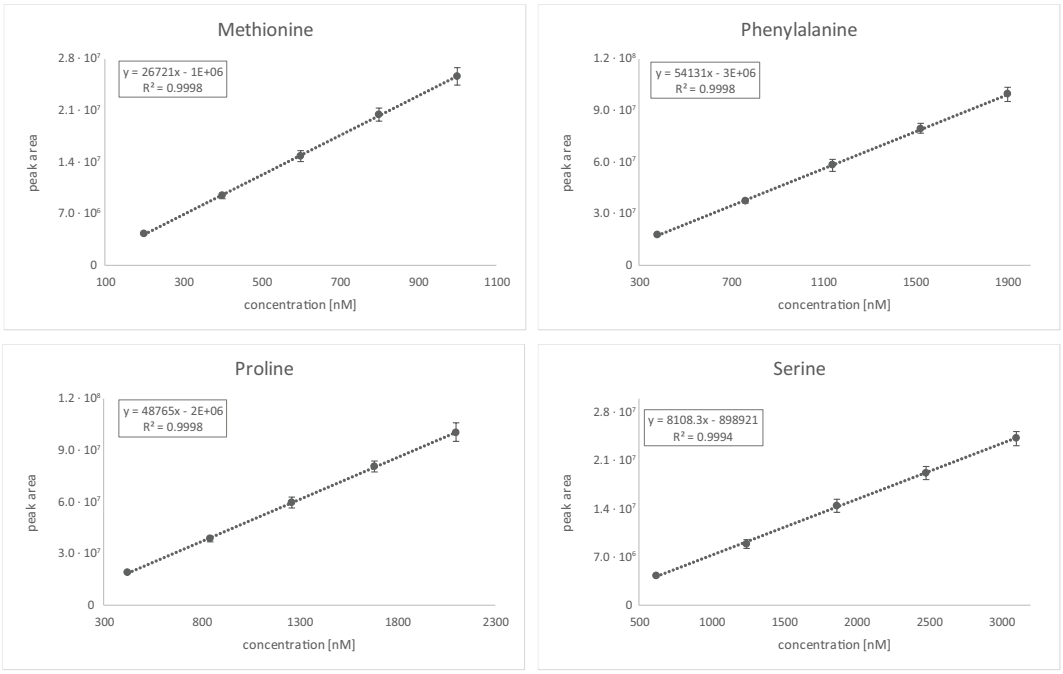

Figure A.1.4.: Calibration curves of the amino acids methionine, phenylalanine, proline and serine.

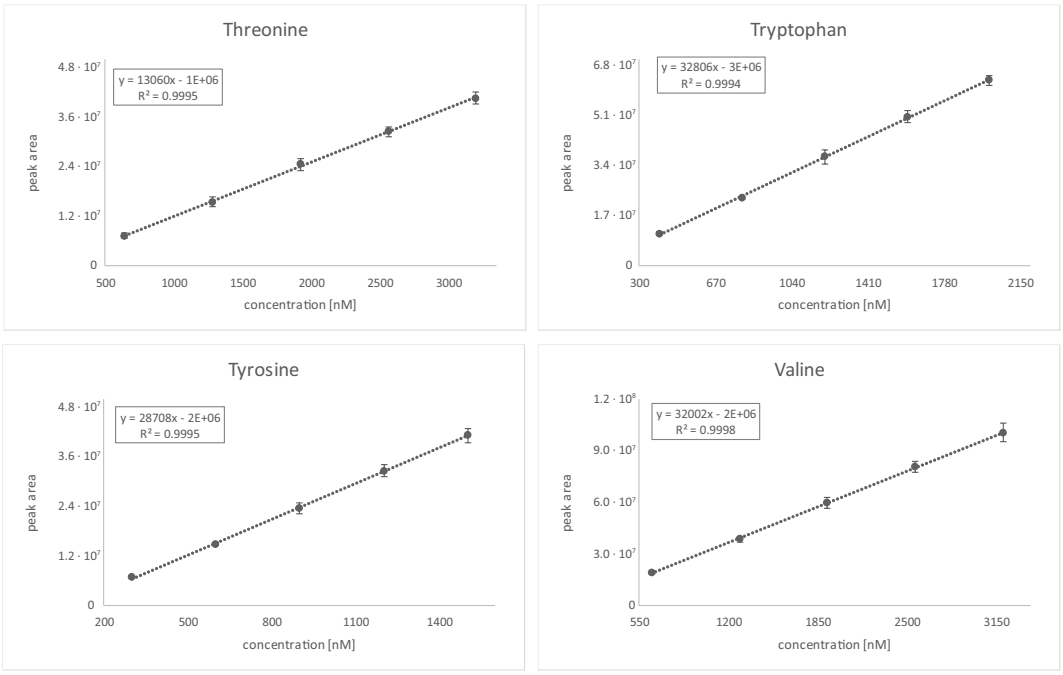

Figure A.1.5.: Calibration curves of the amino acids threonine, tryptophan, tyrosine and valine.

Table A.1.2.: Amino acid concentrations in each fraction after the whole separation process. (*) For Isoleucine and Leucine, the exact quantities can differ slightly because of an incomplete baseline separation.

Amino acid concentration in each fraction [µM]							
Amino Acid	Frac. A	Frac. B	Frac. C	Frac. D	Frac. E	Frac. F	Frac. G
Retention time [min]	0 - 7	7 – 8.5	8.5 – 11.5	11.5 - 15	15 - 27	27 - 39	39 - 50
Alanine	238.18	-	0.16	-	-	-	-
Arginine	0.07	-	0.08	63.70	-	0.62	-
Asparagine	61.16	-	-	-	-	-	-
Aspartic acid	195.56	-	-	-	-	-	-
Cysteine	96.74	-	5.02	-	-	-	-
Glutamine	17.15	-	-	-	-	-	-
Glutamic acid	146.79		-	-	-	-	-
Glycine	170.95	-	-	-	-	-	-
Histidine	-	-	9.64	0.04	-	-	-
Isoleucine*	-	-	51.61	-	-	-	-
Leucine*	-	-	72.80	-	-	-	-
Lysine	-	-	65.98	0.27	-	-	-
Methionine	-	-	19.75	0.04	-	-	-
Phenylalanine	-	-	-	-	-	32.43	-
Proline	71.68	-	-	-	-	-	-
Serine	102.18	-	-	-	-	-	-
Threonine	110.97	-	-	-	-	-	-
Tryptophan	29.01	0.17	-	-	-	-	-
Tyrosine	-	-	10.32	40.49	-	0.11	-
Valine	0.18	-	68.96	-	-	-	0.11

Table A.1.3.: The parameters used for the quantification of the amino acids with the UPLC/MS.

Amino acid	Molecular formula	Retention time [min]	Theoretical exacte mass [u]
Alanine	$^{13}C_3H_7^{15}NO_2$	1.34	94.06205
Arginine	$^{13}C_6H_{14}^{15}N_4O_2$	1.29	185.12722
Asparagine	$^{13}C_4H_8^{15}N_2O_3$	1.33	139.06826
Aspartic acid	$^{13}C_4H_7^{15}NO_4$	1.35	139.05524
Cysteine	$^{13}C_3H_7^{15}NO_2S$	1.34	126.03413
Glutamine	$^{13}C_5H_{10}^{15}N_2O_3$	1.34	154.08726
Glutamic acid	$^{13}C_5H_9^{15}NO_4$	1.36	154.07424
Glycine	$^{13}C_2H_5^{15}NO_2$	1.32	79.04305
Histidine	$^{13}C_6H_9^{15}N_3O_2$	1.29	165.08799
Isoleucine	$^{13}C_6H_{13}^{15}NO_2$	2.26	139.11907
Leucine	$^{13}C_6H_{13}^{15}NO_2$	2.01	139.11907
Lysine	$^{13}C_6H_{14}^{15}N_2O_2$	1.26	155.12700
Methionine	$^{13}C_5H_{11}^{15}NO_2S$	1.70	156.07213
Phenylalanine	$^{13}C_9H_{11}^{15}NO_2$	4.48	176.11348
Proline	$^{13}C_5H_9^{15}NO_2$	1.39	122.08441
Serine	$^{13}C_3H_7^{15}NO_3$	1.32	110.05697
Threonine	$^{13}C_4H_9^{15}NO_3$	1.35	125.07597
Tryptophan	$^{13}C_{11}H_{12}^{15}N_2O_2$	8.19	218.12813
Tyrosine	$^{13}C_9H_{11}^{15}NO_3$	2.06	192.10840
Valine	$^{13}C_5H_{11}^{15}NO_2$	1.53	124.10006

Table A.1.4.: The concentrations used in the feeding experiments for the pathway elucidation of BrCN.

Amino acid	**$^{13}C^{15}N$-labelled amino acid mix**	**Fraction A**	**$^{13}C^{15}N$-labelled Glycine**
	Concentration [µM]		
Alanine	1.46	4.76	
Arginine	0.48		
Asparagine	0.38	1.22	
Aspartic acid	1.16	3.91	
Cysteine	0.40	1.93	
Glutamine	0.40	0.34	
Glutamic acid	0.94	2.94	
Glycine	1.00	3.42	1.30
Histidine	0.10		
Isoleucine	0.48		
Leucine	1.02		
Lysine	0.42		
Methionine	0.20		
Phenylalanine	0.38		
Proline	0.42	1.43	
Serine	0.62	2.04	
Threonine	0.64	2.22	
Tryptophan	0.40	0.58	
Tyrosine	0.30		
Valine	0.64		

Table A.1.5.: Theoretical exact masses of the molecular ion peak, the bromine isotope peak and the proton adducts each of cyanogen bromide and ^{13}C-, ^{15}N-labelled BrCN in u.

	^{79}BrCN	**^{81}BrCN**	**H^{79}BrCN**	**H^{81}BrCN**
BrCN	104.92086	106.91880	105.92870	107.92660
Br^{13}C	105.92422	107.92217	106.93200	108.93000
BrC^{15}N	105.91790	107.91585	106.92572	108.92368
Br^{13}C^{15}N	106.92125	108.91921	107.92908	109.92703

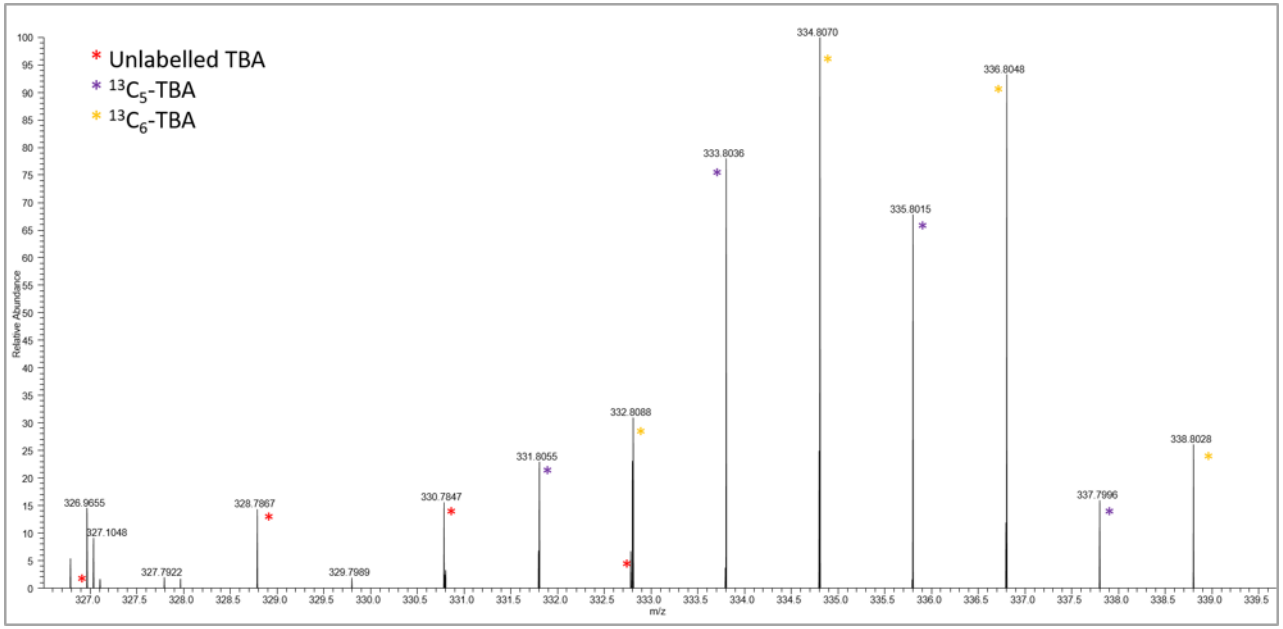

Figure A.1.6.: Mass spectrum at t_R 17.76 min of an extraction of *N.* cf. *pellucida* cultures grown in $NaH^{13}CO_3$-enriched medium after 23 days. The peaks of the most abundant TBA species are marked with stars.

A.2. TBA

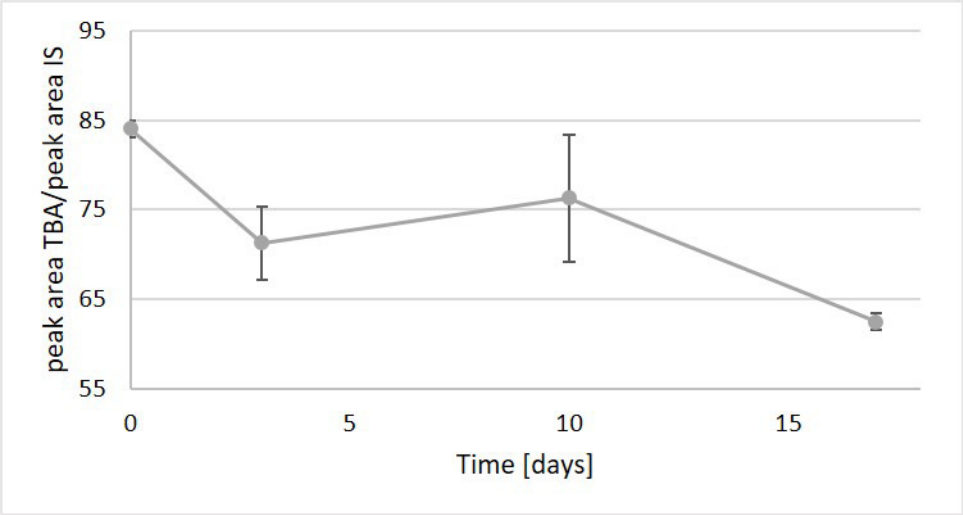

Figure A.2.7.: Results of the stability test of TBA with the internal standard methyl stearate.

Table A.2.6.: Theoretical exact masses of the molecular ion peak and the bromine isotope peaks of tribromoaniline and ^{13}C-, ^{15}N- and *D*-labelled TBA in u.

	$\mathbf{C_6H_4N^{79}Br_3}$	$\mathbf{C_6H_4N^{79}Br_2^{81}Br}$	$\mathbf{C_6H_4N^{79}Br^{81}Br_2}$	$\mathbf{C_6H_4N^{81}Br_3}$
$\mathbf{C_6H_4NBr_3}$	326.78884	328.78679	330.78475	332.78270
$\mathbf{C_6^1H_3DNBr_3}$	327.79512	329.79307	331.79102	333.78898
$\mathbf{C_6^1H_2D_2NBr_3}$	328.80139	330.79935	332.79730	334.79525
$\mathbf{^{12}C_5^{13}CH_4NBr_3}$	327.79219	329.79015	331.78810	333.78605
$\mathbf{^{12}C_4^{13}C_2H_4NBr_3}$	328.79555	330.79350	332.79145	334.78941
$\mathbf{^{12}C_3^{13}C_3H_4NBr_3}$	329.79890	331.79686	333.79481	335.79276
$\mathbf{^{12}C_2^{13}C_4H_4NBr_3}$	330.80226	332.80021	334.79816	336.79612
$\mathbf{^{12}C^{13}C_5H_4NBr_3}$	331.80561	333.80357	335.80152	337.79947
$\mathbf{^{13}C_6H_4NBr_3}$	332.80897	334.80692	336.80487	338.80283
$\mathbf{C_6H_4^{15}NBr_3}$	327.78587	329.78383	331.78178	333.77973
Theoretical ratio	1	3	3	1

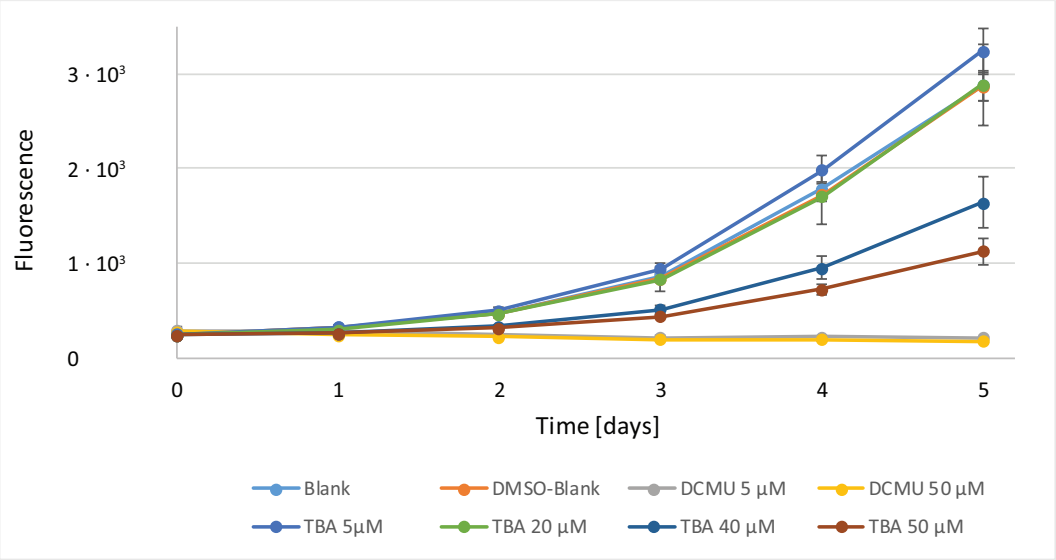

Figure A.2.8.: Growing curve of the *Cylindrotheca closterium* cultures in the third bioassay (n=9).

Zusammenfassung

Der Hauptaspekt dieser Masterarbeit war die Untersuchung zweier halogenierter Metaboliten von *Nitzschia* cf. *pellucida*. Bromcyan (BrCN), der erste Metabolit, ist bereits bekannt. Die allelopathischen und toxischen Wirkungen von BrCN auf umliegende Organismen im Biofilm wurden untersucht. Die bisher völlig unbekannte Biosynthese von Cyanid hingegen wurde im Rahmen dieser Masterarbeit untersucht. Frühere Arbeiten hatten bereits gezeigt, dass in Fütterungsversuchen mit einem $^{13}C^{15}N$-markierten Aminosäuren Gemisch eine Markierung des BrCN erfolgt. So wurde angenommen, dass die Biosynthese eine oder mehrere Aminosäuren als Vorläufer benötigt. Diese Ergebnisse wurden wiederholt, um die beteiligten Aminosäuren weiter einzugrenzen. Zu diesem Zweck wurde das markierte Aminosäuren Gemisch präparativ in Fraktionen mit weniger Aminosäuren getrennt. Es wurden Fütterungsexperimente mit den abgetrennten Aminosäuren durchgeführt, um festzustellen, welche davon der potentielle Vorläufer ist.

Für den Nachweis von BrCN wurde eine etablierte Methode mit Festphasen Microextraktion (SPME) und Gaschromatographie gekoppelt mithochauflösender Massenspektrometrie (GC/HRMS) eingesetzt. Bei der genauen Nachbildung der Versuchsbedingungen konnte jedoch kein BrCN festgestellt werden. Durch die Optimierung der Extraktionsmethode konnte dies vorerst korrigiert und BrCN aus normalen Kulturen nachgewiesen werden. Andererseits zeigten Fütterungsversuche große Schwankungen in Bezug auf den Nachweis und das Vorhandensein von BrCN und markiertem BrCN. Während der Zeit dieser Arbeit konnte keine stabile Markierung erreicht werden. Eine korrekte BrCN- oder markierte BrCN-Erkennung wurde nur bei wenigen Experimenten beobachtet. Um die Biosynthese von Cyanid genauer verstehen zu können, muss die SPME verbessert werden. Da die Menge ein limitierender Faktor ist, können die Fraktionen der Aminosäureauftrennung in den Fütterungsversuchen erst getestet werden, wenn die bereits vorliegenden Ergebnisse kontinuierlich repliziert werden. Experimentelle Bedingungen, die weiter verbessert werden könnten, sind die Konzentration, die Zeit von der Zuführung bis zur Extraktion und auch die Extraktionsdauer selbst.

Der zweite Metabolit, der in dieser Arbeit näher untersucht wurde, ist Tribromoanilin (TBA). Diese Verbindung war bisher nicht als Naturstoff bekannt, somit gibt es

wenig Informationen über das natürliche Vorkommen und die ökologische Bedeutung dieser Verbindung. Als synthetische Verbindung wird TBA hauptsächlich als Flammschutzmittel verwendet. Dies ist wahrscheinlich auch der Grund, warum TBA teilweise in den Blanks, also den Negativkontrollen, erkannt werden konnte. Markierungsexperimente mit stabilen Isotopen von ^{15}N und ^{13}C in Kombination mit GC/HRMS-Analysen bestätigten jedoch eindeutig den biosynthetischen Ursprung. Darüber hinaus konnte eine klare Unterscheidung zwischen dem synthetischen TBA, das als Verunreinigung in die Extrakte eingebracht wurde, und dem natürlichen TBA von *Nitzschia* cf. *pellucida* getroffen werden. Die ^{15}N-Kennzeichnung zeigte deutlich, dass die überwiegende Mehrheit der TBA im Medium natürlichen Ursprungs war.

In einem weiteren Schritt wurde die Biosynthese von TBA untersucht. In einem ersten Schritt beschränkten sich die Experimente auf die aromatischen Aminosäuren als Vorläufer als eine der möglichen Biosynthesewege. In Fütterungsexperimenten wurden den Kulturen von *Nitzschia* cf. *pellucida* ^{13}C-markiertes Phenylalanin und *D*-markiertes Tyrosin zugesetzt, das Endometabolom extrahiert und anschließend durch GC/HRMS analysiert. Es konnte keine Markierung von TBA beobachtet werden, obwohl frühere Studien gezeigt hatten, dass die Kieselalge diese Aminosäuren aus dem Medium aufnehmen konnte. Über die aromatischen Aminosäuren ist nicht der einzige mögliche Biosyntheseweg. Shikimisäure und Chorisminsäure, Vorläufer der aromatischen Aminosäuren, sind ebenfalls vielversprechende mögliche Zwischenprodukte in der Biosynthese von TBA. Ob diese beiden an der Biosynthese beteiligt sind, muss in weiteren Experimenten untersucht werden. Vorab muss jedoch auch geklärt werden, ob diese Verbindungen von den Zellen aufgenommen werden können. In diesem Fall werden Fütterungsversuche ähnlich denen mit Phenylalanin und Tyrosin durchgeführt.

Die intrazelluläre und extrazelluläre Konzentration von TBA wurde in verschiedenen Experimenten bestimmt. Das Endometabolom und das Medium wurden extrahiert und mit GC/HRMS analysiert. Die Änderung der TBA-Konzentration über die Zeit wurde in zwei Zeitreihen bestimmt. Im ersten Experiment war die Zeitspanne zu kurz und die Extraktion zu früh, so dass die Konzentrationen meist unter der Nachweisgrenze lagen. Beim zweiten Versuch dagegen lieferte die Extraktion von Zellen im späten exponentiellen Wachstum positive Ergebnisse. Sowohl die intrazellulären als auch die extrazellulären TBA-Konzentrationen sind im Laufe der Zeit deutlich gestiegen. Die intrazelluläre Konzentration war signifikant höher als die Konzentration im Medium. Am Ende konnten Konzentrationen im mikromolaren Bereich in Zellen und im picomolaren Bereich für die Umgebung bestimmt werden.

Ein weiteres Experiment wurde durchgeführt, um die kurzfristige Freisetzung von TBA im Medium zu untersuchen. Es wurde untersucht, wie viel TBA innerhalb von

48 Stunden in das Medium abgegeben wird. Leider wurde kein signifikanter Unterschied zwischen der Konzentration zu Beginn und nach 48 Stunden festgestellt. Um die kurzfristige TBA-Freigabe weiter zu charakterisieren, müssen einige Verbesserungen am Versuchsaufbau vorgenommen werden. Zum einen sollte die Zeitspanne leicht erhöht werden, damit der Unterschied deutlicher wird. Eine weitere Maßnahme wäre es, die Unterschiede zwischen den biologischen Replikaten zu reduzieren. Anstatt zu den beiden Zeitpunkten wie bisher verschiedene Kulturen zu extrahieren, wäre es sinnvoll, jeweils die Hälfte einer Kultur zu extrahieren. So ist es möglich, die direkte Konzentrationsänderung innerhalb einer Kultur zu bestimmen.

Da Tribromoanilin als Metabolit auch eine potenzielle Allelochemikalie ist, wurde die allelopathische Wirkung auf verschiedene Organismen, die auch mit dem marinen Biofilm in Verbindung gebracht werden können, untersucht. *Littorina obtusata* als Biofilm-Grazer, mehrere Meeresbakterien und *Cylindrotheca closterium* als weitere konkurrierende Kieselalge wurden untersucht. Für die ersten beiden Organismen konnte kein Effekt in den durchgeführten Bioassays beobachtet werden. Dagegen zeigte sich bei *C. closterium* eine starke Aktivität gegen TBA. Dabei induzierte TBA morphologische Zellveränderungen und hemmte das Wachstum, selbst bei einer Konzentration von 5 μM, welche nur wenig über der intrazellulären Konzentration liegt. Die zelluläre Veränderung kann das Ergebnis einer Stressreaktion sein. Ab einer Konzentration von 40 μM kam es auch zu einer deutlichen Wachstumsverringerung. Diese vielversprechenden Ergebnisse sollten weiterverfolgt und durch weitere Experimente mit anderen Kieselalgen bestätigt werden.

Zusammenfassend lässt sich sagen, die Biosynthese, Konzentration und Aktivität von halogenierten Metaboliten gibt einen weiteren Einblick in die chemische Ökologie einzelliger Mikroalgen. Während der Versuchsaufbau für BrCN weiter optimiert werden muss, wurden viele neue Erkenntnisse über TBA gewonnen. Damit wurde der biosynthetische Ursprung eindeutig nachgewiesen und die Konzentration der TBA in verschiedenen Wachstumsphasen von *N.* cf. *pellucida* bestimmt. Die allelopathische Wirkung dieser Verbindung wurde in einem Bioassay bestimmt.

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Declaration of Authorship

I hereby declare that I have written this thesis on my own and that I did not use any sources than those noted. This work has not been submitted to any examination committee up to now, neither in the same nor in a similar form.

Selbstständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und keine weiteren Quellen als die genannten verwendet habe. Diese Arbeit wurde bisher weder in dieser, noch in einer ähnlichen Fassung einem Studien- oder Prüfungsamt vorgelegt.

Ort, Datum	Unterschrift